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# Evaluation of commercial chromatographic adsorbents for the direct capture of polyclonal rabbit antibodies from clarified antiserum☆

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**Abstract**

We have carried out a rigorous evaluation of eight commercially available packed bed chromatography adsorbents for direct capture and purification of immunoglobulins from clarified rabbit antiserum. Three of these materials featured rProtein A (rProtein A Sepharose Fast Flow, Mabselect, Prosep rProtein A) as the affinity ligand, and differed from one another primarily with respect to the underlying base matrix. The remaining five matrices comprised various synthetic low molecular weight ligands immobilised on hydrophilic porous supports and these included: MEP HyperCel, MabSorbent A1P, MabSorbent A2P, FastMabsA and Kaptiv-GY. The general experimental approach taken was to sequentially challenge packed beds of each matrix with a series of different strengths of a clarified antiserum; beginning with the weakest and ending with the strongest. Marked differences in performance (principally evaluated on the basis of dynamic binding capacity, recovery, and purity) were obtained, which allowed clear recommendations concerning the choice of adsorbents best suited for antibody capture from rabbit antisera, to be made.


*Keywords:* Biomimetic ligands; Hydrophobic charge induction; Immunoglobulin purification; Mixed mode chromatography; Protein A; Lipoproteins

## 1. Introduction

Currently the large-scale purification of polyclonal antibodies is, for the most part, carried out by the plasma fractionation industry, with production being geared towards generation of a wide range of plasma proteins (including albumin, Factor VIII, Factor IX, Protein C and von Willebrand Factor) rather than single antibody products [1–3]. Since the first reports on the use of immobilised Protein A for the affinity purification of antibodies over 30 years ago [4,5]. Protein A affinity chromatography has become the industrial standard for the purification of clinical grade monoclonal antibodies [3,6–8], but is rarely applied in the plasma fractionation industry [1,2], largely for reasons of cost and reservations concerning Protein A's chemical stability, adsorbent shelf-life and cleaning [9]. For the production of diagnostic antibody products such concerns are less serious; thus Protein A chromatography currently represents one of most potent separation tools available to the diagnostic industry.

Not withstanding the success of Protein A chromatography in the purification of antibodies, adsorbents based on this biological ligand suffer numerous drawbacks (alluded to immediately above), which have stimulated the search for stable synthetic low molecular weight ligand alternatives to [10–12] or mimics of Protein A [13,14]. As adsorbents based on such ligands allow rigorous cleaning without loss in ligand or binding capacity, their use with mildly conditioned, or even unconditioned, feedstocks (so called 'dirty' feedstocks) would appear to offer highly attractive prospects for bioprocess intensification.

In parallel with the development of new synthetic alternatives to Protein A, others have sought to improve Protein A adsorbents, largely through applying recombinant DNA techniques to: improve the ligand's tolerance to cleaning with sodium hydroxide [15,16]; lower the binding affinity to enable use of milder elution conditions [17]; and allowing

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Table 1
Description of adsorbents employed in this study

| Adsorbent | Immobilised ligand | Description of base particle | Manufacturer |
|---|---|---|---|
| rProtein A Sepharose FF | Recombinant Protein A[a] (lot no. 279973, 6 mg mL$^{-1}$) | 60–165 μm highly cross-linked 4% agarose | GE Healthcare, Uppsala, Sweden |
| MabSelect | Recombinant Protein A[a] (lot no. 286511, 5 mg mL$^{-1}$) | 40–130 μm (av. 85 μm) highly cross-linked rigid 'HF' 3.5% agarose base matrix[b] | GE Healthcare, Uppsala, Sweden |
| ProSep rProtein A | Recombinant Protein A[c] (lot no. 113112724) | 75–125 μm controlled pore high silica glass | Millipore, Billerica, MA, USA |
| MEP HyperCel | 4-mercapto-ethyl-pyridine (lot no. A112, 100 μmol mL$^{-1}$)[d] | Av. 90 ± 10 μm cellulosic base matrix | Pall Life Sciences, BioSepra AS, Cergy-Saint-Christophe, France |
| MabSorbents A1P & A2P | Mimics of key dipeptide motif in Protein A[e,f] (lot no's. FA0415 & FA0485, respectively) | 75–125 μm highly cross-linked 6% agarose base matrix | Prometic Biosciences Ltd., Cambridge, UK |
| Kaptiv-GY | 'Peptidomimetic'-branched synthetic peptide of 15 amino acids[g] (lot no. 0005021150, 8–10 mg mL$^{-1}$) | 50–80 μm bis-acrylamide/azalactone copolymer beaded matrix | Tecnogen, Piana di Monte Verna (CE), Italy |
| FastMabsA | A 'mixed mode' ligand[h] (lot no. XAES-A12, 73 μmol g$^{-1}$) | 80–250 μm highly cross-linked agarose base matrix | UpFront Chromatography A/S, Copenhagen, Denmark |

[a] Engineered to include C-terminal cysteine allowing oriented coupling via thioether linkage through a stable 12-atom epoxide spacer arm to the base matrix (ensuring low ligand leakage).
[b] Rigid agarose support cross-linked with new technique (not disclosed) to allow high flow rates under process conditions.
[c] Engineering of rProtein A, the ligand density and coupling chemistry employed are proprietary to the producer. The stereochemistry of rProtein A immobilisation is designed to maximise antibody binding.
[d] Binding relies on salt-independent hydrophobic interaction, whereas elution is mediated by pH induced electrostatic repulsion, hence the term 'hydrophobic charge induction' [10,19–22].
[e] Ligands (structures not disclosed) comprise a triazine scaffold with two spatially oriented substituents that mimic the helical twist of the key dipeptide in Protein A [14].
[f] The ligand density is proprietary to the producer.
[g] Found by screening a peptide library by measuring ability to interfere with interaction of Protein A and biotinylated immunoglobulins [13].
[h] Low molecular weight synthetic ligand (structure not disclosed) having hydrophilic and hydrophobic functionalities within the same molecule [12].

for chemoselective oriented immobilisation to the base matrix through stable linkage chemistries [18].

Here, eight commercial adsorbents specifically intended for IgG purification were directly compared, for their ability to recover polyclonal antibodies from clarified rabbit antisera feedstocks of varying strength. Three of these materials were new improved rProtein A-linked chromatographic matrices, and the remainder comprised five low molecular synthetic ligand-based adsorbents (for descriptions of the ligands employed in this work the reader is referred to Table 1). The principal criteria evaluated in this work, of dynamic immunoglobulin (Ig) binding capacity, recovery and purity, provided the framework for selection of adsorbents well-suited to the task of antibody purification from complex antiserum feedstocks.

## 2. Materials and methods

### 2.1. Materials

The chromatography feedstocks (i.e. rabbit anti-human transferrin antiserum) and goat anti-rabbit serum used in the preparation of gels for crossed immunoelectrophoresis, were produced internally at DakoCytomation A/S (Glostrup, Denmark) following published procedures [23]. Pure rabbit albumin was prepared in house at DakoCytomation A/S using proprietary procedures, while rabbit Ig was available as a commercial product (X0903 concentrate and commercial product strength 20 g L$^{-1}$) at DakoCytomation A/S. The various chromatographic media employed, i.e.: MabSelect$^{TM}$ and rProtein A Sepharose FF (GE Healthcare, Amersham Biosciences, Uppsala, Sweden); ProSep rProtein A (Millipore, Billerica, MA, USA); MEP HyperCel (Pall Life Sciences, BioSepra SA, Cergy-Saint-Christophe, France); MabSorbent A1P and MabSorbent A2P (Prometic BioSciences Ltd., Cambridge, UK); FastMabsA (UpFront Chromatography, Copenhagen, Denmark); and Kaptiv-GY$^{TM}$ (Tecnogen, Piana di Monte Verna (CE), Italy), were obtained as gifts from the manufacturers (see Table 1 for detailed descriptions). High performance thin layer chromatography (HP-TLC) plates (Silica gel 60) and HAS 1000 protein grade agarose were acquired from Merck (Darmstadt, Germany) and Medinova Scientific A/S (Hellerup, Denmark), respectively. The staining reagents used on crossed immunoelectrophoretograms, i.e. Coomassie Brilliant Blue R250, Sudan Black and zinc acetate, were supplied by Kem-En-Tech (Copenhagen, Denmark), Sigma (St. Louis, MO, USA) and Merck (Whitehouse Station, NJ, USA), respectively, and Primuline stain for HP-TLC was purchased from Sigma (St. Louis, MO, USA). All reagents for reducing SDS–PAGE (NuPage® 10-well pre-cast '4–12%' polyacrylamide gradient gels, Mark 12$^{TM}$ molecular weight marker, Simply Blue$^{TM}$ Safe Stain, Novex Gel-Dry Solution, NuPage$^{TM}$ sample and MOPS running buffer systems) were acquired from Invitrogen (Carlsbad, CA, USA). All other chemicals were of AnalaR or equivalent grade, and were obtained from registered suppliers.

### 2.2. Preparation of feedstocks for packed bed chromatography experiments

The chromatographic feedstock used in all chromatography experiments detailed within this paper, was a rabbit anti human

transferrin anti-serum that received no conditioning other than clarification and dilution. Two hundred millilitre lots of crude rabbit antiserum pools from 'Danish Whites' were first filtered free of particulate matter by passage through a Nalgene disposable dead end membrane 0.45 μm filters (VWR International, Buffalo Grove, IL, USA) to yield clarified undiluted sera (hereafter designated '100% serum strength'). The mean Ig concentration from seven different batches of this feedstock was unusually high ($21.2 \pm 1.1$ mg mL$^{-1}$ cf. typically quoted value of 13.6 mg mL$^{-1}$ for IgG [24]). In some experiments these feedstocks were applied directly onto equilibrated packed beds, but in most cases they were diluted 2, 3, 5, 6 or 9-folds with the appropriate column equilibration buffer (see Table 2) to yield feeds with serum strengths of 50%, 33.3%, 20%, 16.7% or 11.1%, respectively.

### 2.3. Chromatography

All chromatography was performed at ambient temperature (~21 °C unless stated otherwise) employing an Äkta Explorer 100 system equipped with flow-through pH and conductivity probes, and a fraction collector (GE Healthcare, Uppsala, Sweden). Approximately 5 mL packed beds of chromatographic media contained in 1 cm internal diameter columns (HR10/10, GE Healthcare) were operated under downward flow during packing, using flow rates similar to those recommended by the manufacturer of each matrix (Table 2). The flow adapters were then lowered onto the gels before copiously washing the beds with the appropriate equilibration buffer (see Table 2) until the UV absorbance, conductivity, and pH of the liquid exiting the column reached that of the incoming equilibration buffer. The flow adaptors were then re-lowered onto the packed beds to give final bed heights of between 5.5 and 7.2 cm. The buffer systems employed (Table 2) for media equilibration, product elution, and column regeneration of packed beds of the non-Protein A-based media were those suggested by the individual media manufacturers. In the absence of clear recommendations from the maker's on buffer systems to use for any of the Protein A-based matrices (i.e. MabSelect$^{TM}$, rProtein A Sepharose FF and ProSep rProtein A), the following simple buffers were selected: 25 mM potassium phosphate, pH 7.0 for equilibration and washing; and 0.1 M sodium citrate, pH 3.5 for elution. The first rProtein A-based matrix evaluated in this study was rProtein A Sepharose FF and the purpose of the first chromatographic run with this material (Table 3) was to identify a suitable pH for efficient stepwise elution of captured Igs from rProtein A-linked supports. Following loading of 20% (v/v) antiserum and chasing unbound material out of the column, the bed was developed with a falling pH gradient, i.e. from pH 7.0 to 3.0 (formed with the buffers 25 mM potassium phosphate, pH 7.0 and 100 mM sodium citrate, pH 3.0), over 20 CVs. In general accordance with the finding of others [7] rabbit Igs were desorbed between pH 5.5 and 3.5 in ~4.5 CV (results not shown). The binding affinity of rabbit IgG for Protein A is equally as strong as that shown by human $IgG_1$, $IgG_2$, and $IgG_4$ [8,24–26], and these IgG species are eluted from Protein A-linked adsorbents over

Table 2
Packing and chromatographic conditions[a] used for the matrices under test

| Adsorbent | Packing/ equilibration (cm h$^{-1}$) | Bed volume (mL) | Equilibration, loading[b] and washing buffer | Elution buffer | Regeneration solution |
|---|---|---|---|---|---|
| rProtein A Sepharose FF | 382/229 | 4.6 | 25 mM K-phosphate, pH 7 | 100 mM Na-citrate, pH 3.5 | 100 mM Na-citrate, pH 2.5 |
| MabSelect | 611/229 | 5.1 and 5.2[c] | 25 mM K-phosphate, pH 7 or 25 mM K-phosphate, 150 mM NaCl, pH 7.4[d] | 100 mM Na-citrate, pH 3.5 | 100 mM Na-citrate, pH 2.5, or 10 mM NaOH[e] |
| ProSep rProtein A | 611/229 or 316[f] | 4.8 | 25 mM K-phosphate, pH 7 | 100 mM Na-citrate, pH 3.5 | 100 mM Na-citrate, pH 2.5 |
| MEP HyperCel | Settling[g]/229 | 5.7 | 50 mM Tris–HCl, pH 8 | 50 mM Na-acetate, pH 4 | 1 M NaOH |
| MabSorbent A1P | Settling[g]/153 | 5.0 | 25 mM K-phosphate, pH 7 | 10 mM Na-citrate, pH 3 | 0.5 M NaOH |
| MabSorbent A2P | 309/153 | 4.3 | 25 mM K-phosphate, pH 7 | 50 mM Na-citrate, pH 3 | 0.5 M NaOH |
| FastMabs | Settling[g]/229 | 4.7 | 10 mM Na-citrate, pH 5.7 | 25 mM K-phosphate, 1 M NaCl, pH 6.5, or 10 mM NaOH | 1 M NaOH |
| Kaptiv-GY | 229/76 | 5.2 | 65 mM bis-Tris–HCl, pH 6.5 | 100 mM acetic acid | (i) 0.1 M acetic acid, 0.5 M NaCl, pH 4; ⇒ (ii) 0.1 M Tris–HCl, 0.5 M NaCl, pH 8 |

The flow rates employed at all other stages (wash, elution, regeneration and test of theoretical plates) were the same as those employed for equilibration of the beds.
[a] Unless indicated otherwise, separations were conducted at ~21 °C.
[b] In all cases loading was performed at 38 cm h$^{-1}$, ensuring an on-column time of 8.6–11.5 min.
[c] Employed in experiments conducted at 4–8 °C.
[d] For run 3 (at 4–8 °C; Table 3) only.
[e] The harsher cleaning regime was employed in experiments performed at 4–8 °C (see Table 3).
[f] For run 5 (Table 3) only.
[g] Beds were first allowed to form by settling, before being packed down further at the equilibration flow rate.

Table 3
Combined influence of feedstock complexity and number of cycles on the chromatographic purification of rabbit immunoglobulins on packed beds of rProtein A-based matrices operated under conditions presented in Table 2

| Adsorbent | Run | Feedstock antiserum strength (%, v/v) | Feedstock conductivity ($mS\,cm^{-1}$) | Feedstock applied (mL) | Equivalents of '100% antiserum' applied ($mL\,mL^{-1}$ bed) | Ig applied ($mg\,mL^{-1}$ bed) | Ig bound[a] ($mg\,mL^{-1}$ bed) | Ig eluted ($mg\,mL^{-1}$ bed) | Ig elution efficiency[b] (%) | Eluted Ig concn ($mg\,mL^{-1}$) | Fold volume expansion[c] | Ig Purity[d] (%) | Ig mass balance (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| rProtein A Sepharose FF | 1[e] | 20 | 5.5 | 45 | 2.0 | 42.2 | 39.6 | 35.7 | 90 | 8.2 | 2.6 | 110 | 90 |
| | 2 | 33.3 | 6.4 | 50 | 3.6 | 73.0 | 47.0 | 29.1 | 62 | 8.4 | 2.6 | 104 | 75 |
| | 3 | 50 | 7.5 | 26 | 2.8 | 62.6 | 50.3 | 49.8 | 99 | 14.3 | 1.5 | 116 | 100 |
| MabSelect | 1 | 20 | 5.5 | 50 | 2.0 | 39.2 | 32.5 | 20.8 | 64 | 13.3 | 1.6 | 90 | 70 |
| | 2 | 33.3 | 6.4 | 38 | 2.5 | 52.4 | 35.1 | 28.4 | 81 | 18.1 | 1.2 | 106 | 87 |
| | 3 | 50 | 7.5 | 22 | 2.2 | 46.5 | 39.8 | 27.8 | 70 | 11.8 | 1.8 | 91 | 74 |
| | 4 | 100 | 10.8 | 15 | 2.9 | 59.6 | 35.3 | 31.4 | 89 | 13.3 | 1.6 | 102 | 93 |
| | 5 | 100 | 10.8 | 5 | 1.0 | 20.4 | 19.9 | 15.9 | 80 | 11.6 | 1.9 | 81 | 80 |
| MabSelect @ 4–8 °C | 1 | 50 | 7.4 | 22 | 2.1 | 46.7 | 38.0 | 34.2 | 90 | 12.7 | 1.7 | 99 | 92 |
| | 2 | 50 | 7.4 | 2 | 0.2 | 4.4 | 4.3 | 3.3 | 76 | 2.1 | 10.3 | 98 | 77 |
| | 3[f] | 50 | 13.8 | 35 | 3.4 | 73.5 | 34.9 | 30.4 | 87 | 13.2 | 1.6 | 95 | 94 |
| | 4[g] | 50 | 7.5 | 22 | 2.1 | 45.2 | 37.3 | 29.8 | 80 | 12.9 | 1.7 | 96 | 83 |
| | 5[g] | 100 | 10.8 | 5 | 1.0 | 21.0 | 19.2 | 17.1 | 89 | 8.9 | 2.4 | 86 | 89 |
| | 6 | 100 | 10.8 | 15 | 2.9 | 60.8 | 35.4 | 31.2 | 88 | 11.6 | 1.9 | 86 | 93 |
| | 7 | 50 | 7.5 | 22 | 2.1 | 40.0 | 30.6 | 32.7 | 107 | 10.6 | 2.0 | 90 | 105 |
| ProSep rProtein A | 1 | 20 | 4.8 | 42 | 1.8 | 40.6 | 32.1 | 30.2 | 94 | 12.1 | 1.8 | 79 | 95 |
| | 2 | 33.3 | 6.4 | 27 | 1.9 | 45.8 | 31.9 | 28.8 | 90 | 11.5 | 1.9 | 72 | 93 |
| | 3 | 50 | 7.5 | 18 | 1.9 | 37.5 | 26.8 | 26.0 | 97 | 10.4 | 2.1 | 63 | 98 |
| | 4 | 100 | 10.8 | 10 | 2.1 | 42.5 | 26.1 | 26.7 | 102 | 10.7 | 2.0 | 75 | 102 |
| | 5[h,i] | 100 | 10.8 | 1 | 0.2 | 4.2 | *ND* | 2.9 | *ND* | 1.8 | 12.0 | 88 | ND |
| | 6[h] | 100 | 10.8 | 1 | 0.2 | 4.2 | *ND* | 2.9 | *ND* | 1.8 | 12.0 | 88 | ND |

[a] After washing and immediately prior to elution.
[b] Elution efficiency $= [(\sum \mathrm{Ig}_{\mathrm{eluted}})/(\sum \mathrm{Ig}_{\mathrm{loaded}} - \sum \mathrm{Ig}_{\mathrm{wash\ fractions}} - \sum \mathrm{Ig}_{\mathrm{run\ through}})]100\%$.
[c] Relative to original '100% antiserum' with an Ig concentration of $21.6 \pm 1.3\,g\,L^{-1}$ ($n = 37$).
[d] Numerical estimates of Ig purity were obtained from measurements of Ig and protein content as described in Sections 2.5–2.7.
[e] Developed with a pH gradient (starting at pH 7 and ending at pH 3). Complete desorption of rabbit Ig required a pH < 3.8.
[f] For equilibration, loading, and washing 25 mM K-phosphate buffer pH 7.4, supplemented with 150 mM NaCl, was used.
[g] Wash after load was reduced from typically ∼14 to ∼5 CV.
[h] Only the main load and elution fractions were analysed.
[i] Equilibration, washing, elution and regeneration were performed at $306\,cm\,h^{-1}$. ND = not determined.

very similar ranges of pH, with complete desorption normally occurring by pH 3.5 to 3.0 [7]. Thus, in subsequent experiments with all of the rProtein A-based supports, a 0.1 M sodium citrate buffer, pH 3.5 was selected for stepwise elution of bound Igs.

A standard chromatographic run was performed in the following way. Packed beds of chromatography media were thoroughly equilibrated at flow rates between 76 and 316 cm $h^{-1}$, depending on the matrix under test (see Table 2), with an appropriate buffer until the UV absorbance, pH and conductivity of the flow exiting the column matched that entering it (i.e. at least 10 CV). Clarified antisera or serum feedstocks were then loaded directly onto the equilibrated packed beds at a linear flow rate of 38 cm $h^{-1}$ in all cases, which guaranteed an 'on-the-column' residence time of 8.6–11.5 min. On completion of sample loading, the columns were irrigated with ~4 CV (20 mL) of equilibration buffer at the same flow rate (i.e. 38 cm $h^{-1}$) and then a further 10 CV of the same buffer at a higher flow rate (i.e. the same as that used for equilibration; Table 2). In most experiments product elution was performed at the equilibrating flow rate (Table 2) in a stepwise fashion using 5 or 10 CV of a suitable elution buffer.

Finally, columns were cleaned-in-place (CIPed) with 3–10 CV of a recommended sanitising solution (see Table 2) at 38–76 cm $h^{-1}$, washed successively with at least 10 CV of equilibration buffer and 6 CV of 20% (v/v) ethanol. Columns were stored in the latter solution until required at either 4–8 °C or ~21 °C, depending on temperature under which the column was to be operated. During CIP upward flow was used. At all other chromatographic stages downward flow was applied. Flow exiting columns was monitored continuously by on-line measurement of UV absorbance, pH and conductivity, and collected fractions (1–4 mL) were assayed for antibody, soluble protein, and lipoprotein contents (Sections 2.5, 2.6 and 2.8, respectively).

For dynamic breakthrough studies and for studying the impact of the feedstock on chromatographic performance/robustness, the experimental approach taken was to sequentially challenge packed beds with a series of different strengths (11–100%, v/v) of a clarified rabbit antiserum; beginning with the weakest and ending with two runs of the strongest. In most cases the beds were 'overloaded' (i.e. well beyond the point of breakthrough) with antiserum; the total amount applied per millilitre of bed in each set of tests was kept relatively constant (i.e. at ~60 ± 10 mg Ig $mL^{-1}$ for beds of Mabselect and rProtein A Sepharose FF; ~40 ± 4 mg Ig $mL^{-1}$ of Prosep Protein A, MEP HyperCel, MabSorbent A1P, MabSorbent A2P and FastMabsA; ~28 ± 6 mg Ig $mL^{-1}$ of Kaptiv-GY and FastMabsA). In these tests dynamic binding capacities ($DBC_{0.5\,g\,L^{-1}}$) were determined from generated breakthrough curves at the point where the Ig concentration at the column outlet reached 0.5 g $L^{-1}$. The final run in each series was conducted with the same feed as that used in the penultimate test (i.e. the highest strength feed), but with a ~2 to 10-fold reduced challenge depending on the matrix under evaluation. After washing and eluting bound Ig the adsorbents were CIPed as described above, and then probed for signs of deterioration in bed quality (Section 2.4) before carrying out the next run. Four of the eight matrices (MabSelect™, ProSep rProtein A, MEP HyperCel, MabSorbent A1P) under test were challenged consecutively with rabbit antisera of 20%, 33%, 50% and 100% strength. The remaining four adsorbents (rProtein A Sepharose FF, MabSorbent A2P, FastMabsA and Kaptiv-GY™) were subjected to fewer (2–3) and less testing (11–50% serum strength) runs.

### 2.4. Residence time distribution

At all stages (i.e. after packing, prior to and after every chromatographic run) packed columns were inspected for possible deterioration in bed quality using residence time distribution (RTD) analysis. For this, small pulses of a low molecular weight tracer (either 50 μL of 5% (v/v) acetone, 50 μL of 0.5 M NaCl, or 100 μL 0.5 M sodium citrate pH 5.7) were injected on to the column under test and the tracer concentration in the column effluent was monitored by measuring either its UV absorbance (at 280 nm for acetone tracers) or conductivity (for NaCl and sodium citrate). A minimum of three such measurements was performed in each test. In this work the number of theoretical plates, $N$, and the 'height equivalent to a theoretical plate', $H$, were calculated from Eqs. (1) and (2) [27,28].

$$N = 5.54\left(\frac{V_R}{w_{1/2}}\right)^2 \quad (1)$$

$$H = \frac{L}{N} \quad (2)$$

where $V_R$ is the retention time, $w_{1/2}$ is the recorded peak width at half of its height, $L$ is the height of the packed bed.

The Unicorn software of the Äkta explorer 100 allowed calculation of $V_R$, $w_{1/2}$ and also another useful parameter known as the symmetry factor, $A_s$ Eq. (3)

$$A_s = \frac{b}{a} \quad (3)$$

where $a$ is the 1st half peak width at 10% of its height and $b$ is the second half peak width at 10% of its height. $A_s$ should be as close to 1 as possible, but for short columns acceptable values of $A_s$ are 0.8 to 1.8 [28]. Tubing dead volumes were compensated for in calculation of $V_R$.

### 2.5. Immunoturbidimetric determination of rabbit immunoglobulin content

The development of the robust assay for rabbit immunoglobin applied in this study has been described in detail elsewhere [29] and is only briefly outlined here. 'Purified immunoglobulin' (X0903, DakoCytomation A/S, Glostrup, Denmark) from non-immunised rabbits was diluted with 'S2005 buffer' (DakoCytomation A/S) to generate a series of standards with concentrations between 6.6 and 500 mg $L^{-1}$, and samples were appropriately diluted with the same buffer so as to lie within this range. Duplicate portions (50 μL) of diluted samples and standards were pipetted into the wells of a microtitre plate (96-well

Polysorb, Nalgene Nunc Int., Rochester, NY, USA), followed by 90 μL aliquots of 'S2008 reaction buffer' (DakoCytomation A/S). After 5 s of brief mixing, the plate was incubated for ∼300 s at 30 °C in a pre-heated $Thermo_{max}$ or Spectra Max 250 microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA) and then read at 340 nm. Next, 210 μL aliquots of an antibody mixture consisting of 2-fold diluted goat anti-rabbit immunoglobulins (Z0421, DakoCytomation A/S) mixed in a 4:3 ratio with S2008 reaction buffer, were added rapidly to each well with a BioHit Proline 12-channel automated pipette (50–1200 μL, Helsinki, Finland). After incubating at 30 °C for 300 s the plates were re-read at 340 nm.

### 2.6. Estimation of protein content

In some early chromatographic studies, protein content was determined by refractometry. However, in most cases the protein content in samples was estimated by spectrophotometric analysis at 280 nm in an UltroSpec III UV–vis Spectrophotometer (GE Healthcare, Uppsala, Sweden) assuming an extinction given by a 1% (w/v) solution of a pure protein when the light path is 1 cm ($E^{1\%}_{1\,cm}$) to be 13.8 for 'highly pure eluates' or 10 for samples of lesser purity (i.e. feedstocks and 'dirty eluates'). Pure human IgG, albumin and transferrin have $E^{1\%}_{1\,cm}$ values at 280 nm of 13.8, 5.8 and 11.2, respectively [30].

### 2.7. Numerical estimation of Ig purity

Numerical estimates of Ig purity in eluted fractions were simply obtained by dividing the immmunoglobulin content of the fractions by their total protein contents.

### 2.8. Determination of cholesterol content

Analysis of the cholesterol concentration in selected samples was used to provide an indirect measure of their lipoprotein content. Samples (900 μL) were extracted with 15 mL of a chloroform/methanol (2:1) mixture at 4 °C for 0.5 h, before adding 1.5 mL portions of a 0.8% (w/v) potassium chloride solution. After mixing overnight at 4 °C, the lower phases were transferred to clean glass tubes, dried under nitrogen, and then dissolved in 100 μL portions of chloroform/methanol (1:1). Nine microlitre aliquots of these extracts were applied to Silica gel 60 HPTLC-plates using a Desaga AS-30 HP-TLC-applicator (Desaga, Wiesloch, Germany). Cholesterol standards (either 50 ng and 500 ng for semi-quantitative analysis or six standards ranging from 100 to 3000 ng for quantitative purposes) were applied to the same plates, which were subsequently developed in a Camag Horizontal Development Chamber (Muttenz, Switzerland), using chloroform/methanol/ acetic acid (190:9:1). For semi-quantitative analysis, plates were dipped in heptane/isopropanol/acetic acid (95:5:1), sprayed with Primuline reagent, and then viewed under a short wavelength (205 nm) UV lamp. For quantitation of cholesterol content, dried plates were first soaked with an aqueous solution of 15.6% (w/v) copper sulphate in 8% (w/v) phosphoric acid, then air-dried and charred at 170 °C for ∼480 s. The HP-TLC plates were then scanned at 420 nm in a CD-60 HP-TLC densitometer equipped with ProQuant software (both from Desaga, Wiesloch, Germany).

### 2.9. Crossed immunoelectrophoresis

Crossed immunoelectrophoresis was used both qualitatively (i.e. for visual estimations of purity and for detection of contaminating lipoprotein in samples) and semi-quantitatively (e.g. for determining the relative abundance of a principle contaminant, namely albumin), and was performed essentially as described by Høiby and Axelsen [31] using the 'macro' technique. In the first dimension ∼100 μg of total protein was applied to a 10 cm × 10 cm glass plate with 15 mL of 1% (w/v) agarose gel (made up in a proprietary running buffer, pH 8.3–8.9). The gel was then electrophoresed at 10–15 V $cm^{-1}$ for typically 0.75 h using the running buffer. The 2nd dimension of the gel contained 12.5 μL $cm^{-2}$ of a goat anti-rabbit serum immunoglobulin fraction purified by the method of Harboe and Ingild [23] from goats immunised with rabbit serum. Electrophoresis in the 2nd dimension was conducted for at least 16 h at a constant voltage of ∼2 V $cm^{-1}$. Following electrophoresis, gels were subjected to the following sequential operations: (i) pressing between two sheets of Whatman filter paper; (ii) drying at 70 °C; (iii) washed twice with 0.1 M NaCl and then once with deionised water (for 600 s each); (iv) drying once again; (v) and finally staining for either protein or lipoprotein. Protein staining was performed by immersion for at least 0.25 h at room temperature in a solution composed of 0.75% (w/v) Coomassie Brilliant Blue R250 in acetic acid/ethanol. Gels were de-stained in acetic acid/ethanol (3 cycles of 600 s each), before drying at 70 °C. Lipoprotein was stained in a similar manner using an aqueous solution of 2 g $L^{-1}$ Sudan Black and 20 g $L^{-1}$ zinc acetate. After 24–48 h the gels were de-stained in 70–90% (v/v) ethanol and then dried at 70 °C. Digital images of crossed immunoelectrophoretograms were obtained by scanning gels into a PC with the aid of a 1200 × 1200 dpi optical resolution Canon CanScan D660U flat bed scanner (Canon Inc., Tokyo, Japan) or 1200 × 1200 dpi optical resolution Hewlett-Packard Scanjet 3500c flatbed scanner (Hewlett-Packard, Palo Alto, CA, USA). In some cases the ratio of the albumin to Ig peak areas was calculated in the following way. Images of Coomassie Blue stained crossed immunoelectrophoresis plates were blown up and printed out in triplicate. The Ig and albumin peaks from each were then cut out, weighed on an analytical balance (model LA230S, Sartorius Instruments, Göttingen, Germany) and ratio-ed.

### 2.10. SDS–PAGE

Protein compositions in samples were also analysed by reducing SDS–PAGE [32]. Electrophoresis was performed on 20–25 μL samples in Bis–Tris 4–12% (w/v) polyacrylamide gels with a MOPS running buffer, exactly as recommended by the manufacturers. Protein bands were visualised by staining with Simply Blue™ Safe Stain.

Table 4
Combined influence of feedstock complexity and number of cycles on the chromatographic purification of rabbit immunoglobulins on packed beds of low molecular weight synthetic ligand-based matrices operated under conditions presented in Table 2

| Adsorbent | Run | Feedstock antiserum strength (% v/v) | Feedstock conductivity (mS $cm^{-1}$) | Feedstock applied (mL) | Equivalents of '100% antiserum' applied (mL $mL^{-1}$ bed) | Ig applied (mg $mL^{-1}$ bed) | Ig bound[a] (mg $mL^{-1}$ bed) | Ig eluted (mg $mL^{-1}$ bed) | Ig elution efficiency[b] (%) | Eluted Ig concn (mg $mL^{-1}$) | Fold volume expansion[c] | Ig purity[d] (%) | Ig mass balance (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MEP HyperCel | 1[e] | 20 | 6.2 | 48 | 1.7 | 37.9 | 22.7 | 20.9 | 92 | 6.0 | 3.6 | 86 | 95 |
| | 2[e] | 33.3 | 6.9 | 29 | 1.7 | 37.9 | 21.4 | 17.4 | 81 | 5.0 | 4.3 | 86 | 89 |
| | 3[e] | 50 | 7.9 | 19 | 1.7 | 37.5 | 19.2 | 16.0 | 83 | 4.6 | 4.7 | 88 | 91 |
| | 4 | 100 | 10.8 | 10 | 1.8 | 39.6 | 26.7 | 18.4 | 69 | 5.3 | 4.1 | 86 | 79 |
| | 5 | 100 | 10.8 | 5 | 0.9 | 19.1 | 15.7 | 8.8 | 56 | 3.1 | 7.0 | 68 | 63 |
| MabSorbent A1P | 1 | 20 | 5.5 | 46 | 1.8 | 40.2 | 8.6 | 9.0 | 105 | 3.8 | 5.7 | 72 | 101 |
| | 2 | 33.3 | 6.3 | 28 | 1.9 | 41.0 | 8.8 | 8.8 | 100 | 2.8 | 7.7 | 68 | 100 |
| | 3 | 50 | 7.5 | 18 | 1.8 | 39.0 | 10.1 | 8.4 | 83 | 2.6 | 8.3 | 68 | 95 |
| | 4 | 100 | 10.8 | 10 | 2.0 | 39.8 | 11.4 | 7.2 | 63 | 1.8 | 12.0 | 65 | 89 |
| | 5 | 100 | 10.8 | 1.5 | 0.3 | 6.4 | 4.0 | 4.0 | 99 | 1.3 | 16.6 | 83 | 99 |
| MabSorbent A2P | 1 | 20 | 5.5 | 32 | 1.5 | 36.5 | 14.0 | 14.2 | 101 | 3.1 | 7.0 | 55 | 100 |
| | 2 | 33.3 | 6.4 | 27 | 2.1 | 47.9 | 14.0 | 14.0 | 100 | 3.0 | 7.2 | 55 | 99 |
| FastMabsA | 1[f] | 20 | 4.2 | 45 | 1.7 | 44.3 | 40.6 | 26.4 | 65 | 2.3 | 9.4 | ND | 68 |
| | 2[g] | 20 | 4.1 | 155 | 6.6 | 141.7 | 31.7 | 26.6 | 84 | 3.9 | 5.5 | 31 | 96 |
| Kaptiv-GY | 1 | 11.1 | 1.9 | 50 | 1.1 | 22.3 | 5.1 | 3.5 | 68 | 2.3 | 9.4 | 25 | 92 |
| | 2[h] | 16.6 | 2.3 | 50 | 1.6 | 34.4 | *ND* | 4.0 | *ND* | 2.6 | 8.3 | 38 | ND |

[a] After washing and immediately prior to elution.
[b] Elution efficiency $= [(\sum \mathrm{Ig}_{\mathrm{eluted}})/(\sum \mathrm{Ig}_{\mathrm{loaded}} - \sum \mathrm{Ig}_{\mathrm{wash\ fractions}} - \sum \mathrm{Ig}_{\mathrm{run\ through}})]100\%$.
[c] Relative to original '100% antiserum' with an Ig concentration of $21.6 \pm 1.3\ \mathrm{g\ L^{-1}}$ ($n = 37$).
[d] Numerical estimates of Ig purity were obtained from measurements of Ig and protein content as described in Sections 2.5–2.7.
[e] ~14 CV wash step was followed with 5 CV of MilliQ water prior to elution.
[f] Elution performed with 10 mM NaOH.
[g] Elution performed with 25 mM K-phosphate, 1 M NaCl, pH 6.5.
[h] Only the main load and elution fractions were analysed; ND = not determined.

## 3. Results and discussion

### 3.1. Experimental approach

In ascertaining the suitability of various commercial rProtein A and low molecular weight ligand-based media (Table 1) for the task of recovering Ig directly out of clarified rabbit antiserum, the general experimental approach we have undertaken here has been to consecutively challenge fixed beds of each adsorbent with a series of rabbit antisera feedstocks of increasing difficulty (i.e. combined increases in antiserum strength and ionic strength), and study the impact of these treatments on: (i) Ig dynamic binding capacity; (ii) bed integrity; (iii) Ig elution efficiency; and (iv) purity. The conditions selected for packing and operation of fixed beds of these materials are shown in Table 2, and the results from 37 chromatographic runs conducted with these, and clarified rabbit antiserum feedstocks, are summarised in Tables 3 and 4, and Figs. 1–5.

In most cases chromatographic beds were heavily 'overloaded' with immunoglobulins (i.e. well past the point of breakthrough), which allowed breakthrough curves to be generated and the dynamic binding capacities for Ig to be calculated. After washing and eluting adsorbed Igs, the matrices were CIPed, and then subjected to RTD analysis. The final run in each series was typically performed with a markedly reduced volume (i.e. not loaded to breakthrough) of the same feed as that employed in the penultimate 'overloaded' run, and will hereafter be referred to as 'underloaded' runs. Half of the matrices (MabSelect, ProSep rProtein A, MEP HyperCel and Mabsorbent A1P) under trial were subjected to the following five-run series of antiserum feedstocks: 20% ⇒ 33.3% ⇒ 50% ⇒ 100% ⇒ 100% (v/v) strength. The remaining adsorbents (rProtein A Sepharose FF, MabSorbent A2P, FastMabsA and Kaptiv-GY) experienced fewer (i.e. 2–3) and generally less taxing (11–50% antiserum) runs.

### 3.2. General observations

Examples of typical chromatograms obtained from runs performed at room temperature are presented in Fig. 1a and b, for representatives of the rProtein A and low molecular weight synthetic ligand-based groups of supports, respectively. Although the ligands on the latter materials are very different from one another, a common feature observed in all of their chromatograms was the presence of a sizeable stripping peak during the regeneration phase (discussed later in Section 3.5). Such peaks were absent from separations performed at ambient temperature on the agarose-based rProtein media and were very small with the controlled pore glass rProtein A matrix. That said, in low temperature runs performed with MabSelect, the use of a harsher cleaning regime (i.e. 10 mM sodium hydroxide cf. 100 mM sodium citrate, pH 2.5) yielded tiny strip peaks. Additional generalisations concerning Ig separations in this work that can be gleaned from inspection of Fig. 1a and b are that as a group the rProtein A-linked materials yielded eluates of significantly higher Ig concentration and purity, than their low molecular weight synthetic ligand-based counterparts.

### 3.3. Dynamic Ig binding capacity

As alluded to earlier, the front part of 'overloaded' chromatograms enabled breakthrough curves to be constructed. Fig. 2 shows Ig breakthrough profiles corresponding to the 'overloaded' runs documented in Tables 3 and 4, while Fig. 3 summarizes the combined impact of feedstock strength and number of cycles of operation on the dynamic binding capacity ($DBC_{0.5\,g\,L^{-1}}$) at an Ig concentration in the flow exiting the column of $0.5\,g\,L^{-1}$, which corresponds to ∼5 and ∼2.5% Ig breakthrough with 50% (v/v) and 100% (v/v) antiserum feedstocks, respectively.

As a group the rProtein A-based matrices exhibited much higher dynamic capacities than the low molecular weight synthetic ligand support types. The highest $DBC_{0.5\,g\,L^{-1}}$ of

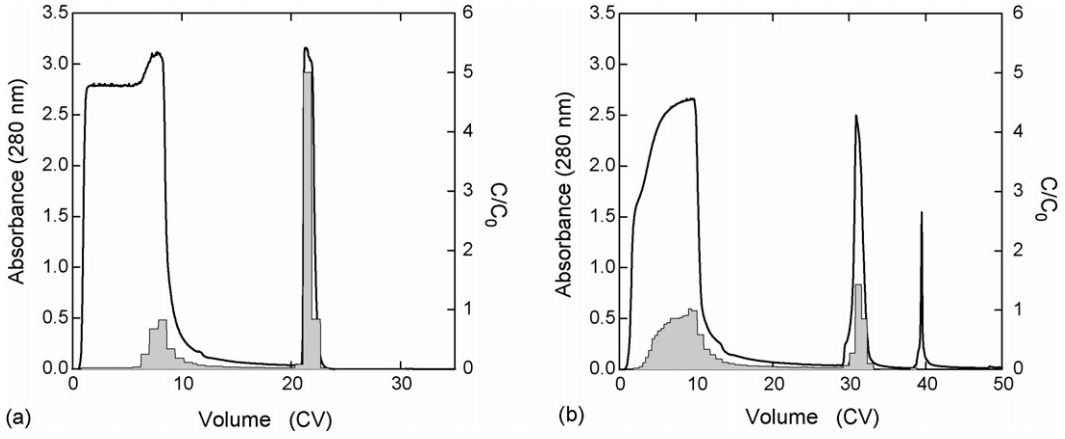


Fig. 1. Typical profiles from 'overloaded' runs performed on 'rProtein A' and 'low molecular weight synthetic ligand'-based media. The examples shown are from separations performed at ∼21 °C on (a) MabSelect (run 2, Table 3) and (b) MabSorbent A1P (run 1, Table 4). Fractions collected during loading, washing, and elution were collected and analysed off-line for Ig and protein contents as detailed in Sections 2.5 and 2.6, respectively. The solid lines represent the $UV_{280\,nm}$ traces, and the shaded histograms indicate the normalised Ig concentrations, expressed as the ratio $C/C_0$, where $C$ is the concentration in each fraction and $C_0$ is that of the feedstock applied to the columns. Strip peak fractions were not assayed for Ig content.

>50 mg mL$^{-1}$ of adsorbent was determined in run 3 on rProtein A Sepharose FF (Fig. 3), which was conducted with a 50% (v/v) antiserum feedstock. The other rProtein A–agarose adsorbent employed in this study, MabSelect, features exactly the same ligand as that on rProtein A Sepharose FF, attached at a slightly lower ligand density (5 mg mL$^{-1}$ cf. 6 mg mL$^{-1}$; Table 1) to a newly developed agarose base matrix (Table 1). The lower capacity observed for MabSelect cf. rProtein A Sepharose FF correlates well with its reduced ligand density.

The rProtein A-linked adsorbent possessing the lowest Ig binding capacity was the controlled porous glass-based support, ProSep rProtein A, which recorded a maximum $DBC_{0.5\,g\,L^{-1}}$ of 36 mg mL$^{-1}$ with 20% (v/v) antiserum (Fig. 3). In stark contrast to the agarose-based adsorbents, increasing the antiserum strength of the feedstock from 20% (v/v) in run 1, through 33.3% (v/v) and 50% (v/v) in runs 2 and 3, respectively, to 100% in run 4, was accompanied by a gradual decline in ProSep rProtein A's level of Ig binding to 30 mg mL$^{-1}$ (Fig. 3).

A very different pattern of behaviour was observed with rProtein A Sepharose FF and MabSelect. At ambient temperature the Ig binding capacities for both supports were seen to rise in response to an increase in antiserum strength from 20%

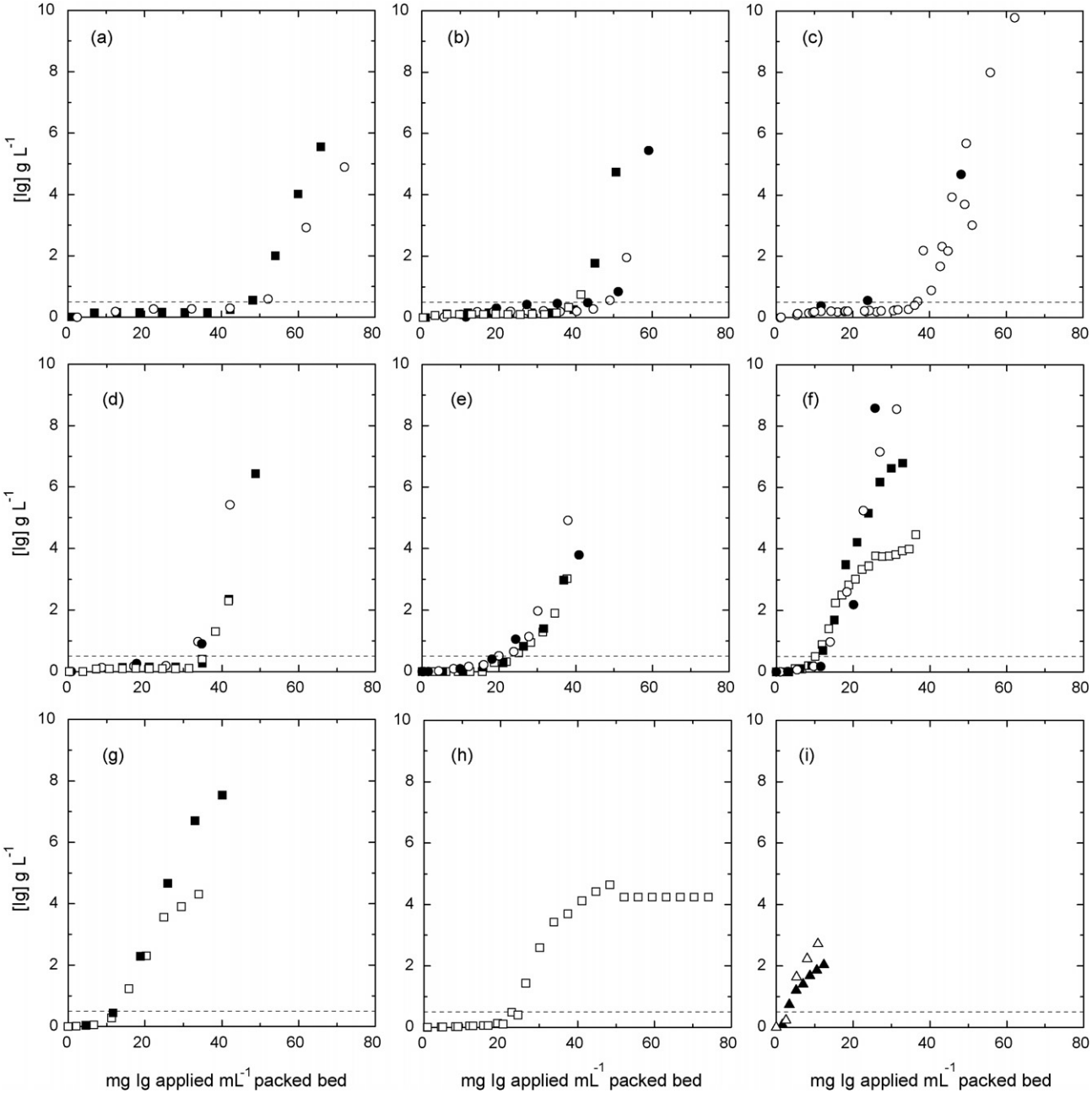


Fig. 2. Effect of antiserum feedstock strength on dynamic binding of Ig to packed beds of: (a) rProtein A Sepharose Fast Flow; (b) MabSelect; (c) MabSelect @ 4–8 °C; (d) ProSep rProtein A; (e) MEP HyperCel; (f) MabSorbent A1P; (g) MabSorbent A2P; (h) FastMabsA; and (i) Kaptiv-GY at a superficial fluid velocity of 38 cm h$^{-1}$. Symbols: (▲) 11.1%; (Δ) 16.7%; (□) 20%; (■) 33.3%; (◯) 50%; (●) 100% (v/v) antiserum in feedstock. The broken horizontal line indicates an Ig concentration at the column outlet of 0.5 g L$^{-1}$. Refer to Tables 3 and 4 for run identification/history.

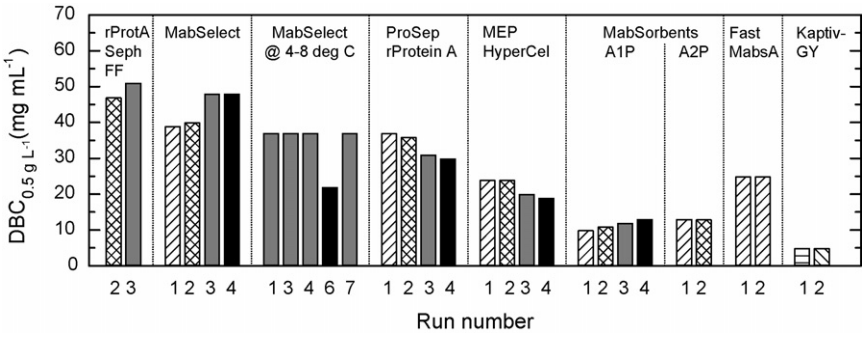


Fig. 3. Combined influence of number of runs and antiserum strength on the Ig dynamic breakthrough capacity ($DBC_{0.5\,g\,L^{-1}}$) of packed beds of rProtein A and low molecular weight ligand-based media at a superficial fluid velocity of 38 cm $h^{-1}$. The first run in each series was performed using a feedstock of low antiserum strength (i.e. 11.1% or 20%, v/v). The antiserum strength was then raised in stepwise fashion for each of the subsequent runs as described in Section 2.3. The feedstock applied in each run is identified by the following key: (▤) 11.1% (1.9 mS $cm^{-1}$); (▧) 16.7% (2.3 mS $cm^{-1}$); (▨) 20% (4.1–6.2 mS $cm^{-1}$); (▩) 33.3% (6.3–6.9 mS $cm^{-1}$); (■) 50% (7.4–7.9 or 13.8* mS $cm^{-1}$); and (■) 100% (10.8 mS $cm^{-1}$) antiserum. The run numbers for each series of tests correspond to those listed in Tables 3 and 4. *Note, the ionic strength in run 3 on MabSelect at 4–8 °C was raised from ~7.5 to 13.8 mS $cm^{-1}$.

(v/v) to 50% (v/v), but a further hike in antiserum strength to 100% (v/v) caused no additional rise in Ig adsorption (Fig. 3), despite the doubling in Ig concentration and increased ionic strength of the latter feedstock (Table 3). The strength and specificity of interaction between immunoglobulins and Protein A is reported to be dependent on ionic strength, where increasing the ionic strength of the feedstock is thought to simultaneously reduce electrostatic repulsion and enhance hydrophobic interaction between the ligand-target pair [7,33]. In this work we have found no evidence for enhanced Ig binding capacity at increased ionic strengths. For example, in a sequence of tests performed with MabSelect and 50% (v/v) antiserum at 4–8 °C, an approximate doubling in feedstock conductivity during run 3 (13.8 cf. 7.4–7.5 mS $cm^{-1}$) yielded an identical $DBC_{0.5\,g\,L^{-1}}$ of 37 mg $mL^{-1}$ to that observed in the preceding and subsequent 'overloaded' runs (Fig. 3). In contrast, the shift from 50% (v/v) to 100% (v/v) antiserum had a profoundly deleterious, but reversible, effect on Ig binding to MabSelect. The $DBC_{0.5\,g\,L^{-1}}$ dropped from 37 mg $mL^{-1}$ in runs 1, 3, and 4 with 50% (v/v) antiserum, to just 22 mg $mL^{-1}$ in run 6 with 'neat' rabbit antiserum, and returned to 37 mg $mL^{-1}$ in the final (7th) run, which again was conducted with 50% (v/v) antiserum. In experiments with 50% (v/v) antiserum, the significant drop in MabSelect's Ig binding capacity from 48 to 37 mg $mL^{-1}$, that

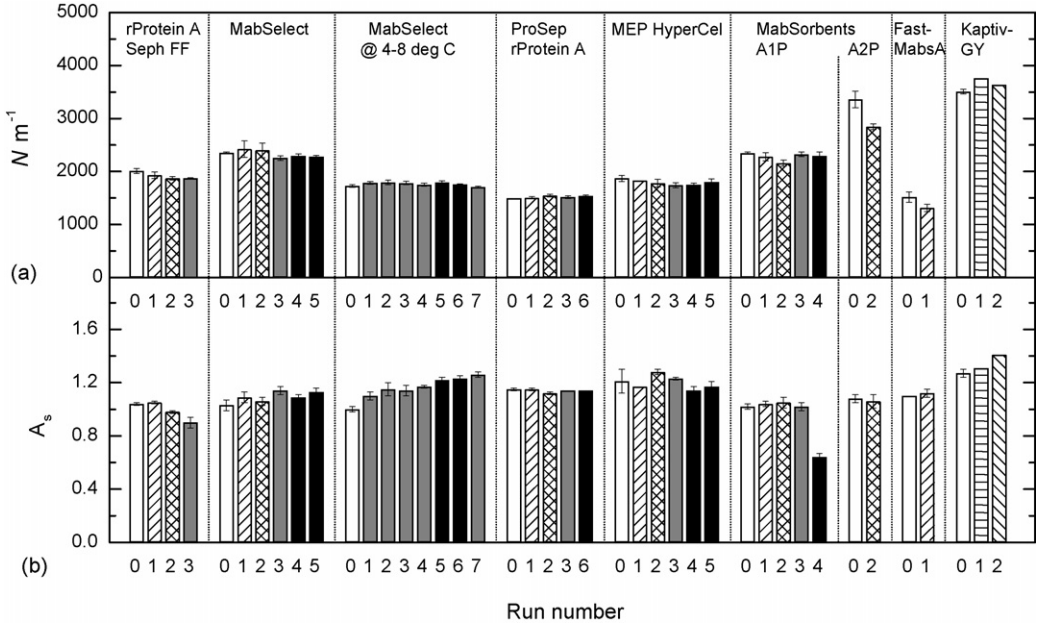


Fig. 4. Combined influence of number of runs and antiserum strength on (a) the number of theoretical plates per metre ($N\,m^{-1}$) and (b) symmetry factor ($A_s$) of packed beds of rProtein A and low molecular weight ligand-based media. After every chromatographic run, packed columns were subjected to a minimum of three residence time distribution (RTD) analyses as described in Section 2.4. The resulting parameters, $N\,m^{-1}$ and $A_s$, provided information on the quality of the bed. The run numbers for each series of tests correspond to those listed in Tables 3 and 4. The feedstock applied in each run is identified by the following key: (□) 0%; (▤) 11.1% (1.9 mS $cm^{-1}$); (▧) 16.7% (2.3 mS $cm^{-1}$); (▨) 20% (4.1–6.2 mS $cm^{-1}$); (▩) 33.3% (6.3–6.9 mS $cm^{-1}$); (■) 50% (7.4–7.9 or 13.8* mS $cm^{-1}$); and (■) 100% (10.8 mS $cm^{-1}$) antiserum. Run '0' indicates previously unused and freshly equilibrated beds. The run numbers for each series of tests correspond to those listed in Tables 3 and 4. *Note, the ionic strength in run 3 on MabSelect at 4–8 °C was raised from ~7.5 to 13.8 mS $cm^{-1}$.

was observed on shifting from ambient to 4–8 °C (Fig. 3), is most likely attributable to reduced mass transfer [34,35] at low temperature combined with an increased tendency for support fouling to occur. It is plausible that the latter effect could, at least in part, account for: (i) MabSelect's further reduction in binding capacity to just 22 mg mL$^{-1}$ on raising the antiserum strength two-fold (i.e. from 50 to 100%, v/v) and (ii) ProSep rProtein A's steady Ig capacity drop with increase in feedstock strength. Support for this assertion comes from the identification of cholesterol, an indirect measure for the presence of lipoproteins, in all strip and elution fractions tested, regardless of the support (see later Section 3.5).

The high level Ig binding exhibited by the rProtein A supports with clarified rabbit antiserum could not be matched by any of the low molecular weight synthetic ligand-based supports, which displayed $DBC_{0.5\,g\,L^{-1}}$ values ranging from a maximum of ~25 mg mL$^{-1}$ for MEP HyperCel and FastMabsA, to as low as 5 mg mL$^{-1}$ (Fig. 3). Only two low molecular weight ligand-based supports, i.e. MEP HyperCel and MabSorbent A1P, were put through a full five run series in which the feedstock strength was raised with every new run (see earlier text comments and Table 4). The remaining adsorbents in this group (MabSorbent A2P, FastMabsA, & Kaptiv-GY) were exposed to lesser challenges on account of their poor overall performance (see later text, Table 4 and Fig. 5) in the first run (Fig. 3) with low strength (11.1 or 20%, v/v) antiserum feeds.

Superficially, MEP HyperCel showed a similar trend, of declining Ig binding capacity with increase in antiserum strength (Fig. 3), to that described previously for ProSep rProtein A. Given the reported salt-independent (up to 1 M NaCl) nature of human polyclonal antibodies binding to MEP HyperCel [10,21], the observed drop in $DBC_{0.5\,g\,L^{-1}}$ from 24 mg mL$^{-1}$ in run 1 with 20% (v/v) antiserum, to 19 mg mL$^{-1}$ in run 4 with neat antiserum, is unlikely to be due to the higher conductivity of the latter feedstock (i.e. 10.8 cf. 6.8 mS cm$^{-1}$). Although no specific data has been published regarding MEP HyperCel's binding capacity towards rabbit antibodies, Schwartz et al. [22] suggested that immunoglobulin binding is independent of species and subclass. Dynamic binding capacities of 32–40 mg mL$^{-1}$ have been reported for the adsorption of pure human polyclonal IgG (HuIgG) [10,21]. These values are at least 33% higher than those reported herein for the adsorption of rabbit polyclonal antibodies from clarified, but otherwise unconditioned, antiserum (run 4, Fig. 3).

MabSorbent A1P's Ig binding capacity showed a very slight tendency to increase (from 10 mg mL$^{-1}$ in the first run to 13 mg mL$^{-1}$ in the fourth) as the antiserum strength of the feedstock was raised in steps from 20% through to 100%, whereas the $DBC_{0.5\,g\,L^{-1}}$ for MabSorbent A2P's remained unchanged at 12 mg mL$^{-1}$ on switching from 20 to 33.3% rabbit antiserum. The ligands for MabSorbents A1P and A2P were both designed to mimic Protein A in its interaction with IgGs [36]. Although human IgG has so far been the subject of most work with MabSorbents, studies with related ligands have shown substantial species differences [37]. Indeed, in a study applying very similar chromatographic conditions to those reported herein, a capacity of up to 27 mg mL$^{-1}$ was reported using neat ovine serum as the feedstock material [38].

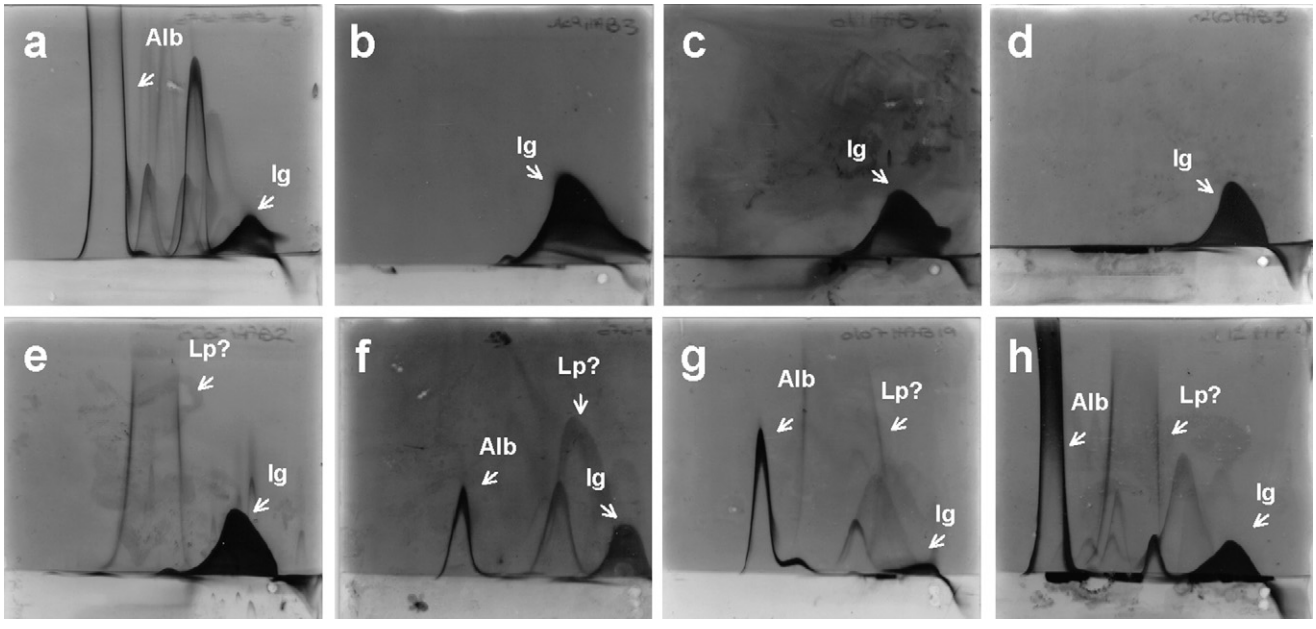


Fig. 5. Crossed immunoelectrophoretic analysis of (a) rabbit antiserum, and eluates from packed beds of (b) rProtein A Sepharose FF (run 1), (c) MabSelect (run 4), (d) MabSelect (4–8 °C, run 1), (e) ProSep rProtein A (run 2), (f) MEP HyperCel (run 3), (g) MabSorbent A1P (run 1), and (h) MabSorbent A2P (run 1). In each case 130–134 μg of protein was loaded in the first dimension. Refractometry was used to measure the protein contents of eluates from MEP HyperCel and MabSorbent A1P, whereas the protein contents of rabbit antiserum and all other eluates were determined by spectrophotometry as described in Section 2.6. The second dimension was cast with goat anti-rabbit serum. Lipoprotein material in crossed immunoelectrophoretograms was identified by staining with Sudan Black (Section 2.9). Peaks corresponding to immunoglobulins, albumin and lipoprotein are indicated by the abbreviations 'Ig', 'Alb' and 'Lp?', respectively. Eluates from MabSelect, ProSep rProtein A, and MEP HyperCel were also analysed for the presence of cholesterol and free fatty acids as described in Section 2.8; only the latter two were enriched relative to the antiserum starting material. The cholesterol level in the antiserum starting material was determined to be 479 ng per mg of total protein.

The manufacturers claim dynamic IgG binding capacities that are very much higher (i.e. up to 50 mg mL$^{-1}$) than those recorded in this study (10–13 mg mL$^{-1}$), but again (as above for MEP HyperCel), the former values were obtained with pure HuIgG, whereas the latter are for the recovery of rabbit polyclonal antibodies from clarified antisera.

FastMabsA displayed a reasonable Ig binding capacity of 25 mg mL$^{-1}$ with 20% (v/v) antiserum, but its binding was largely non-specific. For example, >60% of the applied protein in run 1 was adsorbed and the bed turned pink, presumably due to adsorption of haemoglobin from the antiserum. Moreover, under the manufacturer's recommended elution conditions, a heavily contaminated 'pink' eluate was obtained (discussed in Section 3.5).

The lowest $DBC_{0.5\,g\,L^{-1}}$ for rabbit Igs was recorded by another PAM-linked adsorbent, Kaptiv-GY. In 'overloaded' runs with 9- and 6-fold diluted rabbit antiserum Kaptiv-GY's dynamic binding capacity (5 mg mL$^{-1}$, Fig. 3) was five times lower than that obtained using purified HuIgG [39] and rabbit antiserum [40] on a related ligand immobilised on a composite material.

### 3.4. RTD analyses

Evidence for deterioration in the quality of packed beds of all of the adsorbents employed in this work was sought by routine visual inspection of the beds and monitoring of two parameters derived from RTD measurements, i.e. $N\,m^{-1}$ and $A_s$. The combined influence of number of runs and antiserum strength on $N\,m^{-1}$ and $A_s$ is summarised in Fig. 4. For freshly packed beds (i.e. run 0) the magnitude of $N\,m^{-1}$ (Fig. 4a) was seen to be a function of adsorbent particle size (cited in Table 1), while initial $A_s$ values of 1.0 to 1.3 for all supports (Fig. 4b) were indicative of well-packed beds [28].

Out of the 37 runs performed, visual evidence for perturbations in bed integrity was found in just two cases, namely after the third run on rProtein A Sepharose FF and following the fourth run on MabSorbent A1P, both involving high strength antiserum feedstocks. Contraction of the bed occurred in both instances and reduced $A_s$ values (significant in the case of Mabsorbent A1P) were obtained, but $N\,m^{-1}$ remained relatively unaffected.

### 3.5. Ig purification

The combined influence of feedstock strength and number of cycles on the chromatographic purification of rabbit immunoglobulins on packed beds of rProtein A and low molecular weight synthetic ligand-based matrices is summarised in Tables 3 and 4, respectively.

Under the conditions recommended by the various manufacturers, the recovery of bound Ig (i.e. elution efficiency) from packed beds of rProtein A and low molecular weight synthetic ligand linked supports met with few difficulties. In 25 out of 34 runs elution efficiencies were >80%, and in only five cases were they 65% or less. No one matrix could be singled out as delivering poor elution efficiency, and although no rigorous attempts to assay or confirm Ig's presence in strip fractions were made, a general inverse correlation between elution efficiency and the size of the stripping peak was not observed, indicating that 'irreversible' adsorption of Ig was not a significant problem affecting any of the matrices tested in this work. In light of this, the general observation made earlier (Section 3.2) that strip peaks were observed in chromatograms from all low molecular weight synthetic ligand-based adsorbents, but not from all rProtein A-linked materials, is most likely explained by more efficient removal of non-specifically adsorbed substances, afforded by the use of much harsher cleaning regimes than those employed for rProtein A-based supports.

The ability to achieve concentration of the product relative to its level in the feedstock is directly related to the capacity of the adsorbent and the ease with which it is subsequently desorbed. Given the higher Ig binding capacity of the rProtein A linked media cf. low molecular weight synthetic ligand-based matrices, combined with roughly similar elution efficiencies, it is not surprising that the former materials consistently delivered the highest Ig concentration on elution. For example, in 'overloaded' runs the range of Ig concentrations in eluates from rProtein A derivatised supports was 8.4–18.1 mg mL$^{-1}$ (Table 3). From the low molecular weight synthetic ligand-based group of media, the highest eluate Ig concentrations (4.6–6 mg mL$^{-1}$) were observed in runs with MEP HyperCel. That said, none of the matrices tested was able to deliver product concentration with respect to the original Ig concentration in neat antiserum of 21.6 ± 1.3 mg mL$^{-1}$ ($n = 37$). Indeed, in all cases considerable volume expansion was observed (Tables 3 and 4), ranging in 'overloaded' runs from 1.2-fold (for run 3 on MabSelect at ambient temperature) in the best case, through to 12-fold (run 4 on MabSorbent A1P) in the worst.

All eluates examined in this study tested positive for cholesterol, and are therefore likely to contain lipoprotein. Additionally, eluates from MEP HyperCel, MabSorbent A1P and Kaptiv-GY were turbid. Lipoproteins are known to precipitate in low strength acidic buffers [23], similar to those employed in this work for desorbing Ig elution off beds of these adsorbents, and thus it is possible that lipoproteinaceous material accounts for the turbidity in these eluates.

Numerical estimates of Ig purity in eluates from all of the chromatographic materials under test are presented in Tables 3 and 4, and crossed immunoelectrophoretic analysis of eluates from some of the runs described in Tables 3 and 4 are shown in Fig. 5. A general correlation between these two data sets was observed, although an apparent discrepancy relating to comparison of ProSep rProtein A and MEP HyperCel was noted (discussed below). Numerically, the highest purities were determined for rProtein A Sepharose FF, closely followed by MabSelect. Crossed immunoelectrophoretograms of eluates from beds of these materials were virtually identical (regardless of operating temperature in the case of MabSelect). Interestingly, the purities of eluates from 'overloaded' runs performed on MEP HyperCel were generally higher than those originating from ProSep rProtein A. At face value, comparison of crossed immunoelectrophoretograms from representative ProSep rProtein A and MEP HyperCel derived eluates (Fig. 5e and f) indicates that the reverse is true, i.e. that ProSep rProtein A eluates are purer

than those from MEP HyperCel, given the presence of three distinct contaminant peaks cf. only one in the analysis of the eluate from ProSep rProtein A. Rabbit albumin is strongly immunogenic, so that trace amounts give rise to strong signals, whereas larger amounts of less immunogenic contaminants elicit weaker responses. For example, a clear peak albumin was detected in crossed immunoelectrophoretograms obtained with 1 μg of pure rabbit albumin; a level corresponding to <0.8% of the protein loading typically applied in the analyses shown in Fig. 5. Comparison of the areas under albumin peaks in electrophoretograms of samples in Fig. 5 with the pure albumin standard, allowed the amount of albumin in each to be estimated. In this way the albumin content in an eluate (Fig. 5f) from MEP HyperCel was found to represent only 3% of the total protein.[1]

The appearance of the large contaminant peak in ProSep eluates, tentatively identified as a lipoprotein by staining with Sudan Black, was only noted in 'overloaded' runs. Crossed immnoelectrophoretograms of eluates from two 'underloaded' runs, i.e. 5 and 6 (data not shown) appeared identical to those from rProtein A Sepharose FF and MabSelect (Fig. 4b–d). Additionally, the determined Ig purities of eluates from runs 5 and 6 were noticeably higher than those observed for eluates from four previous 'overloaded' runs (see Table 3).

No obvious changes in purity (Tables 3 and 4) and contaminant profiles (Fig. 5), with increase in 'challenge' (i.e. 'mL equivalents of 100% antiserum applied per mL of bed'), feedstock strength, and number of runs, were observed with any of the materials, save for MEP HyperCel and MabSorbent A1P. Faint contaminating bands of albumin were detected in heavily 'overloaded' Coomassie Blue stained SDS-polyacrylamide gels of eluates from all runs on MEP HyperCel (data not shown) and the same species was detected as the main contaminant in crossed immunoelectrophoretograms (Fig. 5f). Comparison of the latter analyses from the five runs performed on MEP HyperCel indicated a tendency for the level of albumin to increase as the antiserum feedstock strength was raised.

In the case of MabSorbent A1P, a slight drop in purity from 72 to 65% was detected in 'overloaded' runs (Table 4) as the antiserum strength was raised from 20% (v/v) to 100% (v/v). Much better purity (i.e. 83%) was recorded when the 'challenge' was reduced from ∼2.0 to 0.3 mL equivalents of 100% antiserum applied per mL of bed in the final 5th run. Crossed immunoelectrophoresis from all five MabSorbent A1P runs appeared identical, showing a strong albumin peak and 4–5 fainter peaks, in addition to that for Igs (see Fig. 5g), but eluates from two 'overloaded' runs performed on MabSorbent A2P were pink and judged to be less pure (see Table 4 and Fig. 5h). Significantly higher purities than those reported here have been obtained when using less challenging human plasma, 'conditioned'[2] rabbit antiserum and near hyperimmunized ovine serum [38] feedstocks. In this study albumin was the main contaminant of Igs eluted off MabSorbents A1P and A2P. In studies with human plasma and ovine serum [38] the eluates from these supports, as well as those from others derivatised with a closely related ligand [37,42], contained significantly less albumin. Comparative studies of differences between hydrophobic drug binding sites [43], chemical and thermal stability [44], and thermal transitions [45] of serum albumins from five mammals, showed significant differences between those from rabbit and human. In light of this, it is conceivable that rabbit albumin could bind more strongly than human albumin to MabSorbents A1P and A2P.

Ig eluates from FastMabsA and Kaptiv-GY were pink, highly impure (i.e. 31% for FastMabsA and 25–38% for Kaptiv-GY) and little improved relative to the applied antiserum (Ig purity ∼29%). Crossed immunoelectrophoretic analysis of Kaptiv-GY eluates showed multiple contaminant peaks (including a massive lipoprotein peak), but a complete absence of albumin. The manufacturer of FastMabsA recommended two elution buffers (Table 2). When elution was performed with 10 mM sodium hydroxide buffer (run 1, Table 4), the bulk of Ig elution from FastMabsA occurred at pH 10 over 11 CV. Switching to a 20 mM potassium phosphate, 1 M NaCl pH 6.5 elution system (in run 2, Table 4) resulted in a more concentrated product. However, SDS–PAGE of eluates from both runs on FastMabsA confirmed the presence of large amounts of albumin.

## 4. Conclusions

The performance of eight commercial chromatography media has been analysed with respect to how they cope with direct capture and purification of Igs from a complex feedstock, namely clarified rabbit antiserum. The approach of varying a combination of factors simultaneously (rather than systematically study the effects of single variables at a time) enabled, in the space of just a few chromatographic experiments, rapid grading of the suitability of chromatographic matrices for the specific task of recovering rabbit antibodies directly out of clarified antisera. Two adsorbents (one from each class) can be singled out as giving good overall performance in this study, namely MabSelect and MEP HyperCel.

A significant problem affecting the performance of all of the media in this work, to greater or lesser extents, was the presence of lipoproteins in rabbit antisera. The latter were deemed causal agents for at least part of the observed loss in binding capacity with increasing antiserum strength (and lowering of operating temperature in the case of MabSelect), and direct evidence for non-specific accumulation of lipoproteinaceous material on supports came from detection of cholesterol in all eluates and strip fractions that were tested.

MabSelect delivered high Ig binding capacities (second only to rProtein A Sepharose FF), good desorption efficiency, and high eluate purity, as judged by immunoturbidimetric assay, SDS–PAGE and crossed-immunoelectrophoresis. Moreover, with the exception of the challenge presented in run 6 at 4–8 °C with 100% antiserum, none of these properties appeared unduly compromised by stepwise increase in strength of the rabbit antiserum feedstock. Aspects of the performance of ProSep rProtein A and rProtein A Sepharose FF made these materials

[1] Albumin represents ∼56% of the total protein in human plasma [36,41].

[2] i.e. Employing precipitation and albumin removal steps (Confidential report from Prometic Biosciences Ltd. who were supplied with the feedstock used in this study).

inferior to MabSelect for the particular application discussed herein. For instance, although possessing the highest binding capacity, beds of rProtein A Sepharose FF were compromised when challenged with higher strength antiserum feedstocks, whereas Igs eluted from ProSep rProtein A were significantly less pure than those originating from either agarose-based Protein A matrix, and were also judged inferior to those arising from the hydrophobic charge induction adsorbent, MEP HyperCel.

Although the stalwart chromatographic technique for monoclonal antibody purification, Protein A affinity chromatography has yet to make its mark in the plasma fractionation industry and in the purification of antibodies for immunodiagnostic and immunoassay purposes [1]. This is mainly due to high cost of the ligand and resulting support, low adsorbent stability during sanitization, and requirements for extensive validation of support cleaning and monitoring of Protein A leakage into the product.

In some cases Protein A affinity media are operated at low temperatures in order to ensure long term stability of the ligand. As has been shown in this study, low temperature operation with clarified antiserum feeds is not without its fair share of problems. For example, the viscosity of the mobile phase increases and the solubility of lipoproteins and other colloidal materials within it drops, causing a greater tendency for foulants to accumulate within the bed. The net result is considerably reduced chromatographic performance and a requirement for more stringent cleaning of the bed, than would be needed when operated at ambient temperatures. In studies with Protein A Sepharose FF, Hale et al. [9] showed that routine cleaning with 0.5 M NaOH induced a loss in monoclonal antibody binding capacity of 1% per cleaning cycle. In subsequent work by Johansson et al. [46] with MabSelect, the combined use of >0.25 h contact time with a milder cleaning cocktail (1 M NaCl and 50 mM NaOH) delivered an irreversible 11% capacity loss after 300 cycles. The generation of 'NaOH tolerant' rProtein A species [15,16] and improved methods for stable and chemoselective anchoring of these to appropriate supports, promises to address many of the above concerns[3], but is unlikely to eliminate all of them.

Despite initial promises of increased robustness in crude feeds over Protein A-linked supports, as a group, low molecular weight synthetic ligand-based adsorbents were generally compromised to a much greater extent by dissolved 'problem-species' present in rabbit antiserum. For example, comparison of the dynamic Ig binding capacity data determined here for Kaptiv-GY and 11% (v/v) rabbit antiserum (the lowest strength tested), with reported values obtained using pure Ig and/or highly conditioned feedstocks, indicated a >80% loss in capacity. The best of these materials, MEP HyperCel suffered a 33% loss in capacity when challenged with antisera relative to that obtained with pure human IgG.

Of the five small synthetic ligand-based adsorbents tested, only MEP HyperCel was up to the task of capturing Ig from rabbit antisera. This low cost matrix (roughly a quarter the price of Protein A-linked adsorbents based on figures for 1 L quantities) delivered about half the capacity of MabSelect, the purity of its eluates was judged superior to that from ProSep rProtein A, and second only to that emanating from both of the rProtein A-linked agarose media. In contrast to sterile injectable therapeutic products, immunodiagnostics and/or immunoassays employ significantly less pure products. Indeed, the low-level contamination of MEP HyperCel eluates with serum albumin is entirely acceptable judged on the basis of head-to-head comparison in an immunoturbidimetric application with a commercial DakoCytomation product, involving 100 human serum samples. In this context, the extra purification afforded by MabSelect may be considered surplus to requirements. The issues that plague the current crop of Protein A-linked adsorbents (i.e. high cost, low tolerance to NaOH, extensive cleaning validation) are not matters for concern with MEP HyperCel. For example, in a 204 cycle study, with a 1 M NaOH wash after each cycle, Guerrier et al. [21] demonstrated that monoclonal antibody binding capacity was reduced by 25%, but could be fully restored to its original value after a single wash with 2 M urea.

The limited number of runs applied in current tests, combined with changes in the antiserum feedstock from run to run, do not allow predictions of the long term performance of either lead adsorbent to be made. Based solely on the performance criteria investigated here (i.e. dynamic binding capacity, Ig purity and elution efficiency), of the adsorbents we have tested for the purification of Igs from rabbit antiserum, MabSelect would be the material of choice. However, once issues of cost, capacity loss per cleaning cycle, cleaning validation, and batch cross-contamination are brought into consideration, the gap between MabSelect and MEP HyperCel is expected to close. Moreover, given: (i) the lower purity demands of antibody products for immunodiagnostics; (ii) the fact that MEP HyperCel can meet these; and is (iii) considerably cheaper, the decision to chose MabSelect over MEP HyperCel might be reversed.

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