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Validation studies in the regeneration of ion-exchange celluloses

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Abstract

The effectiveness of a clean-in-place procedure involving treatment with 0.5 *M* NaOH for 16 h at room temperature has been examined following process-scale chromatography of 1.85 kg hen egg-white proteins on the DEAE-cellulose, Whatman Express-Ion Exchanger D and the QA-cellulose, Whatman Express-Ion Exchanger Q in 251 columns operating at flow-rates of 150 cm/h. Treatment of the media with 0.5 *M* NaOH did not affect the performance of the media after re-equilibration. The NaOH treatment was effective for sanitization and depyrogenation of columns of Express-Ion D and Express-Ion Q following gross microbial contamination. Furthermore, hydrolysis of the DEAE functional groups from Express-Ion D during treatment with 0.5 *M* NaOH was beyond the limits of detection, i.e. $<0.01\%$ of the total DEAE content of the exchanger.

1. Introduction

Ion-exchange chromatography is a widely used technique in the downstream processing of commercially important biopolymers. For low-pressure chromatography, ion exchangers are traditionally based on polysaccharide supports including cellulose, agarose and dextran [1,2]. In industrial applications, particularly in the manufacture of biopharmaceuticals, regulatory aspects of the chromatographic process are an important consideration in process development. Process validation is a complex subject and the key issues have been adequately reviewed elsewhere [3]. In the present study we are interested only in the validation of a specific anion-exchange chromatography process, using cellulose-based adsorbents, with emphasis on aspects of sanitization and leachables. Large-scale ion-exchange processes can be carried out using either batch stirred tank or column techniques [4] although the latter technique may be more desirable where validation is important since it is a closed system and consequently much easier to manage and control compared with an open batch stirred tank unit operation.

During the chromatographic step it is necessary, in the validated process, to demonstrate that the product eluted during the process is of the desired quality with regard to sterility, endotoxin content and contaminants arising from the chromatographic medium itself (leachables). The medium would typically undergo a clean-in-place protocol (CIP) periodically during use, and this CIP protocol is an opportune step to effect sanitization, but may adversely affect the process if it were to result in chemical attrition of the medium, i.e. generation of leachables. We have

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previously reported that storage of the anion-exchange celluloses DE52 [5], QA52 [6], DE92 [7] and DEAE-cellulose/D856 [8] in 0.5 *M* NaOH for a period of 12–16 h represents typical conditions for an effective CIP. Furthermore we have shown that these conditions effect simultaneous sterilization and depyrogenation of heavily contaminated columns of DE52 and QA52 following challenge with ca. $1 \cdot 10^9$ *Escherichia coli* per ml [6,9,10]. It has been reported that agarose-based ion exchangers for example can be cleaned using dilute NaOH [11] and these conditions have been investigated for use in sanitization. Storage of various chromatographic media in dilute NaOH at concentrations of up to 1 *M* and the use of dilute ethanol have been shown to be effective for sanitization using a range of microorganisms [3,11–13].

The question of leachables is a key issue in validation [3] and if leachables derived from the chromatographic medium were to coelute with the product then the consequences could be quite severe. It could be argued that if leachables were generated during column regeneration (CIP) then provided they were washed out prior to product elution they would not pose a problem. While this argument may be justified, the effects on chromatographic performance including capacity and resolution would likely be significant, thereby rendering the process irreproducible. There is little published information on leachables although the subject has received some attention in the field of affinity chromatography where it is referred to a ligand leakage [14–16]. A study was recently reported [17] on leachables arising from the anion-exchange agarose, DEAE-Sepharose Fast Flow, where extended storage in 1 *M* NaOH gave rise to limited hydrolysis of the functional groups from the matrix.

In the present study we examine validation aspects of a CIP procedure used in conjunction with the process-scale chromatography of hen egg-white proteins on two fast flowing anion-exchange celluloses derivatised with either DEAE or QA functional groups. The CIP procedure which is effective in column regeneration has been examined for chromatographic performance, sanitization and also for media stability in terms of leachables.

2. Experimental

2.1. Materials

Cell debris remover (CDR), Express-Ion D (formerly named DEAE-cellulose/D856) and Express-Ion Q (formerly named QA-cellulose/D856) were obtained from Whatman International (Maidstone, UK). A G450 × 500 glass chromatography column fitted with 30-μm polypropylene bed supports was obtained from Amicon (Stonehouse, UK). N,N-Diethylethanolamine was obtained from Aldrich (Gillingham, UK). 2,3-Dihydroxypropyltrimethylammonium chloride was a generous gift from Degussa (Hanau, Germany). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Merck (Poole, UK). All other chemicals were of analytical-reagent grade. Fresh size 2 hen eggs were obtained from Barradale Farms (Headcorn, UK).

2.2. Feedstock preparation

Egg whites were separated from 600 fresh hen eggs and diluted to 14% (v/v) with 0.025 *M* Tris–HCl buffer (pH 7.5). The egg-white suspension was clarified using a total of 22 kg of pre-equilibrated CDR in a batch mode. Spent CDR was removed by centrifugation through a 1.6 × 0.6 mm slotted screen (EHR 500 basket centrifuge; Robatel and Mulatier, Lyons, France) and the sample was clarified through a Grade 541 filter paper (Whatman International). The clear solution (200 l) containing 9.24 mg/ml of total protein was used for chromatography on Express-Ion Q.

2.3. Process-scale chromatography

Express-Ion Q (15.9 kg) was equilibrated with 0.025 *M* Tris–HCl buffer (pH 7.5) to give a final slurry concentration of ca 30% (w/v). The slurry was transferred to the 50 cm × 45 cm I.D.

column barrel section and the bed consolidated at a pressure of ca. 10 p.s.i. (1 p.s.i. = 6894.76 Pa) according to the column manufacturer's guidelines. The packed column (16 cm × 45 cm I.D.) had a volume of ca. 25 l and a packing density of 0.214 kg/l. The ion exchanger was used with the egg-white feedstock accordingly: (i) analytical loading, (ii) preparative loading, (iii) clean-in-place, (iv) analytical loading. A pressure–flow-rate test was carried out after column packing and after the CIP procedure. All procedures were carried out at room temperature (15–20°C).

(i) Analytical loading

Egg-white feedstock (10 l) was loaded on to the column and non-bound material removed by washing with 0.025 *M* Tris–HCl buffer (pH 7.5) (50 l). Bound material was eluted using a linear gradient of 0–0.5 *M* NaCl in 0.025 *M* Tris–HCl buffer (pH 7.5) (200 l). A flow-rate of 4 l/min was maintained throughout.

(ii) Preparative loading

Egg-white feedstock (200 l) was loaded on to the column and non-bound material removed by washing with 0.025 *M* Tris–HCl buffer (pH 7.5) (100 l). Bound material was eluted using a linear gradient of 0–0.5 *M* NaCl in 0.025 *M* Tris–HCl buffer (pH 7.5) (200 l). A flow-rate of 4 l/min was maintained throughout.

(iii) Clean-in-place procedure

The column of Express-Ion Q was washed in 0.5 *M* NaOH (50 l), depressurized and allowed to stand at room temperature for 16 h. The column was repressurized and washed successively with water (50 l), 0.1 *M* Tris–HCl buffer (pH 7.5) (50 l) and 0.025 *M* Tris–HCl buffer (pH 7.5) (200 l). A flow-rate of 4 l/min was maintained throughout.

(iv) Analytical loading

Egg-white feedstock (10 l) was chromatographed on Express-Ion Q as described in (i).

2.4. Assays

Pooled fractions at various stages of chromatography were assayed for protein content by measuring the absorbance at 280 nm against standard solutions of ovalbumin. Throughout the column procedures the effluent was monitored for absorbance at 280 nm and by conductivity.

2.5. Sanitization testing

Studies on sanitization of Express-Ion D and Express-Ion Q were carried out as an external contract with Safepharm Labs., Derby, UK, using their standard operating procedures. For the microbial challenge, cultures of *E. coli* NCIMB 8545, *Staphylococcus aureus* NCIMB 9518, *Pseudomonas aeruginosa* NCIMB 8626, *Aspergillus niger* IMI 149007, *Candida albicans* NCPF 3179 and *Bacillus subtilis* NCIMB 3054 were grown, and mixed to give a suspension of microorganisms containing ca. $1 \cdot 10^7$ colony forming units (cfu)/ml.

(i) Column studies

Express-Ion D and Express-Ion Q were equilibrated with 0.025 *M* sodium phosphate buffer (pH 7.4) and packed into 15-ml columns (19 cm × 1 cm I.D.). The suspension of microorganisms (45 ml) was loaded onto each column and the final 15-ml fractions collected as the pre-CIP samples. The beds were washed with 0.5 *M* NaOH (45 ml) and stood at room temperature for 12–24 h. The columns were washed successively with sterile endotoxin-free water (45 ml) sterile endotoxin-free 0.1 *M* sodium phosphate buffer (pH 7.4) (45 ml) and sterile endotoxin-free 0.025 *M* sodium phosphate buffer (pH 7.4) (90 ml). The final 15-ml fractions were collected under aseptic conditions as the post-CIP samples. Flow-rate was maintained at 1 ml/min throughout.

The pre-CIP samples were tested for total viable counts (TVCs), sterility and endotoxin, using the Limulus Amoebocyte Lysate test (LAL). The post-CIP samples were tested for TVCs, sterility, LAL and pyrogens using the rabbit pyrogen test.

(ii) Batch studies

Express-Ion D and Express-Ion Q were equilibrated with 0.025 *M* sodium phosphate buffer (pH 7.4). Equilibrated Express-Ion D and Express-Ion Q (10 g) were each mixed with the suspension of microorganisms (10 ml) and a solution of NaOH (45 ml) added to give a final concentration of 0.5 *M*. The suspension was incubated at room temperature for 12–24 h. A sample of each suspension (5 ml) was withdrawn, adjusted to pH 7.4 using 1 *M* HCl, and tested for TVCs.

2.6. Leachables testing

Express-Ion D and Express-Ion Q were equilibrated with 0.025 *M* sodium phosphate buffer (pH 7.4) and packed into 15 ml columns (19 cm × 1 cm I.D.). 0.025 *M* sodium phosphate buffer (pH 7.4) (45 ml) was passed through each column and the final 15-ml fractions collected as sample 1. Each bed was washed with 0.5 *M* NaOH (45 ml) and stood in 0.5 *M* NaOH at room temperature for 16 h. Each column was washed with 0.5 *M* NaOH (45 ml) and the first 30-ml fractions collected as sample 2. Each column was washed successively with water (45 ml), 0.1 *M* sodium phosphate buffer (pH 7.4) (45 ml) and 0.025 *M* sodium phosphate buffer (pH 7.4) (90 ml). The final 15-ml fractions were collected as sample 3. Flow-rate was maintained at 1 ml/min throughout.

For Express-Ion D, samples 1, 2 and 3 were analysed for N,N-diethylethanolamine by GC–MS. Samples 1, 2 and 3 (10 ml) were extracted with dichloromethane (2 ml). The dichloromethane samples were analysed using a Hewlett-Packard Model 5890 gas chromatograph coupled to a Model 5972 mass-selective detector. The column used was a Hewlett-Packard HP5-MS capillary 30 m × 0.25 mm I.D. with a 0.25-μm film thickness. Injection volume was 1 μl, via a splitless injector, at a temperature of 180°C, using helium carrier gas at a constant flow-rate of 1 ml/min. The oven temperature programme was 35°C for 2 min, then 10°C/min to 100°C, then 30°C/min to 250°C then 250°C for 0.5 min. The interface temperature to the MS system was 280°C and masses scanned were 86, 58 and 117 with a dwell time of 50 ms/mass. The instrument was calibrated using standard aqueous solutions containing 1, 10 and 50 mg/l N,N-diethylethanolamine and extracted as described above. The abundance of the 86 ion was used for quantification and masses 58 and 117 used as qualifier ions.

For Express-Ion Q, samples 1, 2 and 3 were analysed for 2,3-dihydroxypropyltrimethylammonium chloride by ion chromatography. The samples were analysed using a Dionex Model DX100 ion chromatograph equipped with a Dionex CS12 column. The mobile phase was 20 m*M* methanesulphonic acid at a flow-rate of 0.8 ml/min. Peaks were detected by suppressed conductivity. The system was calibrated using standard solutions of 2,3-dihydroxypropyltrimethylammonium chloride in either 0.025 *M* sodium phosphate buffer, pH 7.4 or 0.5 *M* NaOH.

3. Results and discussion

We have previously reported that Express-Ion D exhibits rapid binding kinetics facilitating its operation at high flow-rates for process-scale chromatography [8]. In our earlier work, we reported that Express-Ion D had a dynamic binding capacity of 84 mg protein/ml column volume following a saturation loading of 9.26 mg/ml hen egg-white proteins at a flow-rate of 140 cm/h [8]. Under similar conditions the capacity of Express-Ion Q was found to be 80 mg protein/ml column volume [18]. For the preparative loading of hen egg-white proteins on Express-Ion Q a total of 1.848 kg of protein was loaded onto the column which represents a submaximum loading of ca. 73 mg protein/ml packed column volume. The absorbance profile of the column eluate during the process run is represented in Fig. 1. The protein mass balance data for this study are summarised in Table 1. In order to assess the capture efficiency of the adsorptive process during a single pass through the column a binding efficiency is calculated according to:

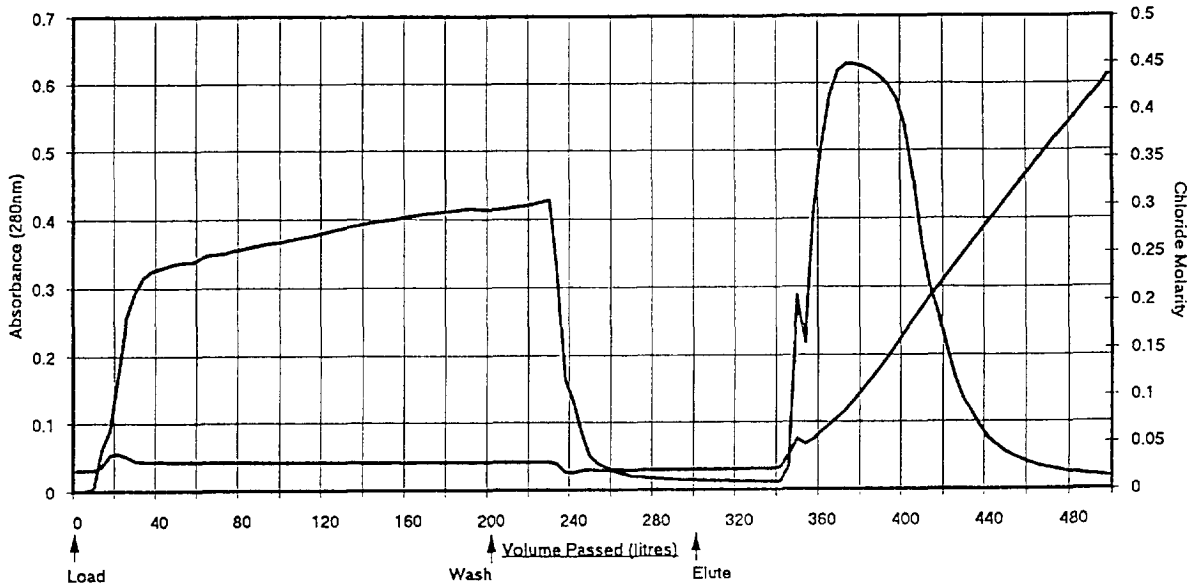


Fig. 1. Absorbance profile of column eluate during preparative chromatography of 9.24 mg/ml hen egg-white proteins on Express-Ion Q on a process scale (16 cm × 45 cm I.D.) using 0.025 *M* Tris–HCl buffer (pH 7.5), at a flow-rate of 150 cm/h at 15–20°C.

$$\text{binding efficiency (\%)} = \frac{\text{mass of protein adsorbed}}{\text{mass of protein loaded}} \cdot 100 \quad (1)$$

The data in Table 1 demonstrate that 1.593 kg of protein bound to Express-Ion Q during the loading stage which is equivalent a protein capacity of 62.7 mg protein/ml column volume. This reflects utilisation of ca. 86% of the maximum theoretical capacity of the Express-Ion Q and compares well with the data reported for Express-Ion D [8]. In this study we appeared to recover 100% of the adsorbed protein (Table 1) whereas for Express-Ion D recovery was ca 93% [8]. We have previously reported that a CIP using 0.5 *M* NaOH for 12–16 h (overnight) is effective at restoring column performance for DE52 [5], QA52 [6], DE92 [7] and Express-Ion D [8] in each case following preparative loadings. The analytical loading of egg-white on the Express-Ion Q after CIP is represented by the chromatogram in Fig. 2b. The elution profile is similar, if not slightly improved, over that seen after column packing (Fig. 2a), indicating that the NaOH treatment has no detrimental effect on the chromatographic performance of the medium. This is in keeping with our previous studies on Express-Ion D [8]. The pressure–flow-

Table 1
Protein mass balance during preparative chromatography of hen egg-white proteins on Express-Ion Q

Stage of chromatography	Feedstock total protein (g)	Total protein (g)		Binding efficiency (%)
		In mobile phase	Adsorbed on Express-Ion Q	
Loading	1848	236	1612	
Wash		19	1593	86.2
Elution		1640	0	

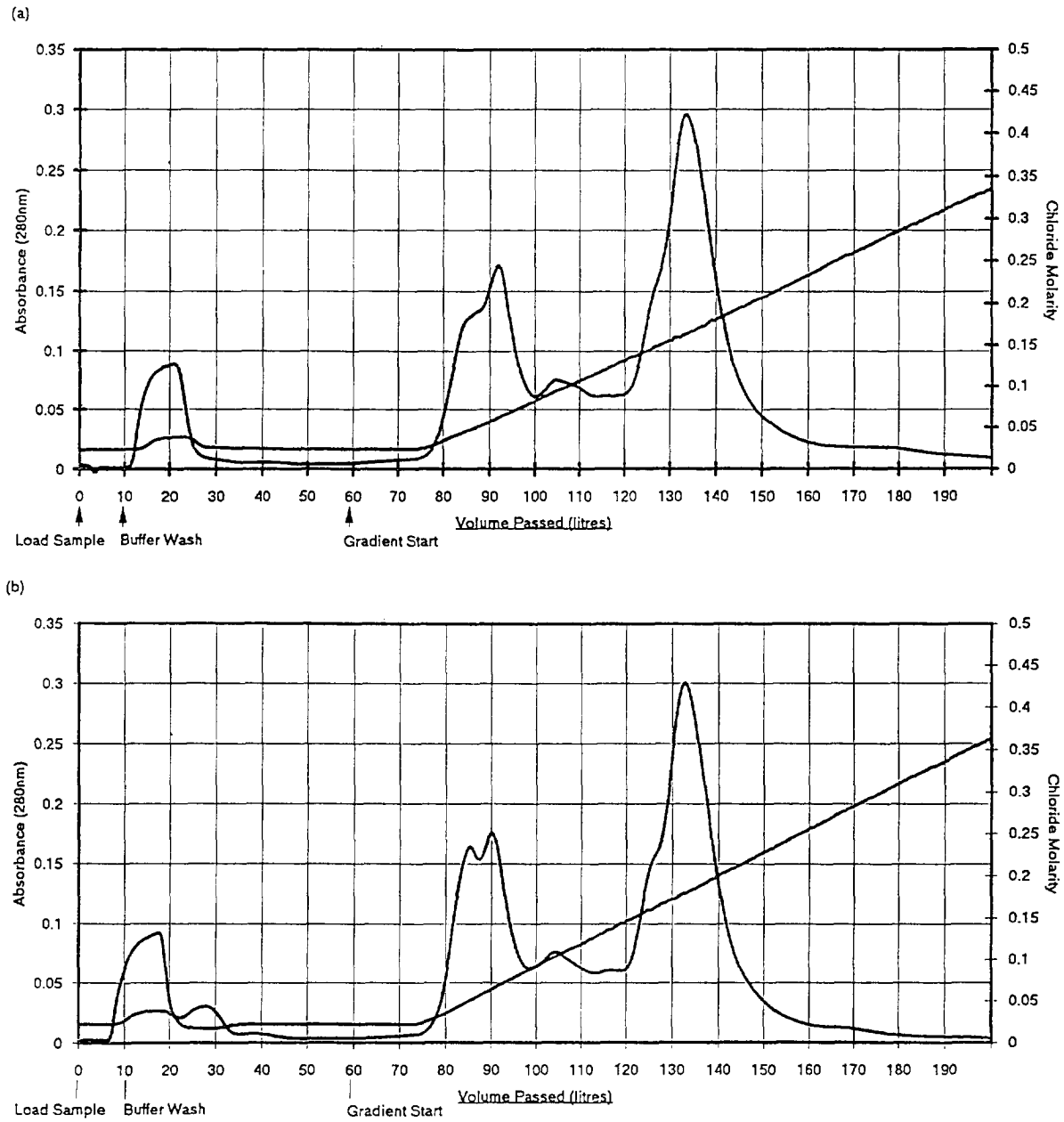


Fig. 2. Column chromatography of hen egg-white proteins on Express-Ion Q on a process scale (16 cm × 45 cm I.D.) using 0.025 *M* Tris–HCl buffer (pH 7.5). (a) Analytical loading (100 g) before preparative run, (b) analytical loading (100 g) after CIP; flow-rate 150 cm/h at 15–20°C.

rate data obtained before and after the CIP procedure are summarised in Table 2 and there was no significant effect on flow performance of the Express-Ion Q following overnight storage in 0.5 *M* NaOH.

Having demonstrated that storage of columns

Table 2
Pressure–flow-rate relationship during process-scale chromatography of hen egg-white proteins on Express-Ion Q

Pressure (p.s.i.)	Flow-rate (cm/h)	
	After column packing	Post-CIP
5.0	134.7	131.7
7.5	173.6	173.6
10.0	212.4	217.0

of Express-Ion D and Express-Ion Q in 0.5 *M* NaOH for 12–16 h is an effective bed regeneration following preparative chromatography of hen egg-white proteins, the effectiveness of such a treatment for bed sanitization was investigated. In this study a similar CIP was carried out using sodium phosphate buffer as the mobile phase as this was considered to be more suitable for sustaining the viability of microorganisms than a Tris buffer. Following a challenge with a mixed suspension of *E. coli*, *S. aureus*, *P. aeruginosa*, *A. niger*, *C. albicans* and *B. subtilis* the column effluents had a high bioburden with a significant endotoxin content (Table 3). Following the CIP and subsequent bed re-equilibration using sterile endotoxin-free buffers, the column effluents gave negative responses in the total viable count test, were sterile and contained very low levels of endotoxin giving a negative response in the rabbit pyrogen test (Table 3). The data presented in this study demonstrate that overnight treatment with 0.5 *M* NaOH is an effective regime for sanitization of packed columns of Express-Ion D and Express-Ion Q following a very heavy microbial challenge using a mixed suspension of microorganisms which are recommended for use in validation studies [3]. In this study a total of 15 bed volumes of various mobile phases were passed through the columns following the microbial challenge. It is therefore possible that sanitization has been effected purely by displacement of the microorganisms rather than as a result of their physiological incompatibility with 0.5 *M* NaOH. Consequently the mixed suspension of microorganisms was incubated with 0.5 *M* NaOH, in the presence of Express-Ion D and Express-Ion Q. The results of these batch studies are summarised in Table 4. The data show that following storage in 0.5 *M* NaOH no total viable counts were recorded. These data reinforce the effectiveness of our CIP protocol for simultaneous sanitization of a packed column of Express-Ion D and Express-Ion Q.

The final validation aspect of this study concerns leachables, and specifically hydrolysis of the functional group from the ion exchangers during the CIP procedure. Whatman Internation-

Table 3
Sanitization testing of columns of Express-Ion D and Express-Ion Q

Stage of investigation	TVC (cfu/ml)	Sterility test	Endotoxin (EU/ml)	Rabbit pyrogen test
Express-Ion D				
Challenge	$5.5 \cdot 10^6$	nd[a]	nd	nd
Pre-CIP	$7.7 \cdot 10^4$	Fail	> 60	nd
Post-CIP	< 1	Pass	< 0.06	Pass
Express-Ion Q				
Challenge	$4.6 \cdot 10^6$	nd	nd	nd
Pre-CIP	$7.6 \cdot 10^3$	Fail	> 60	nd
Post-CIP	< 1	Pass	< 0.06	Pass

[a] nd = Not determined.

Table 4
Influence of 0.5 *M* NaOH on viability of microorganisms during batch storage with Express-Ion D and Express-Ion Q

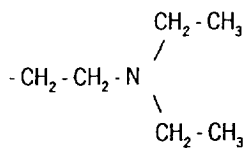
Stage of investigation	TVC (cfu/ml)	
	Express-Ion D	Express-Ion Q
Pre-CIP	$1.1 \cdot 10^7$	$4.2 \cdot 10^6$
Post-CIP	< 1	< 1

Table 5
Leachables testing during CIP of Express-Ion D

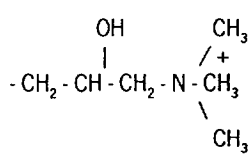
Sample	[N,N-Diethylethanolamine] (mg/l)
1, Pre-CIP	< 1[a]
2, Post-CIP	< 1[a]
3, Re-equilibration	< 1[a]

[a] Limits of detection.

al derivatises the microgranular cellulose matrix with the diethylamineothyl (DEAE) group:



and the 2-hydroxypropyltrimethylammonium (QA) group:



In each case the functional group is covalently attached to the cellulose via ether linkages to the distal carbon atoms [19]. If hydrolysis of the functional groups were to take place then it is reasonable to expect that the groups would be liberated as the alcohols N,N-diethylethanolamine and a 2,3-dihydroxypropyltrimethylammonium salt from Express-Ion D and Express-Ion Q, respectively. If hydrolysis of the functional group were to occur as a result of exposure to NaOH then the liberated amines would be present in the displaced NaOH fraction following the CIP procedure. The first two displaced column volumes of effluent during the post-CIP wash from the Express-Ion D column (sample 2) were collected and analysed for N,N-diethylethanolamine by GC–MS. These data are summarised in Table 5 and the chromatograms represented in Fig. 3. The data demonstrate that no detectable hydrolysis of the functional group had occurred during the CIP and the NaOH fraction (sample 2) and the re-equilibrated media (sample 3) contained no detectable volatile organics under conditions where the alcohol derivative of the functional group can be identified by GC–MS (Fig. 3b). In this investigation the column of Express-Ion D contained 3.05 dry g of media. This is equivalent to 3.0 mmol of DEAE groups. The detection limits for GC–MS were determined to be 1 mg/l for N,N-diethylethanolamine. On the basis of a 30-ml volume of displaced NaOH this corresponds to detection limits of 0.256 μmol of DEAE groups and it may therefore be deduced that hydrolysis of the functional groups represents $< 0.01\%$ of those present within the packed column of Express-Ion D under the CIP conditions used in this study.

While we obtained a sample of the anticipated hydrolysis product of Express-Ion Q, namely, 2,3-dihydroxypropyltrimethylammonium chloride we were unable to separate or identify the material by GC–MS under similar conditions to those used for N,N-diethylethanolamine. Attempts to derivatise the material by either methylation or benzoylation were unsuccessful.

We were able to detect 2,3-dihydroxypropyltrimethylammonium chloride by ion chromatography and the analysis of samples 1, 2 and 3 obtained from the Express-Ion Q study are reported in Table 6. The limits of detection of 2,3-dihydroxypropyltrimethylammonium chloride were 5 mg/l for 0.025 *M* sodium phosphate buffer, pH 7.4 and 25 mg/l for 0.5 *M* NaOH. The data demonstrate that no detectable hydrolysis of the QA groups had occurred during the CIP and both the NaOH fraction (sample 2)

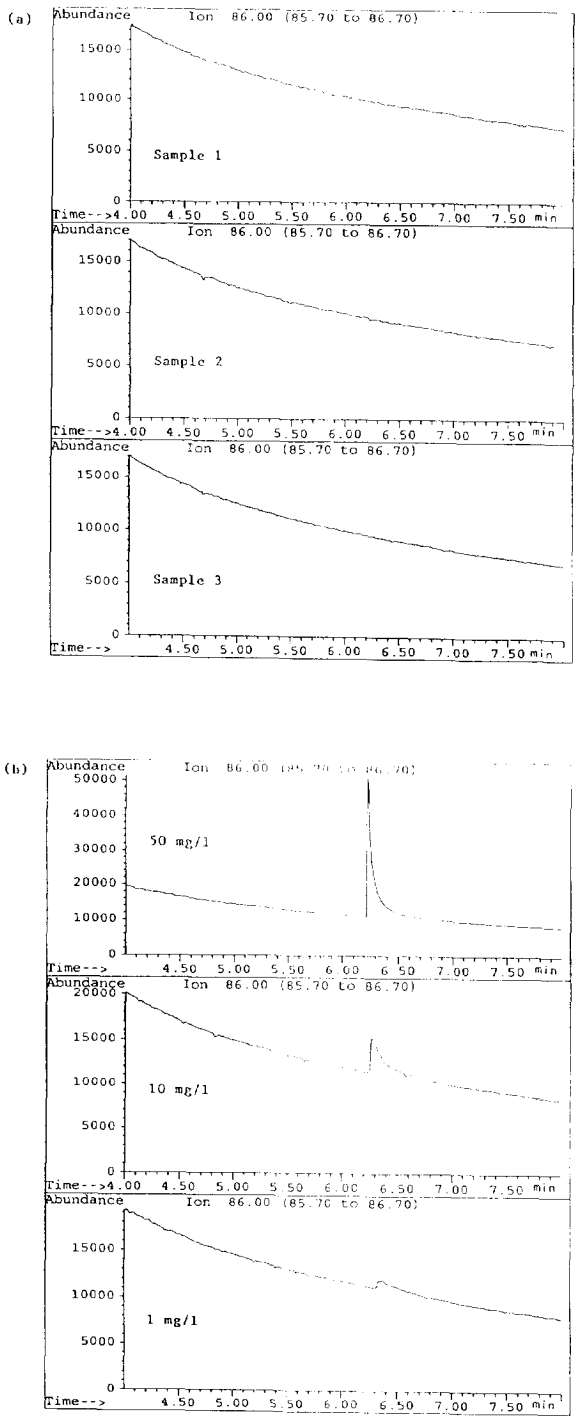


Fig. 3. Analysis of (a) sample 1, sample 2 and sample 3 obtained from the leachables study and (b) standards containing 50, 10 and 1 mg/l N,N-diethylethanolamine. Full analytical details are described in the text.

Table 6
Leachables testing during CIP of Express-Ion Q

Sample	[2,3-Dihydroxypropyltrimenthylamine] (mg/l)
1. Pre-CIP	$<5^a$
2. Post-CIP	$<25^a$
3. Re-equilibration	$<5^a$

[a] Limits of detection.

and the re-equilibrated media (sample 3) contained no ionic species under conditions where the alcohol derivative of the functional group is identifiable by ion chromatography. For the post-CIP sample (30 ml) a detection limit of 25 mg/l corresponds to 4.44 μmol of QA groups (as a chloride salt) within this volume. The 15-ml column of Express-Ion Q contained 3.07 μmol QA groups (3.3 dry g media) and it may therefore be deduced that hydrolysis of the functional groups represents $<0.15\%$ of those present within the packed column of Express-Ion Q under the CIP conditions used in this study.

In this study we have demonstrated that following process-scale column chromatography of hen egg-white proteins on Express-Ion Q a CIP treatment involving storage in 0.5 *M* NaOH for 16 h, had no significant detrimental effect on the chromatographic performance of the bed. This data supports our previous findings for Express-Ion D [8]. The CIP procedure was found to be an effective sanitization protocol for both media following a gross microbial contamination with a mixed culture of six microorganisms. During the CIP, hydrolysis of the functional groups from Express-Ion D and Express-Ion Q was found to be insignificant. Studies of this type are guidelines towards validation of these media in regulated processes and form the basis for extended, process-specific validation studies.

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