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Influence of flow-rate on the chromatographic performance of agarose- and cellulose-based anion-exchange media

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Abstract

The influence of flow-rate on the separation of hen egg-white proteins was investigated using Whatman Express-Ion Exchanger Q and Pharmacia Q-Sepharose Fast Flow. Using 25-ml columns, breakthrough studies demonstrated that Express-Ion Q had an ovalbumin capacity of ca. 70 mg/ml, ca. 20% (w/w) greater than Q-Sepharose Fast Flow. Breakthrough for each exchanger was rapid. Using 25-l columns ca. 3.9 kg of protein was loaded on to each exchanger with Express-Ion Q having a capacity of ca. 2.1 kg compared with ca. 1.8 kg for Q-Sepharose Fast Flow. The desorption kinetics of the agarose were slower than those of the cellulose, as indicated by reduced chromatographic resolution with increasing flow-rate. Express-Ion Q was shown to give consistent chromatography over 100 chromatographic cycles with periodic clean-in-place.

Keywords: Flow-rate; Anion exchangers; Agarose-based anion exchangers; Cellulose-based anion exchangers; Stationary phases, LC; Preparative chromatography; Dynamic capacity; Proteins

1. Introduction

Ion-exchange chromatography is a widely used technique in the downstream processing of commercially important biopolymers. For low-pressure separations, ion exchangers are traditionally based on polysaccharide supports including cellulose, agarose and dextran [1,2]. In order for an industrial ion-exchange process to be commercially viable, it is important to optimize throughput at the required degree of product purity. Throughput in a chromatographic process is affected by capacity, resolution and flow-rate, all of which are interrelated.

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Cellulose is a polysaccharide with a macroporous structure, which in its non-regenerated form offers a very high protein capacity and fast adsorption kinetics [3], features which directly influence dynamic binding capacity and resolution in the chromatographic process [3]. Accordingly, ion-exchange celluloses have been used in various biochemical separations [4–7]. While the capacity and resolution of traditional microgranular ion-exchange celluloses are higher than those of comparable agarose-based exchangers [3,8], the flow performance of the latter product type is superior. In ion-exchange separations where, for example, components of the protein feed are labile or the chromatographic step is rate limiting, then process time and thus flow-

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Table 1
Typical properties of Express-Ion Q and Q-Sepharose Fast Flow

Property	Express-Ion Q	Q-Sepharose Fast Flow
Regains (g/dry g):		
Free base form	1.94	4.86
Hydrochloride form	1.87	4.67
Small-ion capacity (mequiv./dry g)	0.93	1.45
Column packing density (dry g/ml)	0.22	0.12
Protein capacity[a] (mg/dry g)	256	648
Protein capacity[a] (mg/ml)	55	78
Flow-rate (cm/h):		
50 cmH_2O/cm[b]	523	579
75 cmH_2O/cm	751	835

[a] Bovine serum albumin, 0.01 *M* sodium phosphate buffer (pH 8.5).
[b] 1 cmH_2O = 98.0665 Pa.

rate are key factors. In order to address these flow-rate limitations, we developed the Express-Ion range of ion-exchange celluloses, which can sustain high flow-rates, i.e., >150 cm/h in axial flow columns [9], albeit with a reduced protein capacity compared with the traditional micro-granular ion-exchange celluloses [10].

We have previously reported the process-scale chromatography of hen egg-white proteins on 25-l columns containing Express-Ion D [9] and Express-Ion Q [11] and have carried out detailed validation studies on the regeneration of these media following a clean-in-place (CIP) protocol [11,12].

In the present study, we compared the chromatographic performance of Express-Ion Q with the agarose-based anion exchanger Q-Sepharose Fast Flow. Typical properties of these media are summarized in Table 1. In this work we assessed the effect of flow-rate on the separation of hen egg-white proteins in terms of capacity and resolution using 25-ml laboratory columns and 25-l process columns of each medium.

2. Experimental

2.1. Materials

Cell debris remover (CDR) and Express-Ion Q were obtained from Whatman International (Maidstone, UK). Q-Sepharose Fast Flow was obtained from Pharmacia Biotech (St. Albans, UK). A G450 × 500 glass chromatographic column fitted with 30-μm polypropylene bed supports was obtained from Amicon (Stonehouse, UK). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Merck (Poole, UK). Ovalbumin grade V was obtained from Sigma (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 1500 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 50 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 (500 l) containing 7.4–8.7 mg/ml of total protein was used for chromatography on Express-Ion Q and Q-Sepharose Fast Flow.

2.3. Batch kinetics

Express-Ion Q and Q-Sepharose Fast Flow were equilibrated with 0.025 *M* Tris–HCl buffer (pH 7.5). The adsorption of 2.0 mg/ml oval-

bumin was carried out in a batch stirred tank (250 ml) using Express-Ion Q (370 mg dry mass equivalent) and Q-Sepharose Fast Flow (180 mg dry mass equivalent) over a 60-min period with continuous monitoring of the adsorbate solution at 280 nm.

2.4. Dynamic capacity determination

Egg-white feedstock (1000 ml) was applied to columns of Express-Ion Q or Q-Sepharose Fast Flow (15.5 cm × 1.5 cm I.D.) previously equilibrated with 0.025 *M* Tris–HCl buffer (pH 7.5), by which time the absorbance of the eluate at 280 nm was similar to that of the feedstock. Non-bound material was removed by washing with 0.025 *M* Tris–HCl buffer (pH 7.5) (150 ml). Bound material was eluted using a linear gradient of 0–0.5 *M* NaCl in 0.025 *M* Tris–HCl buffer (pH 7.5) (400 ml). The flow-rates were maintained at 1, 2, 4, 6, 8, 10 and 20 ml/min over a series of studies. The protein capacity at 10% and 100% (w/w) of breakthrough were calculated following FPLC analysis using a column of Mono Q as described previously [9].

2.5. Process-scale chromatography

Express-Ion Q (15.4 kg) and Q-Sepharose Fast Flow (35 l) were 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 was 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 of Express-Ion Q (15.5 cm × 45 cm I.D.) had a volume of ca. 24.7 l and a packing density of 0.218 kg/l. The packed column of Q-Sepharose Fast Flow (14.5 cm × 45 cm I.D.) had a volume of ca. 23.1 l and a packing density of 0.129 kg/l. All procedures were carried out at room temperature (15–20°C).

Egg-white feedstock (500 l) was loaded on to the column and non-bound material was removed by washing with 0.025 *M* Tris–HCl buffer (pH 7.5) (150 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) (400 l). CIP was carried out by washing the columns of Express-Ion Q or Q-Sepharose Fast Flow with 0.5 *M* NaOH (50 l). The columns were depressurized and allowed to stand at room temperature for 16 h. The columns were 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). For each medium the flow-rates were maintained at 2, 4 and 6 l/min throughout each chromatographic cycle.

Pooled fractions at various stages of chromatography were assayed for total protein and ovalbumin content by FPLC [9]. Throughout the column procedures the effluent was monitored for absorbance at 280 nm and by conductivity.

2.6. Resolution study

Express-Ion Q and Q-Sepharose Fast Flow were equilibrated with 0.025 *M* Tris–HCl buffer (pH 7.5) and packed into columns (15.5 cm × 1.5 cm I.D.). Egg-white feedstock (10 ml) was loaded on to the columns and non-bound material removed by washing with 0.025 *M* Tris–HCl buffer (pH 7.5) (50 ml). 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 ml). The flow-rates were maintained at 1, 2, 4, 6, 8, 12, 16, 20 and 30 ml/min over a series of studies. Throughout these studies the column effluent was monitored for absorbance at 280 nm and by conductivity.

2.7. 100 cycle study

Express-Ion Q was equilibrated with 0.025 *M* Tris–HCl buffer (pH 7.5) and packed into a column (15.5 cm × 1.5 cm I.D.). Egg-white feedstock (10 ml) was loaded on to the column and non-bound material removed by washing with 0.025 *M* Tris–HCl buffer (pH 7.5) (50 ml). Bound material was eluted using a linear gradient of 0–0.375 *M* NaCl in 0.025 *M* Tris–HCl buffer (pH 7.5) (150 ml). The bed was equilibrated first with 0.1 *M* Tris–HCl buffer (pH 7.5) (30 ml) and subsequently with 0.025 *M* Tris–HCl buffer (pH 7.5) (200 ml). This cycle was repeated a further four times.

Following the fifth elution cycle, a CIP was

performed. The column was washed with 0.5 *M* NaOH (50 ml) and stood for 12 h. The column was washed successively with water (50 ml), 0.1 *M* Tris–HCl buffer (pH 7.5) (50 ml) and 0.025 *M* Tris–HCl buffer (pH 7.5) (300 ml).

The procedure for five egg-white separations followed by a CIP was carried out a total of twenty times, i.e. 100 cycles. Throughout this study the flow-rate was maintained at 4 ml/min and the column effluent was monitored by absorbance at 280 nm and by conductivity. Data were captured on and processed by a Dionex Model AI 450 data handling and processing system. All operations were carried out at room temperature (15–20°C).

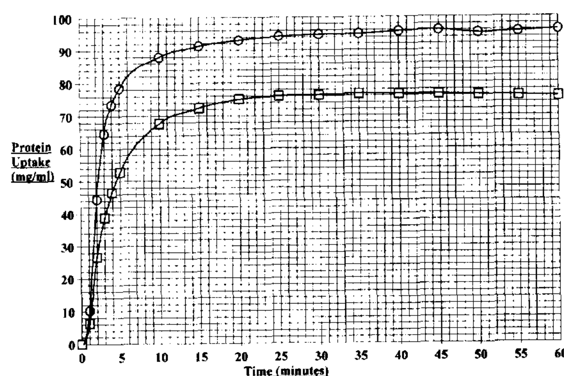

Fig. 1. Kinetic uptake of ovalbumin by (○) Express-Ion Q and (□) Q-Sepharose Fast Flow in 0.025 *M* Tris–HCl buffer (pH 7.5) in a batch stirred tank. Data are expressed as column volume equivalents based on the packing densities in Table 1.

3. Results and discussion

We have previously described the flow performance advantages of Express-Ion media [9,11] compared with more traditional microgranular ion-exchange celluloses. We have demonstrated that these latter products, namely DE52 and QA52, have superior kinetics of adsorption of bovine serum albumin than the agaroses DEAE-Sepharose Fast Flow and Q-Sepharose Fast Flow [3], but their sustainable flow-rates in process columns are significantly lower. The uptake of ovalbumin by Express-Ion Q and Q-Sepharose Fast Flow is represented in Fig. 1, with data expressed in terms of mg ovalbumin/ml column volume for ease of interpretation. The data demonstrate that Express-Ion Q has slightly faster kinetics than Q-Sepharose Fast Flow with 50% uptake for ovalbumin complete in 80 s compared with 130 s for Q-Sepharose Fast Flow. In a packed chromatographic column of bed height 16 cm, assuming 75% (v/v) voidage, then a linear velocity of 150 cm/h equates to a maximum residence time of adsorbate within the column of ca. 5 min. As the flow-rate increases, so the residence time decreases and it may be inferred that kinetic diffusion effects within the matrix of the ion exchanger should have an effect on its chromatographic performance, particularly as flow rates are increased.

Protein capacity is a key parameter influencing throughout and will vary depending on the specific conditions of the separation. A column breakthrough curve obtained using a similar feedstock and operated under conditions representative of those used for preparative chromatography gives a good estimation of the capacity of the exchanger and thus the scale of the process. Egg-white is a multi-component protein mixture [13] and we have reported displacement effects during the anion-exchange purification of the ovalbumin component using Express-Ion D [9] and DE92 [14]. Analysis of the eluting fractions during a breakthrough study by FPLC generates relevant protein binding capacity information [9]. The protein capacity data at 100% (w/w) breakthrough for Express-Ion Q and Q-Sepharose Fast Flow over the flow-rate range 30–700 cm/h (1–20 ml/min) are summarized in Table 2. The data demonstrate that Express-Ion Q has a dynamic capacity for the ovalbumin component of hen egg-white ca. 20% (w/w) greater than Q-Sepharose Fast Flow over the flow-rate range tested. Both media demonstrated a decline in capacity with increasing flow-rate, with similar rates of decline for both media types. This would be expected on the basis of their diffusion kinetics, which were similar for each medium (Fig. 1). In an industrial purification it would generally be undesirable, for economic reasons, to load a column to 100% (w/w) of

Table 2
Protein capacities for Express-Ion Q and Q-Sepharose Fast Flow for hen egg-white proteins during breakthrough

Flow-rate (cm/h)	Protein capacity[a] (mg/ml)			
	Express-Ion Q		Q-Sepharose Fast Flow	
	10% Breakthrough	100% Breakthrough	10% Breakthrough	100% Breakthrough
34	75.5	74.3	n.d.[b]	60.2
68	66.1	76.2	70.9	58.6
136	58.5	72.9	54.8	62.5
204	58.8	65.8	54.9	57.9
272	57.0	61.4	63.9	57.4
340	54.2	61.0	59.0	55.7
680	50.9	62.4	52.2	52.3

[a] Determined as ovalbumin by FPLC.
[b] Not determined.

breakthrough [9] and a more typical loading would be to 10% (w/w) of breakthrough, whereby losses of product may be minimized. By extensive FPLC analysis of the column eluate, we were able to monitor the breakthrough of ovalbumin. From these data we determined a 10% (w/w) breakthrough capacity for this ovalbumin purification. These data are summarized in Table 2. Although there was a reduction in capacity with increasing flow-rate, it was evident that the 10% capacities were very similar to the capacities at 100% breakthrough, indicative of sharp breakthrough. This is reinforced by the fast kinetics of each medium (Fig. 1) and indicates that both Express-Ion Q and Q-Sepharose Fast Flow should be capable of efficient protein purification using process loadings at flow-rates of up to 225 cm/h used in the process-scale investigations.

Hen egg-white contains 63.8% (w/w) ovalbumin [15] and in the process-scale evaluations we loaded columns of Express-Ion Q (24.7 l) and Q-Sepharose Fast Flow (23.1 l) with ca. 4 kg of total protein, i.e. ca. 2.5 kg of ovalbumin [15]. This reflects a loading greater than the maximum dynamic capacity of each column, based on our earlier data (Table 2). The protein capacity data for Express-Ion Q and Q-Sepharose Fast Flow are summarized in Table 3. Since we are dealing with a natural feedstock and it would have been impractical for us to produce and store a pooled feed of 3000 l and use this for each of the six runs, there is a variability in the mass of protein loaded and this is reflected in the mass of protein bound. Notwithstanding feedstock variability, two clear conclusions may be drawn from these studies. First, as the flow-rate increased over the range 75–225 cm/h, the capacity of each medium

Table 3
Protein capacities of Express-Ion Q and Q-Sepharose Fast Flow during preparative chromatography of hen egg-white proteins

Flow-rate (cm/h)	Express-Ion Q		Q-Sepharose Fast Flow	
	Feedstock total protein (g)	Protein adsorbed (g)	Feedstock total protein (g)	Protein adsorbed (g)
75	3823	2189	4347	1916
150	3887	2349	3941	1663
225	3703	1991	4032	1769

decreased. Second, the overall binding capacity of Express-Ion Q was ca. 20% (w/w) higher than that of Q-Sepharose Fast Flow, observations in keeping with those found in the breakthrough studies (Table 2). FPLC analysis of the desorbed protein demonstrated it to be predominantly ovalbumin, with some ovomucoid and ovoglobulin content, observations in keeping with those previously reported for Express-Ion D [9]. Following CIP, the performance of each medium was maintained, further demonstrating the effectiveness of treatment with 0.5 *M* NaOH as a suitable column regenerant, and reinforcing previous studies on Express-Ion Q [11] and Q-Sepharose Fast Flow [16].

In order for us to demonstrate the effect of flow-rate on chromatographic resolution, i.e. an evaluation of desorption kinetics, we carried out a series of analytical egg-white separations on laboratory columns of Express-Ion Q and Q-Sepharose Fast Flow over the flow-rate range 30–1000 cm/h. These chromatograms are presented in Fig. 2. It is evident that the resolution of the conalbumin component, eluting at ca. 120 ml, and the ovalbumin component, eluting at ca. 170 ml, is more sensitive to increasing flow-rate for the Q-Sepharose Fast Flow than for Express-Ion Q. In order to quantify these effects, a degree of resolution may be calculated according to

$$\text{Degree of resolution} = \frac{(V_2 - V_1)}{(W_2 + W_1)} \cdot 2$$

where V_2 = elution volume of ovalbumin, V_1 = elution volume of conalbumin, W_2 = peak width of ovalbumin at 0.608 peak height and W_1 = peak width of conalbumin at 0.608 peak height. These data are presented graphically in Fig. 3, which demonstrates the greater sensitivity to high flow-rates for Q-Sepharose Fast Flow compared with Express-Ion Q, presumably reflecting more impaired diffusion of desorbed protein from the pores of the agarose matrix compared with the cellulose.

The final part of this study was an investigation into the reusability of Express-Ion Q. We have previously reported the effectiveness of a CIP

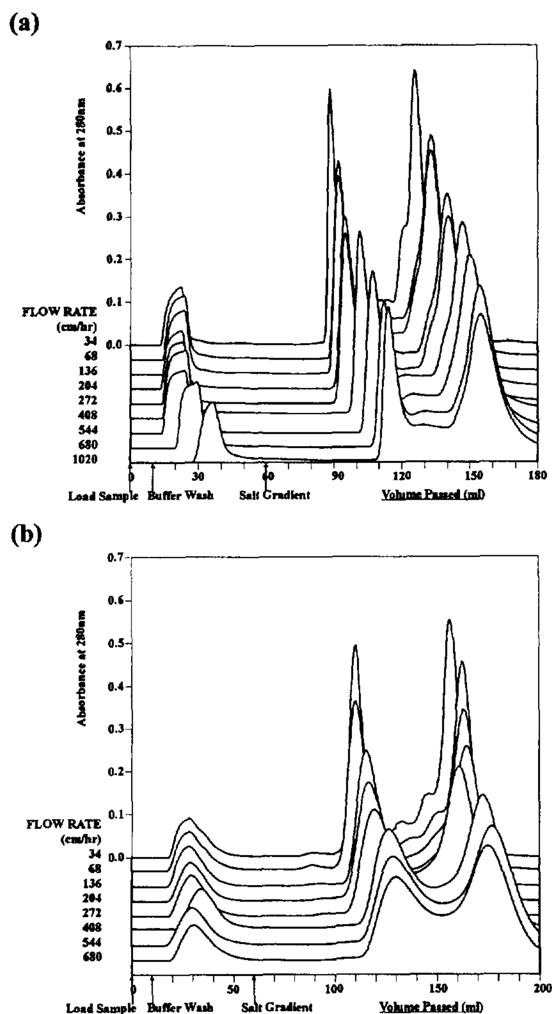

Fig. 2. Column chromatography of hen egg-white protein on (a) Express-Ion Q and (b) Q-Sepharose Fast Flow using 0.025 *M* Tris–HCl buffer (pH 7.5) at flow-rates of 34–1020 cm/h.

using 0.5 *M* NaOH for columns of Express-Ion Q [11] and demonstrated good physical and chemical stability of the medium during the CIP. In the present study we carried out a series of twenty cycles on Express-Ion Q, each involving five consecutive egg-white separations followed by a CIP, maintaining the flow-rate at 136 cm/h. We have superimposed all 100 runs as a single graph in Fig. 4. Although there are minor variations between individual runs, it is evident that the chromatography of hen egg-while feedstock over the 100 cycles is consistent and the CIP pro-

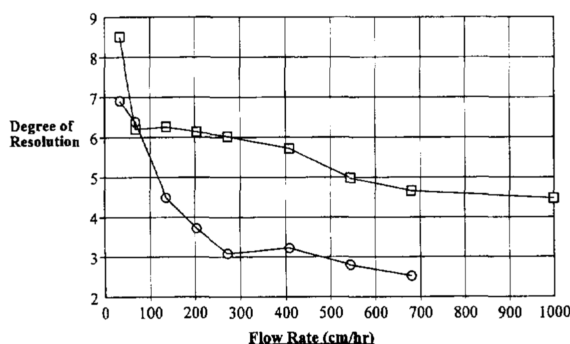

Fig. 3. Influence of flow-rate on the degree of resolution of the conalbumin and ovalbumin components of hen egg-white for (□) Express-Ion Q and (○) Q-Sepharose Fast Flow using 0.025 *M* Tris–HCl buffer (pH 7.5).

cedure has no visible effect on the chromatographic performance of the medium.

In process-scale ion-exchange protein purification, there are a range of adsorbents commercially available. In the present investigation we carried out a comparison of an agarose- and a cellulose-based adsorbent each available for large-scale use. The results demonstrate that both Q-Sepharose Fast Flow and Express-Ion Q are suitable for process-scale protein purification and both give similar chromatographic performance in our hands, with Express-Ion Q having a ca. 20% (w/w) greater binding capacity than Q-Sepharose Fast Flow for ovalbumin. The slightly inferior diffusion kinetics of the agarose matrix may have a disadvantageous effect, but only at flow rates ⩾250 cm/h, particularly with

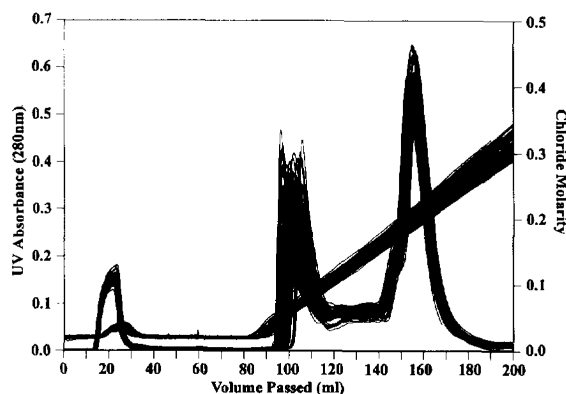

Fig. 4. Column chromatography of hen egg-white proteins on Express-Ion Q using 0.025 *M* Tris–HCl buffer (pH 7.5) at a flow-rate of 136 cm/h over a series of 100 cycles with a CIP after every fifth run.

gradient elution. Over the flow-rate range 75–225 cm/h, a sharp protein breakthrough was observed for each medium, which facilitates efficient adsorptive processes on an industrial scale. It is possible that matrix effects may influence many separations and it was beyond the scope of the present study to investigate these. However, such effects should not be discounted and an appropriate media screening strategy in addition to a flow-rate assessment may be beneficial in throughput considerations.

References

[1] D. Friefelder, Physical Biochemistry, Freeman, San Francisco, CA, 2nd ed., 1982, p. 249.
[2] E.F. Rossomando, Methods Enzymol., 182 (1990) 309.
[3] P.R. Levison and F.M. Clark, Pittsburgh Conference, 1989, Atlanta, GA, Abstracts, p. 754.
[4] P.R. Levison, in G. Subramanian (Editor), Process-Scale Liquid Chromatography, VCH, Weinheim, 1995, p. 131.
[5] W.E. Schwartz, F.M. Clark and I.B. Sabran, LC·GC Mag., 4 (1986) 442.
[6] A. Torres and E.A. Peterson, J. Chromatogr., 499 (1990) 47.
[7] M.E. Osman, A.R. Menzies, B.A. Martin, P.A. Williams, G.O. Phillips and T.C. Baldwin, Phytochemistry, 39 (1995) 409.
[8] P.R. Levison, in J.F. Kennedy, G.O. Phillips and P.A. Williams (Editors), Cellulosics: Materials for Selective Separations and Other Technologies, Ellis Horwood, Chichester, 1993, p. 25.
[9] P.R. Levison, S.E. Badger, D.W. Toome, M. Streater and J.A. Cox, J. Chromatogr. A, 658 (1994) 419.
[10] P.R. Levison, S.E. Badger, D.W. Toome and R.M.H. Jones, in D.L. Pyle (Editor), Separations for Biotechnology 3, Royal Society of Chemistry, Cambridge, 1994, p. 267.
[11] P.R. Levison, S.E. Badger, R.M.H. Jones, D.W. Toome, M. Streater, N.D. Pathirana and S. Wheeler, J. Chromatogr. A, 702 (1995) 59.
[12] P.R. Levison, M. Streater, R.M.H. Jones and N.D. Pathirana, in J.K. Shillenn (Editor), Validation Practices for Biotechnology Products, ASTM STP 1260, American Society for Testing and Materials, Philadelphia, PA, in press.
[13] L. Stevens, Comp. Biochem. Physiol., 100B (1991) 1.
[14] P.R. Levison, S.E. Badger, D.W. Toome, M.L. Koscielny, L. Lane and E.T. Butts, J. Chromatogr., 590 (1992) 49.
[15] W. Bolton, in C. Long, E.J. King and W.M. Sperry (Editors), Biochemists Handbook, Spon, London, 1971, p. 764.
[16] G.K. Sofer and L.-E. Nystrom, Process Chromatography: A Practical Guide, Academic Press, London, 1989, p. 93.