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Rapid ion chromatographic method for the quantitative determination of ligand leakage from ion-exchange media

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Abstract

Ligand leakage is a key issue in the validation of ion-exchange chromatographic processes in many industrial applications. Hydrolysis of ionised functional groups particularly during clean-in-place procedures has been a topic of interest but hitherto generic protocols for identification and quantification of such materials have been unavailable. In the present study we report an ion chromatographic assay suitable for detection of such ionic species. The protocol is robust and is sensitive to levels corresponding to <1% of the total ion-exchange group content of an ion-exchange medium. The effectiveness was demonstrated for a clean-in-place procedure involving 16 h exposure to 0.5 *M* NaOH using the ion-exchange celluloses Whatman Express-Ion Exchangers D, Q, C and S.

Keywords: Ligand leakage; Stationary phases, LC; Ion-exchange media; Diethylethanolamine; Dihydroxypropyltrimethylammonium chloride; Glycolic acid; Isethionic acid

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]. More recently ion exchangers based on composite polymers have been introduced [3]. Studies on the comparative performance of such products have been reported elsewhere [4]. Polysaccharides are hydrophilic with a significant hydroxyl content, providing a surface which can be chemically modified, typically resulting in chemical moieties being ether-linked to the carbohydrate backbone [5]. 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 various aspects have been reviewed elsewhere [6–15].

In the present study we are only interested in the subject of ligand leakage from ion exchangers i.e. the hydrolysis of functional groups during unit operation. The subject of ligand leakage has received some attention in the field of affinity chromatography [16–18], but there has been little reported for ion-exchange media. While the issue of leachables has been mentioned [13] details are limited with some data reported on alkali-promoted hydrolysis of the DEAE group from the agarose DEAE–Sepharose Fast Flow [19]. In this study extended storage in 1 *M* NaOH gave rise to limited hydrolysis of the functional groups from the matrix as determined by

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GC–MS [19]. It is widely accepted that media regeneration protocols are implemented between successive ion-exchange runs with more stringent clean-in-place (CIP) regimes being included periodically [20]. We have reported the effectiveness of 12–16 h contact with 0.5 *M* NaOH in regeneration of columns of the ion-exchange celluloses DE52 [21], QA52 [22], DE92 [23], Express-Ion C [20], Express-Ion D [24], Express-Ion Q [15,25] and the agarose Q-Sepharose Fast Flow [25], when used in process-scale separations.

In the present study we report the development of a rapid ion chromatographic technique for the detection and quantification of potential hydrolysis products of ion-exchange media, and demonstrate its applicability to the assessment of ligand leakage during CIP on a range of anion- and cation-exchange celluloses.

2. Experimental

2.1. Materials

Express-Ion D, Express-Ion Q, Express-Ion C and Express-Ion S were obtained from Whatman International (Maidstone, UK). CG14, CG12 and AG4A guard columns (50 × 4 mm I.D.) and CS14, CS12 and AS4A separator columns (250 × 4 mm I.D.) were obtained from Dionex (Camberley, UK). N,N-Diethylethanolamine, glycolic acid and isethionic acid were from Aldrich (Gillingham, UK). 2,3-Dihydroxypropyltrimethylammonium chloride was a generous gift from Degussa (Hanau, Germany). All other chemicals were of analytical/reagent grade.

2.2. Ion chromatography

Ion chromatography was carried out at room temperature using a Dionex Model 4500 ion chromatograph equipped with a Model II conductivity detector and Model AI-450 data handling and processing system. For N,N-diethylethanolamine a CG14 guard column and CS14 separator column were used. The chromatography was carried out isocratically using 10 m*M* methanesulphonic acid containing 0.15% (v/v) acetonitrile at a flow-rate of 1 ml/min and a 25-μl injection volume. The detector utilised a CMMS-II cation micromembrane suppressor system which was regenerated using 100 m*M* tetrabutylammonium hydroxide. For 2,3-dihydroxypropyltrimethylammonium chloride a CG12 guard column and CS12 separator column were used. The chromatography was carried out isocratically using 20 m*M* trifluoracetic acid containing 1.5% (v/v) acetonitrile at a flow-rate of 1 ml/min and a 25-μl injection volume. The detector utilised a CMMS-II cation micromembrane suppressor system which was regenerated using 100 m*M* tetrabutylammonium hydroxide. For glycolic and isethionic acids an AG4A guard column and AS4A separator column were used. The chromatography was carried out isocratically using 4.9 m*M* sodium tetraborate at a flow-rate of 2 ml/min and a 25-μl injection volume. The column was regenerated using 28 m*M* sodium tetraborate. The detector utilised an AMMS-II anion micromembrane suppressor system which was regenerated using 25 m*M* H_2SO_4.

2.3. Calibration

Calibration data were obtained by duplicate injections at 3 or 4 concentrations ranging from 2.5 to 10 mg/l for diethylethanolamine and dihydroxypropyltrimethylammonium chloride, 0.25–2 mg/l for glycolic acid and 1–5 mg/l for isethionic acid respectively. Each analyte was prepared in 0.025 *M* sodium phosphate buffer (pH 7.4). Calibration curves were computed for each analyte using the Dionex AI-450 data handling system, using a quadratic fit.

In the presence of 0.5 *M* NaOH, the sodium or hydroxyl ions interfered with the ion chromatography of cations or anions respectively. In order to eliminate such interference, solutions of analyte prepared in 0.5 *M* NaOH required dilution to 10% (v/v) with water for diethylethanolamine, dihydroxypropyltrimethylammonium chloride and isethionic acid and to 2.5% (v/v) for glycolic acid, after neutralisation with H_2SO_4. Spike recovery studies were carried out in duplicate by spiking 0.5 *M* NaOH with 25 mg/l diethylethanolamine or dihydroxypropyltrimethylammonium chloride, 10 mg/l glycolic acid or 5 mg/l isethionic acid and

diluting the samples with water as described above, prior to ion chromatography.

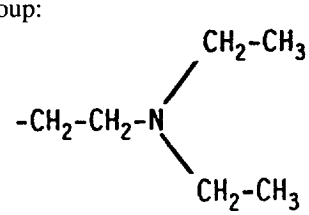
2.4. Ligand leakage studies

Express-Ion D, Express-Ion Q, Express-Ion C and Express-Ion S were equilibrated with 0.025 *M* sodium phosphate buffer (pH 7.4) and packed into 15-ml columns (19 × 1 cm I.D.). Sodium phosphate buffer (0.025 *M*, 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. Samples 1, 2 and 3 were analysed for their respective analyte by ion chromatography as described above.

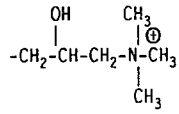
3. Results and discussion

We have previously demonstrated the effectiveness of sodium hydroxide treatment for CIP [15,20–25] and furthermore have reported that storage in 0.5 *M* NaOH is an effective regime for simultaneous sanitization of heavily contaminated columns of ion-exchange media [15,26]. In a typical ion-exchange separation of proteins, the most aggressive mobile phase conditions to which the medium is exposed will be during CIP, where fouled columns require cleaning and regeneration. If leachables derived from the ion exchanger were to co-elute with the product then the consequences could be quite severe. It could be argued that if ligand leakage were to occur during CIP then provided leached material was washed out prior to product elution, it would not pose a problem. While such an argument may be justified, the effects of ligand loss on chromatographic performance including capacity and resolution would likely be significant, rendering the process irreproducible.

Whatman International derivatises the microgranular cellulose matrix with the diethylaminoethyl (DEAE) group:



the 2-hydroxypropyltrimethylammonium (QA) group:



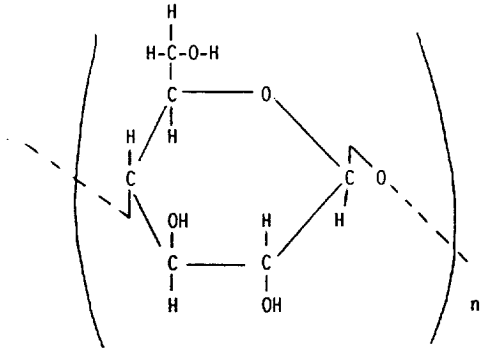
the carboxymethyl (CM) group:

$-CH_2-CO_2H$

and the sulphoxyethyl (SE) group:

$-CH_2-CH_2-SO_3H$

Cellulose is a linear polymer of β-D-glucopyranose with 1,4-glycosidic links:



In each case the functional group is covalently attached to the cellulose in either the 2, 3 or 6 position via an ether linkage to the distal carbon atom [5]. If ligand leakage were to occur then it is reasonable to expect the hydrolysed functional groups to be liberated as alcohols. In the case of Express-Ion D (DEAE) as N,N-diethylethanolamine, in the case of Express-Ion Q (QA) as a 2,3-dihydroxypropyltrimethylammonium salt, in the case of Express-Ion C (CM) as glycolic acid (2-hydroxyacetic acid) and in the case of Express-Ion S (SE) as isethionic acid (2-hydroxyethylsulphonic acid). In a

study on DEAE–Sepharose Fast Flow [19] it was reported that the main ligand leakage product was N,N-diethylethanolamine, a compound readily detectable using GC–MS. In our previous work on Express-Ion D [15] we too reported a GC–MS protocol for determining N,N-diethylethanolamine which was sensitive to 1 mg/l in both buffer and 0.5 *M* NaOH. While this method is suitable it was deemed inappropriate for two reasons. Firstly, the other three proposed leakage products from Express-Ion Q, C and S, respectively, are non-volatile and therefore cannot be readily separated and quantified by GC even following methylation or benzoylation. Secondly, GC–MS systems are relatively expensive instruments to install and use, particularly for only a single application.

We therefore wished to develop a generic separation and quantitation procedure for all four potential leachables. Ion chromatography (IC) is an established ion-exchange technique routinely employed for separation and quantification of small anions and cations. In our earlier work we reported an IC protocol for detecting 2,3-dihydroxypropyltrimethylammonium chloride [15]. In the present study we have improved this method and now report IC methods for the detection and quantitation of all four alcohols described above, using conductivity detection. Typical chromatograms for N,N-diethylethanolamine, 2,3-dihydroxypropyltrimethylammonium chloride, glycolic acid and isethionic acid are represented in Fig. 1a, b, c and d respectively.

Over the concentration ranges tested for each analyte the calibration curves were fitted using a quadratic equation of the form:

$$y = ax^2 + bx + c \quad (1)$$

The quadratic parameters and correlation coefficients computed by the data handling system are summarized in Table 1. For the reasons described above for each analyte present in 0.5 *M* NaOH, the samples required dilution in H_2O. Notwithstanding this dilution, the limits of detection for this assay are summarized in Table 2. The methods are robust and in each case spike recovery studies demonstrate good recovery of each analyte in 0.5 *M* NaOH at the limits of detection (Table 3). Ion chromatograms for each analyte at their detection limits in 0.5 *M* NaOH are presented in Fig. 2.

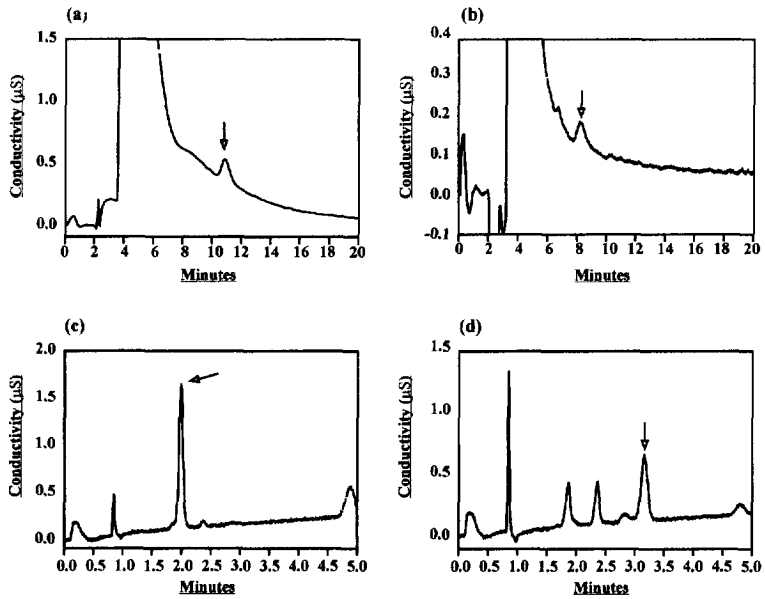


Fig. 1. Ion chromatograms of (a) 5 mg/l N,N-diethylethanolamine, (b) 5 mg/l 2,3-dihydroxypropyltrimethylammonium chloride, (c) 2 mg/l glycolic acid and (d) 2 mg/l isethionic acid. The peak corresponding to each analyte is arrowed respectively. Full analytical details are described in the text.

Table 1
Calibration data for ion chromatography of N,N-diethylethanolamine, 2,3-dihydroxypropyltrimethylammonium chloride, glycolic acid and isethionic acid

Analyte	Quadratic parameters[a]			r^2	n
	a	b	c		
N,N-Diethylethanolamine	$-1.641\cdot10^{-15}$	$8.168\cdot10^{-7}$	0.000	0.9998	6
2,3-Dihydroxypropyl-trimethylammonium chloride	$-4.873\cdot10^{-15}$	$1.364\cdot10^{-6}$	0.000	0.9984	6
Glycolic acid	$2.140\cdot10^{-15}$	$2.657\cdot10^{-7}$	0.000	0.9999	8
Isethionic acid	$4.817\cdot10^{-15}$	$4.817\cdot10^{-7}$	0.000	0.9997	6

[a] $y=ax^2+bx+c$.

Table 2
Detection limits for the ion chromatography of N,N-diethylethanolamine, 2,3-dihydroxypropyltrimethylammonium chloride, glycolic acid and isethionic acid

Analyte	Detection limit (mg/l)	
	Buffer[a]	0.5 *M* NaOH
N,N-Diethylethanolamine	2.5	25
2,3-Dihydroxypropyltrimethyl ammonium chloride	2.5	25
Glycolic acid	0.25	10
Isethionic acid	0.5	5

[a] 0.025 *M* sodium phosphate buffer (pH 7.4).

In the case of isethionic acid there is a contaminating peak in the ion chromatogram (Fig. 1d) eluting at ca. 2.8 min just before the isethionic acid peak, which elutes at ca. 3.1 min. At low levels of isethionic acid i.e. close to the limit of detection, the Dionex AI-450 data handling system was incapable of discriminating between these two peaks and consequently could not integrate them properly. This is illustrated in Fig. 2d. The effect of this is that spike recovery at isethionic concentrations of <10 mg/l may give less than 100% (w/w) recoveries. Problems of this type were not encountered for the other analytes.

The results of the ligand leakage studies for each Express-Ion medium are summarized in Table 4. These data demonstrate that the levels of analyte present in samples 1, 2 or 3 from each ion exchanger were below the limits of detection for that analyte in the respective mobile phase. If chemical hydrolysis of the functional groups were to occur as a result of exposure to NaOH during the column regeneration (CIP) step then the liberated alcohol derivatives should be present in the static NaOH phase present within the column during the CIP, i.e. in the media voidage. This should then be displaced with the post-CIP NaOH flush. The first two displaced column volumes of post-CIP effluent were collected from each column (sample 2) and analysed by IC for their respective leakage product (Table 4). It is evident that no detectable hydrolysis of the functional groups had occurred for Express-Ion D, Q, C or S during the CIP (Table 4), with both samples 2 and 3 giving elution profiles below the limit of

Table 3
Spike recovery data for the ion chromatography of N,N-diethylethanolamine, 2,3-dihydroxypropyltrimethylammonium chloride, glycolic acid and isethionic acid in 0.5 *M* NaOH

Analyte	Spiked concentration (mg/l)	Concentration detected (mg/l)	Spike recovery (%) (w/w)
N,N-Diethylethanolamine	25	24.93	99.7
2,3-Dihydroxypropyl-trimethylammonium chloride	25	24.93	99.7
Glycolic acid	10	10.53	105.3
Isethionic acid	5	3.39	67.8

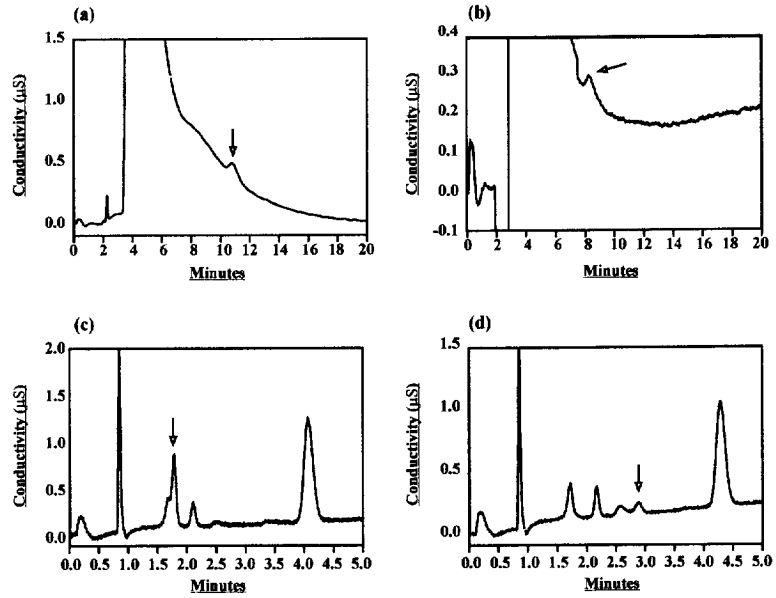


Fig. 2. Ion chromatograms of (a) 25 mg/l N,N-diethylethanolamine, (b) 25 mg/l, 2,3-dihydroxypropyltrimethylammonium chloride, (c) 10 mg/l glycolic acid and (d) 5 mg/l isethionic acid when spiked in 0.5 *M* NaOH. The peak corresponding to each analyte is arrowed. Full analytical details are described in the text.

detection for each analyte. Furthermore spiking of these samples with their respective analyte at levels close to their limits of detection (Table 1) gave recoveries of ca. 100% as described above. Representative ion chromatograms for sample 2 from Express-Ion D and Express-Ion C are presented in Fig. 3a and b, respectively. While these data appear convincing they are in fact ambiguous, at least in terms of validation. In order to reconcile these limits of detection currently expressed in units of concentration, it is necessary to convert them into units of mass such that the percentage hydrolysis of total functional groups bonded to each ion exchanger may be determined. From the detection limits determined for each analyte in 0.5 *M* NaOH (Table 1) it is possible to deduce the mass of analyte above which a signal could be detected in the 30-ml sample 2 obtained for each medium. This is readily converted to moles. From the mass of ion exchanger present in the packed column and the level of functionalisation of the cellulose, as specified in the QC test data supplied by the manufacturer, it is possible to deduce the number of moles of functional groups present in the packed column. On the basis that any hydrolysed analyte must have originated from a finite mass of ion-exchanger contained within the column, it is possible to calculate the minimum percentage hydrolysis of functional groups from the exchanger

Table 4
Ligand leakage studies on Express-Ion D, Q, C and S

Medium	Analyte	[Analyte] (mg/l)		
		Sample 1 pre-CIP	Sample 2 post-CIP	Sample 3 re-equilibration
Express-Ion D	N,N-Diethylethanolamine	<2.5	<25	<2.5
Express-Ion Q	2,3-Dihydroxypropyl-trimethylammonium chloride	<2.5	<25	<2.5
Express-Ion C	Glycolic acid	<0.25	<10	<0.25
Express-Ion S	Isethionic acid	<0.5	<5	<0.5

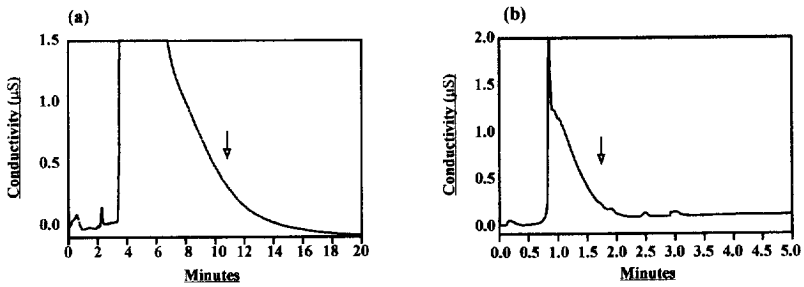


Fig. 3. Ion chromatograms of sample 2 (post-CIP) obtained from columns of (a) Express-Ion D and (b) Express-Ion C. The retention times of N,N-diethylanolamine or glycolic acid are arrowed respectively. Full analytical details are described in the text.

above which analyte is detectable. These calculations are summarized for Express-Ion D, Q, C and S in Table 5. The data demonstrate that the levels of leachables present in the column effluents immediately after a CIP using 0.5 *M* NaOH range from <0.03% (w/w) to <0.21% (w/w) for Express-Ion D, Q, C and S i.e. below their limits of detection.

In this study we report a rapid ion chromatographic method for the detection and quantitation of potential leaked ligands from ion-exchange chromatography media. The method is robust and gives a sensitivity down to less than 1% (w/w) of the total number of functional groups present on a range of cellulose-based ion-exchangers in a typical process situation. Various media manufacturers supply anion and cation-exchangers with similar, but not necessarily identical, functional groups. The IC procedure described in this study should be suitable for use in assaying hydrolysis products of these groups with little, if any, modification. Ion chromatography is a simple technique and as such the procedures developed for this study provide a means by which ligand leakage from ion exchangers can now be routinely measured, and may form the basis for process-specific validation studies.

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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] L.L. Lloyd and J.F. Kennedy, in G. Subramanian (Editor) Process Scale Liquid Chromatography, VCH, Weinheim, 1995, p. 99.
[4] P.R. Levison, C. Mumford and M. Streater, in J.A. Greig (Editor), IEX '96 - Ion Exchange Developments and Applications, 1996, in press.

Table 5
Degree of hydrolysis of functional groups from Express-Ion D, Q, C and S during CIP

Medium	Number of functional groups present in the 15-ml column (µmol)	Detection limit for hydrolysed functional group in 30-ml post-CIP fraction (µmol)	Number of available functional groups hydrolysed during CIP (%) (w/w)
Express-Ion D	3087	6.41	<0.21
Express-Ion Q	2901	4.44	<0.15
Express-Ion C	2834	3.95	<0.14
Express-Ion S	2976	1.01	<0.03

[5] 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.
[6] G.K. Sofer, and L.-E. Nystrom, Process Chromatography. A Guide to Validation, Academic Press, London, 1991.
[7] R.F. Burgoyne, M.C. Priest, K.L. Roche and G. Vella, J. Pharma. Biomed. Analysis, 11 (1993) 1317.
[8] E. Boschetti, X. Pouradier Duteil, C. Nguyen and Y. Moroux, Chim. Oggi, March/April (1993), 29.
[9] N. Adner and G. Sofer, Pharm. Technol. Europe, April (1994), 22.
[10] G. Sofer, Pharm. Technol. Europe, May (1994), 29.
[11] A. Jungbauer and H.P. Lettner, Biopharm, June (1994), 46.
[12] A. Jungbauer, H.P. Lettner, L. Guerrier and E. Boschetti, Biopharm, July/August (1994), 37.
[13] R.J. Seely, H.D. Wight, H.H. Fry, S.R. Rudge and G.F. Slaff, Biopharm, September (1994), 41.
[14] M.O. Budnick, T.A. Cosenza and J. Black, Biopharm, October (1994), 32.
[15] 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.
[16] M. Goldberg, K.L. Knudsen, D. Platt, F. Kohen, E.A. Bayer and M. Wilchek, Bioconjugate Chem., 2 (1991), 275.
[17] M. Baeseler, H. -F. Boeden, R. Koelsch and J. Lasch, J. Chromatogr., 589 (1992), 93.
[18] P. Füglistaller, J. Immunol. Methods, 124 (1989), 171.
[19] M. Anderson, I. Drevin and B. -L. Johansson, Process Biochem., 28 (1993), 223.
[20] P.R. Levison, in M.S. Verrall (Editor), Downstream Processing of Natural Products, John Wiley, Chichester, 1996, p. 179.
[21] P.R. Levison, S.E. Badger, D.W. Toome, D. Carcary and E.T. Butts, in R. de Bruyne and A. Huyghebaert (Editors), Downstream Processing in Biotechnology II, 1989, p. 2.11.
[22] P.R. Levison, M.L. Koscielny and E.T. Butts, Bioseparation, 1 (1990), 59.
[23] P.R. Levison, S.E. Badger, D.W. Toome, M.L. Koscielny, L. Lane and E.T. Butts, J. Chromatogr., 590 (1992), 49.
[24] P.R. Levison, S.E. Badger, D.W. Toome, M. Streater and J.A. Cox, J. Chromatogr. A, 658 (1994), 419.
[25] P.R. Levison, R.M.H. Jones, D.W. Toome, S.E. Badger, M. Streater and N.D. Pathirana, J. Chromatogr. A, 734 (1996) 137.
[26] P.R. Levison and F.M. Clark, Pittsburgh Conference, 1989, Atlanta Abstracts, p. 754.