An automated packed Protein G micro-pipette tip assay for rapid quantification of polyclonal antibodies in ovine serum

Sunil Chhatre [a,*], Richard Francis [b], Daniel G. Bracewell [a], Nigel J. Titchener-Hooker [a]

[a] The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK
[b] Process Science Group, BTG PLC, Blaenwaun, Ffostrasol, Llandysul, Wales SA44 5JT, UK

ARTICLE INFO

ABSTRACT

The demands on the biopharmaceutical sector to expedite process development have instigated the deployment of micro-biochemical engineering techniques to acquire manufacturing insight with extremely small sample volumes. In conjunction with automated liquid handlers, this permits the simultaneous evaluation of multiple operating conditions and reduces manual intervention. For these benefits to be sustained, novel ways are now required to accelerate analysis and so prevent this becoming a throughput bottleneck. For example, although Protein G HPLC is used to quantify antibody titres in bioprocess feedstocks, it can be time-consuming owing to the serial nature of its application. Although commercial options are available that can process many samples simultaneously, these require separate, potentially expensive instruments. A more integrated approach is desirable wherein the assay is implemented directly on a robot. This article describes a high-throughput alternative to antibody HPLC analysis which uses an eight-channel liquid handler to control pipette tips packed with 40 μL of Protein G affinity matrix. The linearity, range, limit of detection, specificity and precision of the method were established, with results showing that antibody was detected reliably and specifically between 0.10 and 1.00 mg/mL. Subsequently, the technique was used to quantify the antibody titre in ovine serum, which is used as feed material by BTG PLC for manufacturing FDA-approved polyclonal bio-therapeutics. The mean concentration determined by the tips was comparable to that found by HPLC, but the tip method delivered its results in less than 40% of the time and with the potential for further, substantial time-savings possible by using higher capacity robots.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The stringent cost and regulatory drivers now facing the pharmaceutical sector have prompted the investigation of a range of approaches to reduce development times and costs. For example, micro scale-down technologies have enjoyed a recent surge in interest and are becoming ever more widely established as viable techniques for accelerating process development [1,2]. When used in conjunction with structured approaches such as factorial design, microscale studies offer the potential for the cost effective and thorough exploration of an experimental space, permitting far higher sample throughput than may be achieved in conventional bench-scale studies [3]. This allows novel products to move through pipelines more quickly while simultaneously enabling the type of thorough process description now encouraged by the Quality by Design initiative [4]. Several advances have been made in the microscale research field for operations such as fermentation

[5,6], microfiltration [7] and chromatography [8,9]. These methods reduce the demands for feed material and increasingly are being implemented alongside automation technologies that enable many experiments to be conducted in parallel, thus allowing far more rapid, accurate and precise operation than that achieved manually. As a result, many data points can be gathered, permitting the generation of comprehensive response surfaces. Furthermore, since the quantities of resources consumed per microscale experiment are negligible (compared with more conventional laboratory- or pilot-scale studies), it becomes easier for a company to tolerate the development costs for studies that fail [10] or for therapeutic candidates that are abandoned following unsuccessful clinical trials.

For the full throughput benefits of these techniques to be realised, however, now requires significant reductions in analytical timescales [11]. Due to the intrinsically slow nature of some assay methods, these can become a major barrier to the high throughput screening of recovery and purification conditions [9] and depending upon the techniques involved, the time periods required to assess product or impurity concentrations can be far longer than the time needed to generate the samples in the first place. For exam-

---

* Corresponding author.
 E-mail address: sunil.chhatre@ucl.ac.uk (S. Chhatre).

ple, in previous work, Chhatre et al. [3] found that the assay times needed to evaluate antibody concentrations in biological samples purified by automated microscale chromatography were an order of magnitude longer than the primary experiment itself. Predominantly this was due to the reliance upon HPLC, which requires extensive manual preparation, consumes significant quantities of buffers and involves long run times [9]. Alternatives to HPLC such as ELISA may require many replicates to achieve adequate confidence in the data and can involve long analysis times (e.g. due to overnight incubations), thus making it difficult to achieve precise and accurate results in a timely fashion. In the long term, the failure to address these concerns will result in analysis becoming a considerable obstacle to the implementation of high throughput process development.

Such issues call for novel high throughput assay techniques which need to be straightforward to use, necessitate little manual intervention and be commensurate with the small sample volumes available from the main scale-down experiment. Potential options available for rapid analysis include the Octet system (FortéBio, CA, USA), the Gyrolab (Gyros, Uppsala, Sweden) and the Bioanalyser (Agilent Technologies, CA, USA), all of which allow evaluation using small analyte volumes. These systems require separate instruments, thus entailing additional capital costs and lead times to train operators and develop robust assay protocols. Although automated liquid handlers can be expensive, if an experiment has been implemented on a robotic platform already, then execution of the assay on the same workstation can avoid further expenditure, thus improving its 'return on investment', and eliminating some of the complexity involved in connecting a robot to standalone analytical equipment. Although a period of familiarisation and training is needed for automation methods, once this understanding has been gained, the close integration of a microscale experiment and its analysis on a robot can reduce turnaround time between studies and so compress development timescales. To exemplify the analytical goals of such an approach, this paper describes a method which uses robotically controlled pipette tips packed with Protein G matrix as an alternative to antibody HPLC. To illustrate the principles involved, the technique was applied for the quantification of the antibody titre in ovine serum samples and the next section describes how the tip method was set-up, along with its comparison with a reference HPLC method.

2. Materials and methods

2.1. Feed material

The crude hyperimmune ovine serum feedstock used in this study was obtained from BTG PLC (Blaenwaun, Ffostrasol, Llandysul, Wales, UK), which uses this material as the feed to manufacture FDA-approved bio-therapeutics such as CroFab[TM] [3]. A 3.6 L bag of serum at −20 °C was supplied by the company and thawed at ambient temperature for 8 h. The serum was agitated gently to ensure a homogenous composition, before being split into separate 150 mL lots for convenience of storage at −20 °C. Whenever needed, serum from each of these lots was thawed, separated into 1.5 mL aliquots in Eppendorf tubes and then refrozen. One of these tubes was then thawed to ambient temperature for every experiment. This prevented the potentially deleterious impact upon protein integrity of repeatedly thawing and refreezing the 150 mL lots or entire 3.6 L bulk whenever serum was required for a study. The antibody concentration in the serum was then determined by HPLC and compared to the result obtained by the Protein G tip method, as described below.
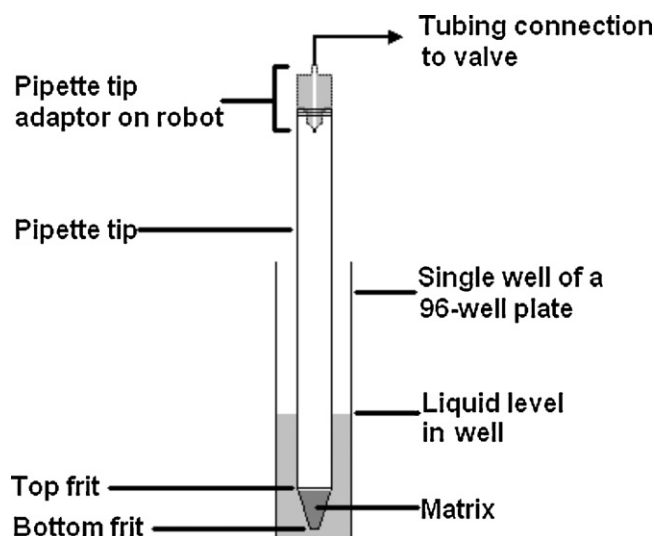

Fig. 1. Schematic outline of the chromatography pipette tip.

2.2. Automated Protein G tip operation

Chromatography pipette tips were obtained from PhyNexus (San Jose, CA, USA), with 40 μL of Protein G affinity matrix packed into the base of the tip between two supporting frits. The tip operation was automated on a Freedom Evo 150 platform (Tecan, Reading, UK) equipped with an eight-channel liquid handling arm capable of pipetting approximately 990 μL per channel, as well as an eccentric plate manipulator and an InfiniTe 200 UV–visible wavelength plate reader (Tecan, Reading, UK). Schematic outlines of the chromatography tip and the robot are shown in Figs. 1 and 2, respectively. The liquid handling arm was set-up with disposable tip (DiTi) adaptors to pick up tips and a 'low DiTi-eject' option to allow tips to be set back in their rack after use. The Protein G tips were operated at 15 μL/s in order to achieve a high throughput and robotic scripts were written in EVOware version 2.1 to control the tips. This included steps to transfer buffers from 100 mL troughs
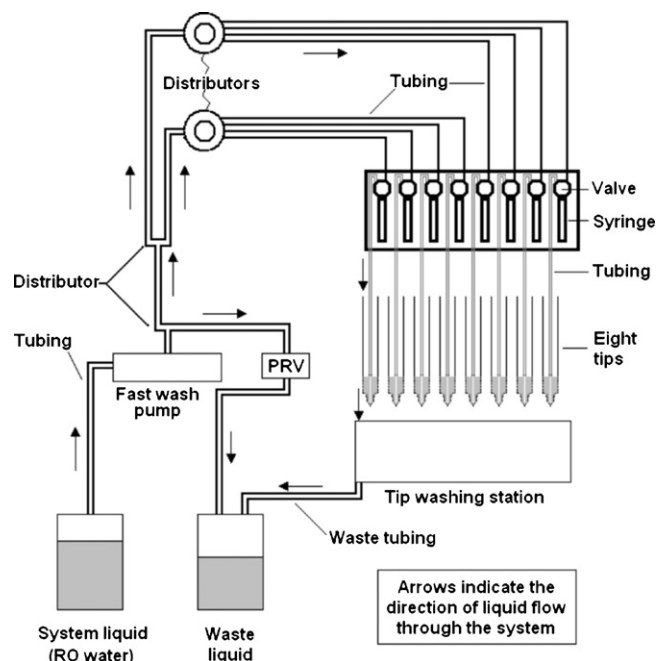

Fig. 2. Schematic layout of the robot. PRV: pressure relief valve.

(Tecan, Reading, UK) into 2 mL deep square 96-well conical-bottom plates (Fisher Scientific U.K. Limited, Loughborough, Leicestershire, UK). Buffers were dispensed into the plates at 300 μL/s using standard 1000 μL non-sterile disposable conductive BioRobotix tips (VWR International, Lutterworth, Leicester, UK). Between pipetting steps for different buffers, flushing steps were employed to clean the tip adaptors and thus reduce the potential for cross-contamination. Gravimetric analysis with between 100 and 500 μL of 10 mM PBS pH 7.40 ± 0.10 (Sigma–Aldrich, Poole, Dorset, UK) confirmed a reproducible ±2% buffer pipetting accuracy of the robot. In the case of the feed, since the serum had already been pre-aliquoted into 1.5 mL lots, it was impractical to hold these volumes in 100 mL troughs and expect the robot to pipette from them accurately and hence serum was transferred into the load plates manually.

2.3. Selection of Protein G tip robotic parameters

The tips required manipulation of robotic parameters in order to achieve efficient contact between the Protein G resin and the liquid. For example, to reduce the chances of exposing the Protein G resin to air at full aspiration, a percentage excess was allowed for the liquid in each of the feed and buffer-containing wells. The excess in each well was set to 10% of the total liquid volume in order to minimise the mass balance error. Based on visual inspection, it was determined that if the tips were positioned to aspirate a few millimetres above the base of each well, the minimum liquid volume in which the tips could reliably remain immersed was 50 μL. Hence the total liquid volume per well was chosen to be 500 μL, with an actual aspirate–dispense volume through the Protein G tips of 450 μL.

It should be noted that unlike the disposable tips used for buffer transfer, the resin bed in the Protein G tips presented a resistance to fluid flow, thus making it necessary to implement measures that allow the pipetted liquid to 'catch-up' with the intended total volume [8]. This was achieved by retaining the tips in the liquid for a further 30 s after every aspirate and after every dispense step using the EVOware timer function, along with the allowance of a 3–5% excess pipetting volume for all feed materials and buffers. Studies with serum and buffers showed that using these settings made it possible to achieve the required 450 μL aspirate–dispense volume (data not shown). The resistance imposed by the matrix also had the deleterious effect of sometimes causing system liquid (water) to drip into the headspace of the tip during dispense steps. This was found to occur due to the use of too low a quality of reverse osmosis water and the problem was avoided almost completely for the duration of the tip run if ultrapure water was used instead. To provide further protection against this problem, an air gap of approximately 500 μL was drawn into the system liquid tubing prior to mounting the PhyNexus tips on to the tip adaptors of the robot. Consequently, it was possible to carry out the complete Protein G tip separation described in the following section with minimal impact from system liquid. It should be noted that for operational convenience, all key liquid handling properties such as flowrate, aspirate–dispense position in the well and system air gap were encoded into a 'liquid class' within the EVOware software. This enabled the robot to re-use exactly the same values for these properties for the Protein G tips during every analytical separation.

2.4. Protein G separation conditions

The buffer operating sequence for the Protein G tips is shown in Table 1 and was the same as that used for the reference HPLC protocol described below. Most of these stages were carried out within individual wells of a 96-well plate, including pre-load equilibration, feed loading, post-load washing, eluting and post-elution water washing. Pre-equilibration water washing for removal of storage buffer and post-run 20% ethanol washing (prior to storage of the Protein G tips in 20% ethanol) were undertaken in 100 mL troughs because these steps did not involve contact with a protein sample and so it was immaterial that the tips shared the same pool of liquid. It should be noted that in these cases, only a small volume of buffer (30 mL) was present in each trough in order to ensure that only the base of the tip was immersed in liquid. This avoided unnecessary entrainment on the side of the tip and so prevented droplets from spilling on to nearby plates when tips were moved around the deck of the workstation. To this end, after completing a pipetting step, tips were also retracted slowly in order to allow liquid to wick off.

It should be noted that although the Protein G tips were re-used, no regeneration steps were employed after each round of processing (e.g. by washing with a dilute basic buffer or a chaotropic salt solution) in order to reduce overall analysis time and also because this mirrors the way in which the HPLC is performed (i.e. there is no such strip between successive rounds of analyte application). Data from the studies described below also indicated that the chosen number of wash and elution steps (three) was adequate for achieving resin washing and desorption. Furthermore, the use of three post-elution water washes and 20% ethanol washes (representing a total of approximately 70 aspiration column volumes across both of these steps) was assumed to be adequate to remove any residual proteins from the matrix prior to subsequent feed application. Similarly, to ensure that the resin was immersed fully during initial water washing and equilibration, these steps also involved three aspirate–dispense cycles in each case. Table 1 summarises the number of aspirate–dispense cycles used for each step. When not in use, the Protein G tips were stored in 20% ethanol at 2–8 °C.

2.5. Antibody quantification in ovine serum by Protein G tips

After setting down the Protein G tips, disposable tips were used to pipette 200 μL of the Protein G elution sample into 96-well polycarbonate plates with a flat, UV-transmissible base (Greiner Bio-One, Gloucester, UK). For convenience, the robotic plate manipulator arm was used to move this plate automatically from its carrier to the UV–visible wavelength plate reader in order to measure the absorbance of each sample at 280 nm. These values were then saved to a spreadsheet. In order to convert these data into antibody concentrations in the serum, the Protein G tips were also used to create calibration graphs using diluted ovine polyclonal IgG. This was produced by reconstituting a 95% pure lyophilised stock into 150 mM sodium chloride (both from Sigma–Aldrich, Poole, Dorset, UK) to an approximate concentration of 50 mg/mL. The exact concentration was determined by the Beer–Lambert law using a 280 nm extinction coefficient of 1.5 mL/(mg cm) in a 1 cm path length UV–visible spectrophotometer. 100 μL stocks of reconstituted IgG were frozen in Eppendorfs and one of these was thawed whenever required for an experiment. The 95% pure IgG stock was then diluted in 150 mM sodium chloride to a range of concentrations to create a set of samples that were loaded on to the tips. The resulting 280 nm elution absorbance values were used to generate a calibration graph in order to quantify the antibody concentration in the unknown samples.

2.6. High performance liquid chromatography

For comparison with the tips, HPLC was used as the reference method to quantify the antibody concentration in the serum [3]. This involved applying a 50 μL sample volume to a 1 mL Protein G HP HiTrap column (GE Healthcare, Buckinghamshire, UK) operated on an Agilent 1200 liquid chromatography system (Agilent Technologies U.K. Limited, Stockport, Cheshire, UK) at a flowrate of 2 mL/min [12]. Samples were held in an autosampler tray in either

Table 1
Sequence of buffers used for operating the Protein G tips. One complete cycle comprises one aspirate and one dispense.

Step	Identity of buffer	Number of aspirate–dispense cycles
Removal of storage buffer (20% ethanol)	Water	3
Pre-load equilibration	25 mM sodium phosphate pH 7.00 ± 0.10	3
Loading	Ovine serum or 95% pure ovine IgG feed	5[a]
Post-load wash	25 mM sodium phosphate pH 7.00 ± 0.10	3[a]
Step elution	100 mM glycine pH 2.70 ± 0.10	3[a]
Wash	Water	3
Storage	20% ethanol	3

All experiments were conducted at ambient temperature.

[a] Value determined by experimentation (see Section 3); other values were chosen to give a large number of column washes for preparing or cleaning the matrix.

sealed 500 μL polypropylene HPLC plates or in 2 mL crimp top vials (both from Essex Scientific Laboratory Supplies, Essex, UK) fitted with 0.1 mL inserts (VWR International Limited, Leicester, UK). The buffers used were the same as those employed for the tips (Table 1) i.e. the equilibrating/washing buffer was 25 mM sodium phosphate pH 7.00 ± 0.10 and the eluting buffer was 100 mM glycine pH 2.70 ± 0.10. Antibody peaks at 280 nm were integrated manually using the Agilent Chemstation software and compared to a standard curve generated by diluting 95% pure ovine IgG into 150 mM sodium chloride (both from Sigma–Aldrich, Poole, Dorset, UK) in the same manner as described above.

2.7. Protein G tip assay characterisation

2.7.1. Determination of the number of Protein G load, wash and elution steps

To achieve adsorption and desorption, samples were aspirated and dispensed through the tips repeatedly in a bidirectional fashion. In order to determine the most suitable number of Protein G tip loading cycles, an uptake curve was created in triplicate for up to eight loading cycles, using 1 mg/mL of 95% pure ovine IgG as the feed. The residual IgG concentration in the load samples was then quantified by HPLC. Following this, the best choice for the number of wash and elution cycles to use in subsequent studies was then determined by applying 0.25, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 mg/mL of pure IgG to the Protein G tips, followed by a total of five wash and five elution steps. Each of these steps was carried out using separate aliquots of wash and elution buffer and the IgG concentration in each sample was calculated by HPLC.

The Protein G assay was then characterised using concepts taken from the Q2 (R1) guidelines for analytical procedures (International Conference on Harmonisation, http://www.ich.org), as outlined below.

2.7.2. Linearity, range and limit of detection

Pure ovine IgG samples of concentrations 3.31×10^{-2}, 6.63×10^{-2}, 1.33×10^{-1}, 2.65×10^{-1}, 5.30×10^{-1}, 1.06, 2.65 and 5.30 mg/mL were prepared in 150 mM sodium chloride, before applying them to the Protein G tips in triplicate. The 280 nm absorbance of 200 μL of the elution sample was read by the plate reader in order to assess the linear range of the response. This experiment was repeated with even more dilute ovine IgG samples (2.07×10^{-3}, 4.14×10^{-3}, 8.28×10^{-3} and 1.66×10^{-2} mg/mL) in triplicate, along with a series of blank samples containing 150 mM sodium chloride alone. The signal to noise ratio was then calculated in order to specify the detection limit and thus the range. For this purpose, a ratio of 2:1 was set in order to define the limit of detection.

2.7.3. Accuracy and precision

In order to determine accuracy and precision, the test sample used was serum and the Protein G tip method was executed to give a total of 96 measurements of antibody concentration, with results compared to those obtained by HPLC. For the Protein G tips, the serum was diluted in 25 mM sodium phosphate pH 7.00 ± 0.10 at a range of factors between 50 and 120 into the linear range of the assay. The diluted serum was applied to the tips and the 280 nm absorbance of the eluate was compared to a calibration curve created using pure ovine IgG. Although spectrophotometric measurements of potentially complex samples would normally overestimate the concentration of a target molecule, in this study the tips were packed with an antibody-specific resin and were operated with a post-load wash (25 mM sodium phosphate pH 7.00) in an attempt to remove any bound impurities from the column prior to elution and thus produce a highly IgG-enriched eluate. This protocol for the tips was similar to that used in a previously described preparative separation for ovine serum, which delivered an antibody eluate that was 95% pure [13]. The ability of the tips to achieve this separation and thus enable accurate IgG quantification was tested as detailed in Section 2.7.4.

To assess intermediate precision of the tips, 32 diluted serum samples were analysed per day for a total of 3 days. To provide an accurate value of the titre for comparison, 90 samples were created by diluting the serum at factors between 4 and 72 into 25 mM sodium phosphate pH 7.00 ± 0.10. The concentrations were then measured by HPLC, with the measurement at each dilution factor repeated a total of five times. All 90 HPLC measurements were made in a single 24-h long period. For both methods, large numbers of samples were used in order to provide an indication of data reliability.

2.7.4. Specificity

As alluded to above, one potential challenge when purifying antibodies from serum is the potential for the co-adsorption of the primary impurity (albumin) with the antibody [14,15]. In the case of the Protein G tips, this could cause some albumin to co-elute with the antibody component, thus affecting assay sensitivity. As indicated above, the operating protocol for the Protein G tips was selected so as to minimise the likelihood of this occurring and to determine the resulting antibody specificity of the Protein G tips, the following studies were undertaken:

(1) *Application of pure albumin.* 96% pure lyophilised bovine serum albumin was obtained from Sigma–Aldrich (Poole, Dorset, UK) and was reconstituted into 10 mM PBS pH 7.40 ± 0.10 (Sigma–Aldrich) to a concentration of approximately 40 mg/mL. The exact concentration was determined using an $\varepsilon_{280\,nm}$ of 0.666 mL/(mg cm) in the UV–visible spectrophotometer and 500 μL stocks of reconstituted albumin were frozen in Eppendorf tubes. Whenever needed for experimentation, one of these was thawed and diluted in 10 mM PBS. 500 μL of albumin at 0.25, 0.50, 0.75, 1.00, 2.00, 3.00, 4.00 and 5.00 mg/mL concentrations were then applied to the Protein G tips. A wide range of albumin concentrations was chosen in
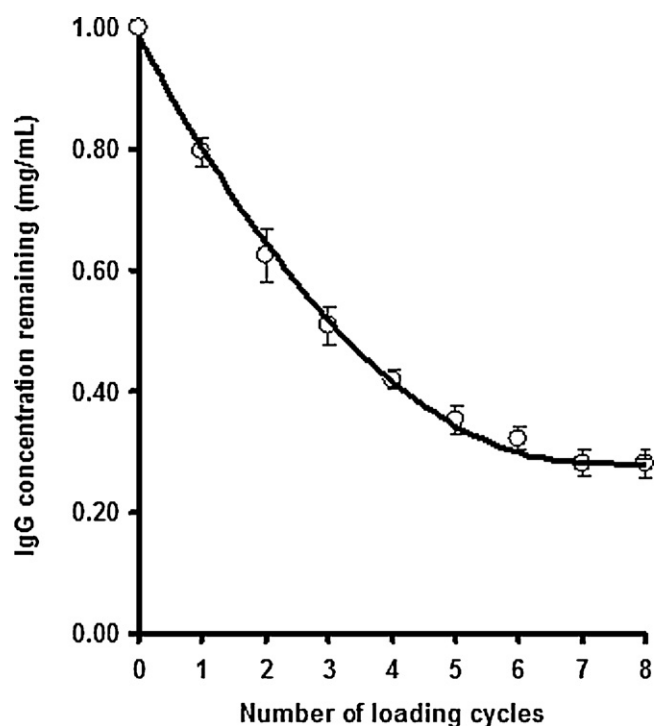
Fig. 3. Pure ovine IgG concentration remaining after applying up to eight rounds of 1 mg/mL feed to the Protein G tips. The y axis plots the IgG remaining free in solution after the number of loading cycles shown on the x axis. Error bars show one sample standard deviation calculated from triplicate values (σ_{n-1}). The majority of uptake occurs within the first five cycles.

Fig. 4. Integrated HPLC elution peak areas for the load, five wash and five elution samples obtained after application of pure ovine IgG to the Protein G tips. The 'Load' samples indicate the residual IgG remaining in the load wells after feed application, while the five wash and elution samples indicate the quantities washed out and eluted in each of those cycles.
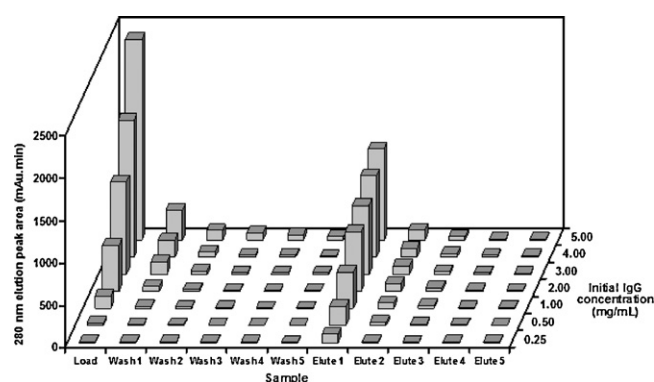
order to test the effect of using both dilute and concentrated samples. The quantities remaining in the load wells were determined by analysing the 280 nm absorbance of 200 μL of those samples in a 96-well polycarbonate plate and comparing those data with a standard curve of 96% pure albumin.

(2) *Albumin spiking study*. Pure albumin at a concentration of 1 mg/mL was spiked into samples containing pure IgG at concentrations varying between 0.2 and 0.9 mg/mL in 0.1 mg/mL increments (i.e. within the linear range of the assay determined by the studies above). The absorbance results from the elution samples were then compared to the values obtained when pure IgG alone was applied to the tips, in order to determine the extent to which co-eluting albumin would interfere with IgG quantification.

3. Results and discussion

3.1. Determination of the number of Protein G load, wash and elution steps

The Protein G uptake curve for up to eight loading cycles with 1 mg/mL purified IgG is shown in Fig. 3. This indicates a steady reduction in the concentration until the fifth cycle, after which the values become more stable, indicating that the matrix is approaching an equilibrium concentration. Only a small reduction was observed between the fifth and eighth cycles and hence for reasons of throughput, it was decided that five loading cycles would be used for all further experimentation. Subsequently, the impact of using up to five washing and elution samples was determined by HPLC (Fig. 4), having first applied feed to the tips for five loading cycles. The figure demonstrates that for all initial IgG concentrations, the residual load and first elution fractions together contained the majority of the antibody. For example, out of every load, wash and elution well at 1 mg/mL, the peak areas for the five wash samples

together constituted only 9% of the total. The residual load material accounted for 19% and the five elution fractions represented a total of 72%, with the first of these samples producing a far larger peak than samples 2–5. Hence for reasons of throughput and accuracy, it was decided that only the first elution fraction would be considered for quantification purposes in the plate reader in all subsequent work. The data in Fig. 4 showed that at every initial concentration, comparatively little protein emerged in the wash fractions and in particular, only a small amount of protein emerged in the fourth and fifth wash and elution cycles. Hence it was decided that subsequently, the operating protocols themselves would employ only three washing and eluting cycles. This was followed by three water washing steps and three 20% ethanol steps before setting down the tips for subsequent re-use.

3.2. Linearity, range and limit of detection

The linearity results are shown in Fig. 5A, with the 280 nm absorbance for the first tip elution sample remaining proportional to the load IgG concentration until 1.06 mg/mL, after which the response becomes non-linear. Subsequently, based upon the blank and extremely dilute samples and using a signal-to-noise threshold of 2:1 in order to specify the detection limit, Fig. 5B was plotted. This shows the ratios for the eight lowest IgG concentrations that were tested and the two points found to lie either side of the threshold corresponded to the 0.0663 and 0.133 mg/mL samples. By interpolating linearly between these two, the IgG concentration which coincided with the 2:1 threshold was found to be 0.1 mg/mL. Linearity was confirmed up to 1.06 mg/mL by a coefficient of determination of 0.99 (Fig. 5C) and hence for practical purposes, the assay range used for subsequent studies was 0.10–1.00 mg/mL.

3.3. Accuracy and precision

The results of the accuracy and precision study are shown in Fig. 6. Out of the 90 samples tested by HPLC, visual inspection suggested that five points were outliers and hence were excluded in order to give a reliable reference value. Similarly for the Protein G tips, one point out of the 96 measurements was judged to be an outlier and was eliminated. From the remaining data, the average concentration determined by HPLC was 37.68 mg/mL, while the outputs from the tips on the 3 days were broadly in line with this result (30.43, 32.47 and 34.94 mg/mL on average). The differences between the tips and the HPLC reference value may be attributed to dilution errors caused by manual pipetting of feed serum and diluent into the 96-well plates. Nevertheless, for all four cases, the
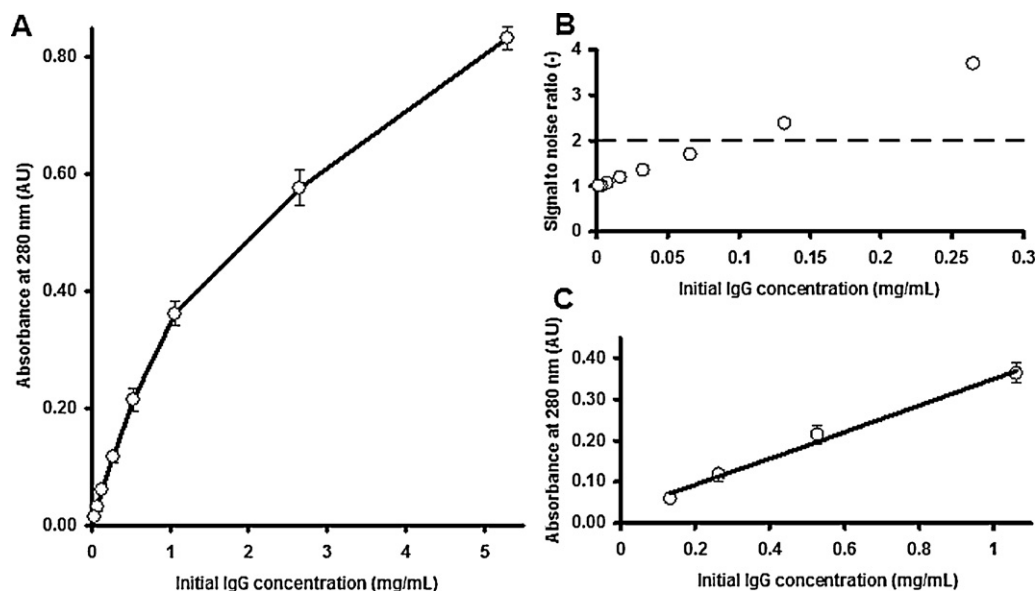
Fig. 5. Linearity data for the tip assay using pure ovine IgG. (A) A_{280} values for the first elution fraction using feed concentrations between 3.31×10^{-2} and 5.30 mg/mL. (B) Elution signal to noise ratios using the eight lowest IgG concentrations (calculated as the average absorbance value after IgG application divided by the average absorbance value after blank sample application). The dashed line indicates the 2:1 detection limit. (C) Subset of the data in (A) ranging from 0.133 to 1.06 mg/mL and the linear trend line ($R^2 = 0.99$ calculated in Microsoft Excel). Error bars show one sample standard deviation of triplicate values.

values were consistent with the 19–41 mg/mL titre range reported previously for this feed material by Newcombe at al. [16]. To quantify the spread of the HPLC and Protein G tip data, the statistics shown in Table 2 were calculated. Considering the full range of the four datasets, there is a wide spread of values which overlap with one another. In the case of the HPLC assay, this may reflect the difficulty associated with manual integration of the elution peak since its lagging edge tails considerably. It is postulated that this is due to the presence of many polyclonal antibody isoforms which have a range of retention times [17], giving rise to an asymmetrical peak and making it difficult to ascertain when the absorbance has returned to baseline. For the tips, it is assumed that the spread of the data is related to errors in the manual dilution and absorbance measurements. Both methods give rise to similar standard deviations, coefficients of variation and 95% confidence intervals. The error bars shown in Fig. 6 represent one standard deviation either side of the mean and the lack of overlap between some of them suggests that there may be a statistically significant difference across the four datasets. Hence for design space characterisation, it was concluded that the tips should be used as a rapid assay for determining the approximate antibody concentration in a high throughput format e.g. for the fast screening of chromatographic separation conditions (e.g. Kelley et al. [18]; Chhatre et al. [3]). Once a good operating region is found, more accurate methods would then be needed to refine the assessment. Although such assays may be more time-consuming, the initial use of Protein G tips to provide an estimate of where operation might be possible could then direct subsequent experimentation and so enable greater value to be extracted from analytical methods that are used later on. In the case of feed-stocks other than polyclonal antibodies (e.g. mAbs from CHO cell culture), this includes assays for both rapid antibody quantification and product quality measurement e.g. the levels of host cell proteins, nucleic acids and product variants such as aggregates or clipped species.

3.4. Specificity

#### 3.4.1. Application of pure albumin

Fig. 7 shows the data obtained after applying 500 μL of 96% pure bovine serum albumin diluted in 10 mM PBS pH 7.40 ± 0.10 at 0.25, 0.50, 0.75, 1.00, 2.00, 3.00, 4.00 and 5 mg/mL to the Protein G tips. The x axis of the graph plots the amount of albumin that was loaded, while the y axis shows the quantity remaining in the load well after being passed through the tips five times. The dashed line indicates the ideal parity relationship between the two quantities (i.e. $y = x$) and not the line of best fit through the data. If there had been any significant deviation from this parity line, then this would have indicated that an appreciable amount of albumin had bound to the resin. Instead, the results show that almost 100% of the loaded albumin remained in the well at all concentrations, again supporting the notion that the method displays adequate specificity for the antibody component. The elution data at all loaded albumin concentrations showed that the absorbance values were indistinguishable from the buffer background (data not shown), indicating that the albumin would be unlikely to interfere with antibody quantification significantly. Especially given that the primary aim of the

Table 2
Statistics for the HPLC and Protein G tips (days 1–3) calculated from the accuracy and precision results to assess the spread of the data.

| Statistic	HPLC	Protein G tips (day 1)	Protein G tips (day 2)	Protein G tips (day 3)
Number of samples	85[a]	32	32	31[a]
Mean antibody concentration (mg/mL)	37.7	30.4	32.5	34.9
Minimum value (mg/mL)	32.6	27.8	28.3	32.4
Maximum value (mg/mL)	42.4	35.3	35.3	38.0
Standard deviation σ_{n-1} (mg/mL)	1.8	1.8	1.7	1.8
Coefficient of variation (%)	4.9	5.9	5.3	5.1
95% confidence interval (mg/mL)	±0.4	±0.6	±0.6	±0.7

[a] For the HPLC and day 3 tip data, a small number of data values were excluded from the original data set because they were judged to be outliers by visual inspection.
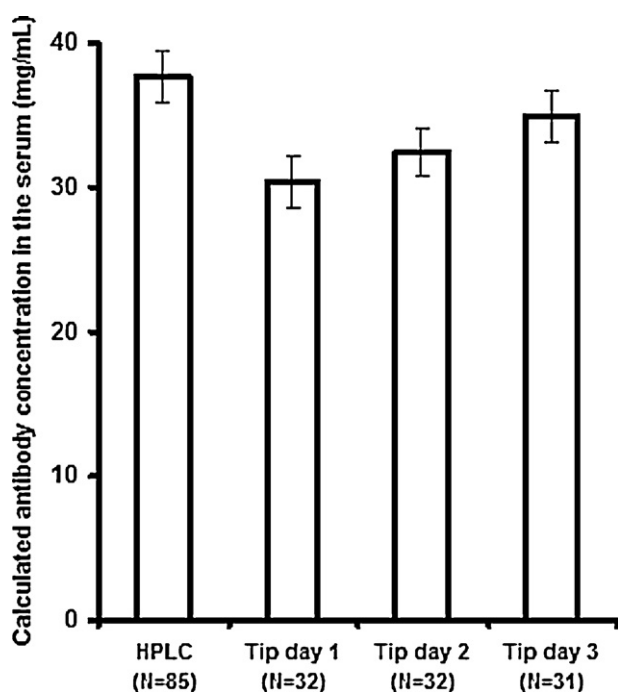
Fig. 6. Accuracy and precision results. Error bars show one sample standard deviation of the values for a total of 85 measurements for the HPLC and for 32 measurements in the first two tip runs (31 in the third). Further statistics quantifying the spread of the data are given in Table 2.
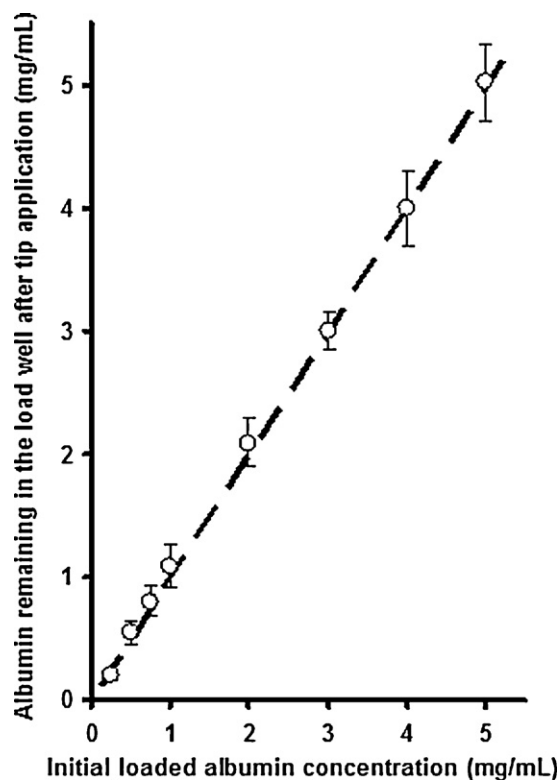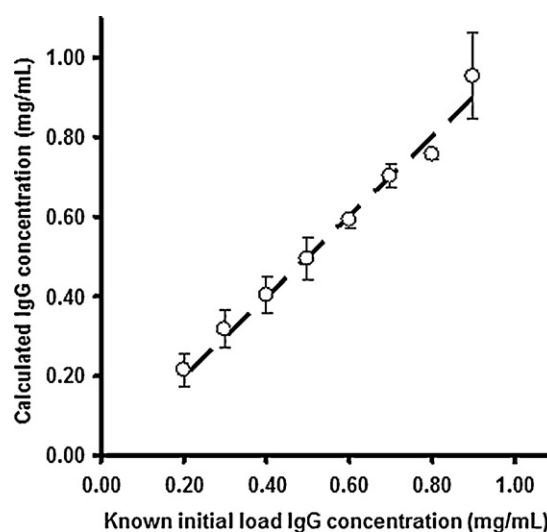
Fig. 8. Impact of spiking albumin into IgG upon Protein G tip specificity. The *x*-axis shows the initial antibody concentration present in spiked samples; the *y*-axis shows the concentration calculated by a calibration curve generated with pure ovine IgG. Error bars show one sample standard deviation of triplicate values. The dashed parity line indicates the ideal proportional relationship between the *x* and *y* axes and not the line of best fit. One of the three values at 0.8 mg/mL IgG was an outlier and therefore was excluded.

studies was to develop a rapid assay for determining good operating regions quickly, the calculated degree of specificity was deemed therefore to be acceptable. To provide further support to this idea, the albumin spiking study was undertaken, as described below.

3.4.2. Albumin spiking study

Fig. 8 shows the results of spiking 1 mg/mL albumin into IgG at concentrations varying between 0.2 and 0.9 mg/mL i.e. within the linear range of the assay determined previously. Absorbance values obtained using pure IgG were used to create a calibration curve and from that, the concentrations of the samples into which albumin had been spiked were then determined. If there was any significant amount of co-elution of albumin with the antibody, then one would expect it to have a significant impact upon the absorbance values and thus lead to artificially higher calculated concentrations. The dashed line on the graph indicates the ideal parity relationship between the *x*- and *y*-axes i.e. that the loaded concentration of antibody in each sample and the amount calculated to be present by the calibration curve were identical. As seen on the graph, virtually all points lie on this line, suggesting minimal interference of albumin with the antibody. In conjunction with the data in Sections 3.4.1 and 3.4.2, it was therefore concluded that the technique displayed adequate specificity for antibody in this feed material.

3.5. Implications of the robotic Protein G tip protocol

3.5.1. Time benefits in using high capacity robotic systems

A comparison of some of the characteristics of the chromatography pipette tip and HPLC methods are given in Table 3. One advantage of the tips lies in running many samples simultaneously (e.g. up to eight samples in parallel on the robot used in this study). For example, the total run time using the tip protocol described above was 60 min. for processing eight samples in parallel (including the time to pipette buffers into plates and perform analysis in the plate reader). By comparison, HPLC is 'serial' in nature and can run only one sample at a time, requiring a 13-min long method to analyse each ovine sample (including 1 min for data evaluation). This results in a total time of 104 min for eight samples and means that the tips could reduce the analytical time by more than 40%.

Fig. 7. Binding of pure albumin to the Protein G tips. The graph shows the relationship between the amount of pure albumin applied and the amount remaining free in the load wells after five loading cycles. The dashed parity line indicates the ideal proportional relationship between the *x* and *y* axes and not the line of best fit. Error bars show one sample standard deviation of triplicate values.

Table 3
Comparison of the tip and HPLC methods.

Characteristic	Protein G tips	HPLC
Number of samples processed simultaneously	8[a]	1
Analysis run time per sample	5 min[a,b]	13 min[b]
Buffer volume requirements	Millilitre-scale	Litre-scale

[a] Based upon an eight-channel liquid handler taking 40 min to carry out the processing steps (using only one elution stage and without the post-run water-washing and 20% ethanol-washing); with higher capacity systems, the processing time per sample could be reduced considerably compared to HPLC, although capital costs would need to be considered (Section 3.5.2).
[b] Including times for analytical separation, collection and storage of data.

This calculation assumes that the tips are cleaned after each run to enable their re-use. If it was decided that the tips would not be re-used (allowing one to forgo the post-run water and 20% ethanol steps and also to use only one elution stage), or if the tips were set back in their rack to await cleaning later when the robot was otherwise unoccupied, this would reduce the processing time to approximately 40 min (i.e. 5 min per sample, thus saving 62% of the time taken by HPLC). Although the processing time for the tips would double when eight calibrated standards are applied, the HPLC time would also double, meaning that the relative time saving achieved by the tips would be maintained.

If a 12- or 96-channel system was used instead of the eight-channel pipettor available for this study, then potentially this could carry out 12 or 96 purifications in parallel in the same 40 min period that it took to perform eight runs. If it is assumed that eight standards were used, taking a total of 40 min for the tips and 104 min for the HPLC, then Table 4 shows the total amount of processing time needed for execution of each technique and the time saving that the tips could make relative to HPLC. Thus, for 12 and 96 samples, respectively, the tips could save 69% and 94% of the analytical time. These time savings are theoretical calculations based upon extrapolation of the durations specified above and do not accommodate any downtime that may occur between different runs (e.g. replenishing buffers troughs or adding new plates to the deck of the robot). Conversely, a few minutes could be saved if buffers were to be pipetted from the troughs into the 96-well plates by the disposable conductive tips at a higher speed than used in this study. Nevertheless, even if the analytical times were slightly different to those given above (e.g. if the HPLC method was shortened slightly and the tips took slightly longer over 96 samples), a conservative estimate for the time saving using the 96-channel system would still be approximately 90%. If such a reduction could be achieved, then this would enable a researcher to carry out a primary experiment, analysis and data evaluation in one working day, decide which experiment to conduct next and perform any manual preparation tasks (e.g. generating any stocks of concentrated buffer solutions that may be required) in readiness to start the study the following morning.

3.5.2. Capital expenditure requirements

Although the order of magnitude saving in analytical time described above would reduce turnaround times between successive experiments considerably, it is important to consider the capital expenditure for the robotic equipment needed. In particular, 96-channel systems are likely to be expensive and will require one plate to be dedicated to every bidirectional cycle for every buffer (e.g. three plates for water washing, three plates for pre-load equilibration, etc.). Hence, a wide robotic platform may also be needed to accommodate the necessary plate carriers and the purchase cost of such equipment could therefore be higher than if a narrower system was acquired. Hence, it would be necessary to determine whether the value gained by such high throughput data acquisition would merit the level of investment required, or whether a lower capacity system would suffice. As outlined above, one mitigating factor is that if a commitment has been made to purchase a robot for microscale development studies, then opting for a highly specified system can facilitate its use for both primary experimentation and analysis, thus avoiding the need for separate high throughput analytical equipment. Although highly specified robots are expensive and one could use that money to purchase a high throughput HPLC system instead, investing in a liquid handler may be more justifiable in those situations where many other types of experimentation are required rather than only dedicated HPLC analysis.

If a high specification robot is deemed worthwhile, then one possibility that can boost throughput even further is to use a system consisting of two liquid handling arms that pipette independently of one another, with a plate manipulator arm positioned in-between. In this set-up, the liquid-handling arm on one side of the platform is dedicated to conduct the main purification study, while the pipetting arm on the other side is used for analysis. The manipulator arm is then used to move plates from one side of the workstation to the other. In this way, the analysis arm can be used to commence preparation activities such as dispensing buffers or equilibrating the Protein G resin while the purification arm is conducting its tasks simultaneously. For example, if an ion exchange pipette tip is being used to screen separation conditions for an antibody and has finished its feed loading step, the manipulator arm could move that plate over to allow access to the Protein G analysis arm while the ion exchange tips enter their post-load wash step. In this way, analysis can begin while the primary experiment is still underway. This could also be achieved if two separate robots were purchased and located side by side, with a shuttle to move plates between the systems. By comparison, if an HPLC is not located physically next to a robot, then manual plate movement is required. Although this is straightforward enough, it may not be operationally convenient if a microscale purification is running overnight, because an operator must be available to move the plate.

3.5.3. Further improvements

Further improvements to the tip method could be achieved if different volumes or types of resin were used. Thus the linear elu-

Table 4
Processing times for HPLC and Protein G tips with 8, 12 and 96-channel liquid handlers, assuming only one elution stage and without the post-run water- or 20% ethanol-washes (i.e. per-sample analysis time = 5 min). For both HPLC and the tips, it was assumed that eight calibrated standards would be employed. The processing time for each HPLC sample was 13 min.

Capacity of the robotic pipetting system (equal to the number of samples being processed)	Protein G tips			HPLC			Percentage time saving for the tips relative to HPLC
	Sample time (min)	Calibrated standards time (min)	Total (min)	Sample time (min)	Calibrated standards time (min)	Total (min)	
8	40	40	80	104	104	208	62
12	40	40	80	156	104	260	69
96	40	40	80	1248	104	1352	94

tion range achieved with the 40 μL bed used in this study could be extended upwards by using more resin (e.g. the 80 or 160 μL matrix volumes that are available from PhyNexus) or by using a higher capacity resin, in order to increase the amount of antibody that is bound. This would reduce the need for sample dilution prior to loading and so increase the operational convenience of the method. As indicated above, manual feed dilution and pipetting into the 96-well plate may contribute to errors in the tip assay. Hence the use of high accuracy-low volume pipetting systems may overcome this constraint and thus improve performance.

One final issue is the method by which elution is achieved. At present, a step elution is employed to recover the product, whereas for other feed types and operating protocols, a gradient elution may be more appropriate. To achieve this in the tips will require the use of a series of elution aliquots of varying strengths in successive steps and may therefore increase the number of cycles for which the elution is carried out. This may therefore increase the processing time and is an issue that would need to be resolved in order to maintain an adequate level of throughput.

4. Conclusions

This paper has described the development of a high throughput alternative to Protein G HPLC for the quantification of polyclonal antibody in ovine serum. The method is based on the use of chromatography pipette tips packed with 40 μL of Protein G resin and automated on an eight-channel liquid handler. The tips were found to be reliable for detecting antibody concentrations in the 0.10–1.00 mg/mL range and the technique was used to calculate the immunoglobulin titre in crude hyperimmunised samples of ovine serum. The calculated titres were found to be broadly similar to those obtained by a reference HPLC method, but were achieved in less than 60% of the time, with up to an order of magnitude reduction in time possible if a 96-channel robotic workstation was used instead. As such, the method has potentially significant value in the bioprocessing field for accelerating early stage development studies.

References

[1] M. Micheletti, G. Lye, Curr. Opin. Biotechnol. 17 (2006) 611.
[2] G.J. Mannall, J.P. Myers, J. Liddell, N.J. Titchener-Hooker, Biotechnol. Bioeng. 103 (2009) 329.
[3] S. Chhatre, D.G. Bracewell, N.J. Titchener-Hooker, J. Chromatogr. A 1216 (2009) 7806.
[4] A.S. Rathore, H. Winkle, Nat. Biotechnol. 27 (2009) 26.
[5] S.R. Lamping, H. Zhang, B. Allen, P. Ayazi Shamlou, Chem. Eng. Sci. 58 (2003) 747.
[6] A. Zanzotto, N. Szita, P. Boccazzi, P. Lessard, A.J. Sinskey, K.F. Jensen, Biotechnol. Bioeng. 87 (2004) 243.
[7] N.B. Jackson, J.M. Liddell, G.J. Lye, J. Membr. Sci. 276 (2006) 31.
[8] M.D. Wenger, P. DePhillips, C.E. Price, D.G. Bracewell, Biotechnol. Appl. Biochem. 47 (2007) 131.
[9] J.L. Coffman, J.F. Kramarczyk, B.D. Kelley, Biotechnol. Bioeng. 100 (2008) 605.
[10] N.J. Titchener-Hooker, P. Dunnill, M. Hoare, Biotechnol. Bioeng. 100 (2008) 473.
[11] S. Chhatre, N.J. Titchener-Hooker, J. Chem. Technol. Biotechnol. 84 (2009) 927.
[12] B. Balasundaram, D. Nesbeth, J.M. Ward, E. Keshavarz-Moore, D.G. Bracewell, Biotechnol. Bioeng. 104 (2009) 134.
[13] A.R. Newcombe, C. Cresswell, S. Davies, K. Watson, G. Harris, K. O'Donovan, R. Francis, J. Chromatogr. B 814 (2005) 209.
[14] E. Boschetti, Trends Biotechnol. 20 (2002) 333.
[15] S. Chhatre, N.J. Titchener-Hooker, A.R. Newcombe, E. Keshavarz-Moore, Nat. Protoc. 2 (2007) 1763.
[16] A.R. Newcombe, C. Cresswell, S. Davies, F. Pearce, K. O'Donovan, R. Francis, Process Biochem. 41 (2006) 842.
[17] S. Chhatre, R. Francis, N.J. Titchener-Hooker, A.R. Newcombe, E. Keshavarz-Moore, J. Chromatogr. B 860 (2007) 209.
[18] B.D. Kelley, M. Switzer, P. Bastek, J.F. Kramarczyk, K. Molnar, T. Yu, J. Coffman, Biotechnol. Bioeng. 100 (2008) 950.