Journal of Chromatography A, 1272 (2013) 56–64

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Improving pH gradient cation-exchange chromatography of monoclonal antibodies by controlling ionic strength

Liangyi Zhang[a], Thomas Patapoff[b], Dell Farnan[a], Boyan Zhang[a,*]

[a] Protein Analytical Chemistry, Genentech Inc., South San Francisco, CA 94080, USA
[b] Early Stage Pharmaceutical Development, Genentech Inc., South San Francisco, CA 94080, USA

ARTICLE INFO

Article history:
Received 25 September 2012
Received in revised form
18 November 2012
Accepted 23 November 2012
Available online 29 November 2012

Keywords:
Ion-exchange chromatography (IEC)
Salt mediated pH-gradient IEC
Monoclonal antibody (mAb)
Ionic strength
pI values

ABSTRACT

Analytical ion exchange chromatography (IEC) is widely used to profile the charge heterogeneity of therapeutic monoclonal antibodies (mAbs). Since conventional salt gradient IEC methods are product-specific and time-consuming to develop, a previously reported alternative pH gradient IEC (pH-IEC) method using a cation-exchange column has been shown to be a multiproduct charge sensitive separation method for mAbs with isoelectric points between 7.3 and 9.0 [1]. In the work presented here, we have extended the application of that pH-IEC method to also profile the charge heterogeneity of mAbs with extreme pI values (e.g. acidic with pI < 7 or basic with pI > 9). A key observation of our work is that for the buffer systems used by Farnan and Moreno [1], the ionic strength of the mobile phase containing multiple polyamine buffers is pH and concentration dependent, and the ionic strength decreases when the pH increases. For the mobile phase with high buffer concentration the ionic strength is high at low pH values, leading to the flow through of acidic mAbs on the cation-exchange column. The basic mAbs may not have an optimal elution profile as the relatively low ionic strength of the mobile phase reduces the resolution of pH-IEC. To modulate the ionic strength, we introduced a salt gradient in addition to the pH gradient. Studies were performed to optimize the buffer and salt concentrations simultaneously to improve the retention of low pI mAbs and the resolution of high pI mAbs. The optimized salt-mediated pH-IEC method was not only applicable to mAbs over a broader pI range from 6.2 to 9.4, but also offered better resolution for mAbs with pI values between 7.3 and 9.0 than the previously reported pH-IEC method. This salt-mediated pH-IEC method was demonstrated to be robust at various chromatography conditions and capable of assessing manufacturing consistency and monitoring degradation of mAbs.

1. Introduction

Proteins like monoclonal antibodies (mAbs) have mostly charged and polar amino acids at the surface in an aqueous environment [2]. Because of molecular interactions with the solution components, the surface residues can undergo multiple chemical and enzymatic modifications, leading to a heterogeneous mixture of protein variants with slight differences on their electrostatic surfaces [3–6]. Ion-exchange chromatography (IEC) is considered the gold standard for profiling the charge heterogeneity of protein therapeutics [5,7–11]. Analytical IEC methods using pH gradients have emerged as alternative techniques to conventional salt gradient IEC for profiling the charge heterogeneity of therapeutic proteins [1,12–16]. In this technique, proteins are typically loaded on a cation-exchange stationary phase and eluted by increasing the pH of the mobile phase. It has recently been demonstrated that a pH gradient IEC (pH-IEC) method with a relatively broad pH window from 6.0 to 9.5 not only provided better resolution than traditional salt-gradient IEC, but also offered multi-product capability through the analysis of 12 monoclonal antibodies (mAbs) with pI values from 7.3 to 9.0 [1]. That pH-IEC method is also highly tolerant to sample matrix with varied ionic strengths (0–250 mM NaCl) and pH values (5.0–8.5) [1]. Furthermore, the reported pH-IEC method is not evidently impacted by the column length and chemistry, so fast separations with shorter columns can be achieved to improve the throughput of protein variant analysis. According to a recent validation report [16], the developed pH-IEC method has shown great robustness and suitability to be used as a quality control system assay in the biotechnology industry.

Despite the many advantages, the reported pH-IEC method was intended primarily for the mAbs with pI values in the studied range of 7.3–9.0. The fact that the elution profile of a mAb can vary with different buffer compositions and concentrations, and the pH values at which the mAbs elute indicates that pH-gradient IEC involves

* Corresponding author at: Protein Analytical Chemistry, MS #96B, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA. Tel.: +1 650 225 3743; fax: +1 650 225 3554.
E-mail addresses: zhang.boyan@gene.com, bzhang@gene.com (B. Zhang).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2012.11.060

a combined ionic-strength and pH-gradient elution mechanism [1]. This is also consistent with the reports published by Anderson, Pabst and their coworkers, respectively, on pH-gradient anion-exchange chromatography (pH-AIEC) [17–20]. With an increasing number of mAbs in the development phase in the biotechnology industry, especially more low-pI mAbs that show potentially longer half-life based on the animal studies [21], it is highly desirable to expand the applicability of pH-IEC methods to a broader range of therapeutic mAbs. Therefore, in order to extend the application of the previously reported pH-IEC method and improve its resolution and robustness, it is imperative to conduct a systematic investigation into how the ionic strength affects the pH-IEC separation of mAbs.

In this work, we first investigated the pH and ionic strength profiles of the published method to examine for analyzing mAbs having extreme pI values. To further expand the pH-IEC, we evaluated the effect of ionic strength on the pH-IEC separation by using different buffer concentrations and the addition of salt at different concentrations to the pH gradient. By modulating the ionic strength, we aimed to develop a robust, salt-mediated pH-IEC method to profile the charge heterogeneity of mAbs over a broad pI range from 6.2 to 9.4.

2. Experimental

2.1. Materials

All mAbs were manufactured in-house at Genentech (South San Francisco, CA) using stable Chinese Hamster Ovary (CHO) cell lines. The pI values for the mAbs used were determined experimentally using an icIEF protocol from the instrument manufacturer [22] employing seven pI markers. Thermal stressed samples were obtained by incubating mAbs at 40 °C for 3 and 6 weeks, respectively. The stressed mAbs were stored at −80 °C before chromatographic analysis.

Propac WCX-10 columns were purchased from Dionex (Sunnyvale, CA). Imidazole was bought from EMD Biosciences (La Jolla, CA). Piperazine (anhydrous) was acquired from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Hydrocholoric acid (6 N), sodium chloride, and Trisma (Tris) were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ).

2.2. HPLC set up

Cation-exchange chromatography experiments were primarily performed on a Waters 2796 BioAlliance liquid chromatography instrument (Milford, MA). The instrument included a low-pressure quaternary gradient pump, an auto-sampler with temperature control capability, a thermal column compartment for precise temperature control, and a dual-wavelength UV detector. At the outlet of the column, an in-line pH sensor (Model S450CD from Sensorex, Garden Grove, CA) and a conductivity sensor (Model 529 from Amber Science, Eugene, OR) were connected in tandem. The pH sensor was controlled by a model Seven Multi pH meter from Mettler Toledo (Columbus, CA); the conductivity sensor was controlled by a model 1056 digital conductivity meter from Amber Science (Eugene, OR). The pH and conductivity readings from the two meters were collected into Chromeleon through a Dionex UCI 50 analog/digital convertor. Instrument control, data acquisition, and data analysis were performed with Dionex Chromeleon software, version 6.8 (Sunnyvale, CA).

2.3. Mobile phase preparation

A stock buffer solution containing 40 mM of piperazine, 40 mM imidazole, and 40 mM Tris (all free bases) was first prepared without adjusting the pH value and stored at room temperature. Prior to chromatographic experiments, a series of the mobile phase buffers containing equimolar concentration of piperazine, imidazole and Tris at 1, 2, 4 or 8 mM were each made by diluting the buffer stock solution with deionized water. The pH values of the buffers were then adjusted using hydrochloric acid to 5.0 (Buffer A) and 10.8 (Buffer B), respectively. A sodium chloride solution of 0.5 M was prepared with deionized water (Salt Solution). The mobile phases were then individually filtered through a 0.2 μm nylon filter prior to use.

The mobile phase buffers with 11.6 mM piperazine, 1.5 mM imidazole and 2.4 mM Tris were prepared as reported in literature [1,16]. A 10-fold concentrated stock solution containing 116 mM piperazine, 15 mM imidazole and 24 mM Tris was first prepared and stored at room temperature. Before each experiment, two aliquots of the stock solution were diluted 10-fold with deionized water and their pH values were subsequently adjusted using hydrochloric acid to 5.0 (Buffer C) and 9.5 (Buffer D). The mobile phases were then individually filtered through a 0.2 μm nylon filter prior to use.

2.4. Cation-exchange chromatography

Unless stated otherwise, the chromatographic conditions were as follows. mAb samples (control and stressed) were diluted to 2 mg/mL with deionized water and were kept at 5 ± 3 °C in the auto-sampler. A 4 mm × 250 mm Dionex Propac WCX–10 column was used for chromatographic separation and placed in the column compartment with the temperature setting at 40 ± 1 °C. For each chromatographic run, 10 μL of protein (20 μg) was injected.

The previously reported pH gradient was established according to Ref. [1] with minor modification. The starting pH of 6.0 in the reference was changed to 5.0 in this work. Buffers C and D were used to establish the pH-gradient elution. The gradient (min, %D) was as follows: 0, 0%; 2, 0%; 47, 100%; 51, 100%; 52, 0%; 59, 0%. The mobile phase flow rate was 1.0 mL/min and proteins were detected by ultraviolet (UV) absorbance at 280 nm.

The salt-mediated pH gradient was established by using a ternary gradient formed on the quaternary pump using buffer A, B and the Salt Solution (0.5 M NaCl) to replace the Buffer C. A linear gradient from 100% A to 96.8% B and 3.2% salt solution in 58 min was delivered to establish a pH gradient from 5.0 to 10.8 (0.1 pH unit/min) and a mediating salt gradient from 0 to 16 mM NaCl (0.28 mM/min). The final gradient (min, %B and %C) was as follows: 0, 0% B and 0% C; 2, 0% B and 0% C; 60, 96.8% B and 3.2% C; 64, 96.8% B and 3.2% C; 65, 0% B and 0% C; 72, 0%B and 0% C. The mobile phase flow rate was 1.0 mL/min. Proteins were detected by ultraviolet (UV) absorbance at 280 nm.

2.5. Modeling of the pH-IEC

The pH of the linearly mixed gradient of two pH buffers was estimated using the Henderson–Hasselbalch (H–H) equation for each of the components based on ideal solution model.

First, the number of available/dissociable protons was determined for each starting buffer and subsequently for each pH value between the two buffers at a step of 0.1 pH unit based on Eq. (1).

$$\mathrm{pH} = \mathrm{p}K\mathrm{a} + \frac{[A^-]}{[HA]} \quad (1)$$

where Ka is the association constant while $[A^-]$ and $[HA]$ represent the concentration of the deprotonated and protonated forms of a buffer component, respectively.

Second, based on the required number of protons, the molar ratio of the two buffers was derived for each pH value. The

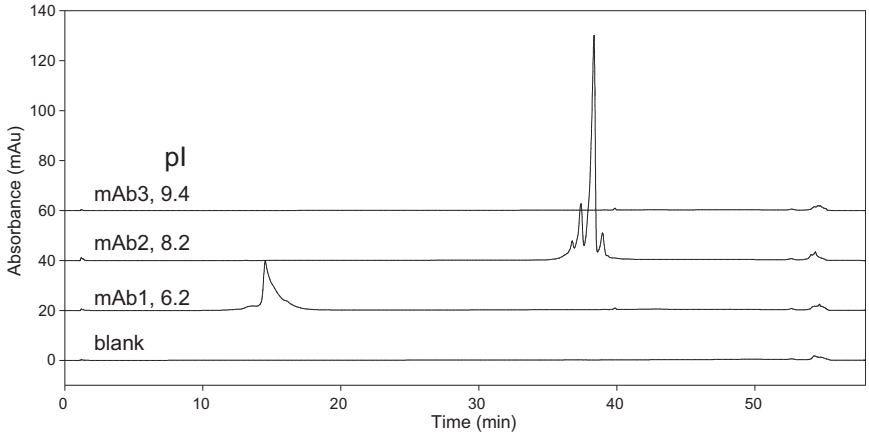

Fig. 1. The charge heterogeneity profiles of mAb1–3 obtained with the reported pH-gradient IEC method.

percentage of each buffer to attain a pH point in the gradient was thus obtained. With this established correlation between buffer percentages and pH values, the pH value at a given percentage of the two buffers was estimated with an accuracy of 0.1 pH unit.

Third, at each pH point, the ionic strength was calculated using the estimated ionic components as shown in Eq. (2).

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i \quad (2)$$

where c_i and z_i represent the concentration and charge of an ionic buffer component. The estimated pH value and ionic strength were plotted as a function of retention time or percentage of buffers.

3. Results and discussion

3.1. Assessment of the reported pH-IEC method

Although the reported pH-IEC method has been demonstrated to be capable of profiling the charge heterogeneity of multiple mAbs, it is intended primarily for mAbs with pI values from about 7.3 to 9.0. For mAbs beyond this range (pI < 7 or pI > 9, also referred to as extreme pI values), the pH-IEC method often yields unacceptable charge heterogeneity profiles. To demonstrate this caveat, we reproduced the reported pH gradient following the procedure that was previously reported with a slight modification [1,16]. The buffers were composed of 11.4 mM piperazine, 1.5 mM imidazole and 2.4 mM Tris, and pH adjusted to 5.0 and 9.5, respectively. The pH range was extended from 6.0–9.0 to 5.0–9.5 so that the low-pI mAbs can be eluted in the linear pH gradient. Three mAbs spanning a wide range of pI values (6.2, 8.2 and 9.4) were analyzed and the resulting chromatograms are shown in Fig. 1. Of these three mAbs, only mAb2 (pI = 8.2) showed an acceptable charge heterogeneity profile characterized by a good separation of charge variants. The charge variants of the low pI mAb1 (pI = 6.2) were not well separated; the high pI mAb3 (pI = 9.4) did not elute during the pH gradient. Even though mAb3 was eluted when the pH gradient was extended to 10.8, the column back pressure was close to the upper pressure limit of the column and the chromatography profiles were inconsistent between different runs. This experiment clearly demonstrated that although the reported pH-IEC method worked well for mAbs with pI values between 7.3 and 9.0, it was not able to profile the charge heterogeneity of mAbs with the extreme pI values.

3.2. Root cause analysis of the limitation of the reported pH-IEC method

To understand why the reported pH-IEC method did not work for mAbs with the extreme pI values, we monitored a series of chromatography parameters, including pH and conductivity at the column exit along with column back pressure. As shown in Fig. 2A, as the pH at the column exit increased from 5.0 to 9.5, and the conductivity of the solvent decreased in a near-linear fashion from 2700 to 800 μS/m (for reference, the conductivity of 5 mM KCl is 720 μS/m while the conductivity of deionized water is 5.5 μS/m). The three pH buffer components are all amines with p*K*a values over a broad range: piperazine with p*K*a1 = 5.68 and p*K*a2 = 9.82, imidazole with p*K*a = 6.95 and Tris with p*K*a = 8.10 (at 25 °C). These compounds are protonated (positively charged) when the solution pH is lower than its p*K*a, but become neutral when the pH is above its p*K*a. When the solvent pH increases, the buffer components gradually become neutral via protonation and thus the conductivity of the buffer decreases. It is noteworthy that the pH profile was concave at pH around 6 because the piperazine was the most abundant component in the buffer so that the pH curve was relatively flat around its p*K*a1 of 5.68.

The pH and ionic strength profiles of the pH gradient were also calculated based on an ideal solution model shown as dashed lines in Fig. 2A. The modeled pH curve is very similar to the experimental pH profile except that the experimental profile was delayed by about 6 min because of the initial 2 min hold at pH 5.0 and the system dwell volume and column volume. Likewise, the modeled ionic strength curve showed similar shape to the conductivity profile observed experimentally. The agreement between the modeling and experimental data suggests that the mixing of amine-based buffer components follows the ideal solution model. The established model can thus be used to estimate experimental pH and ionic strength profiles for other chromatography conditions.

Furthermore, the column back pressure during pH-IEC significantly increased with the pH of mobile phase (Fig. 2B). This is attributed to the decrease of ionic strength, considering that the composition of the mobile phase was constant during the pH gradient. When the ionic strength of the mobile phase is low, the electrostatic potential on the stationary phase surface becomes high, according to the double layer model [23,24]. The high electrostatic potential may change the conformation of the resin (e.g. swelling the resin to reduce the surface charge density), which likely increases the column back pressure [25].

The experimental conductivity and the modeled ionic strength profiles can be used to explain the poor charge heterogeneity

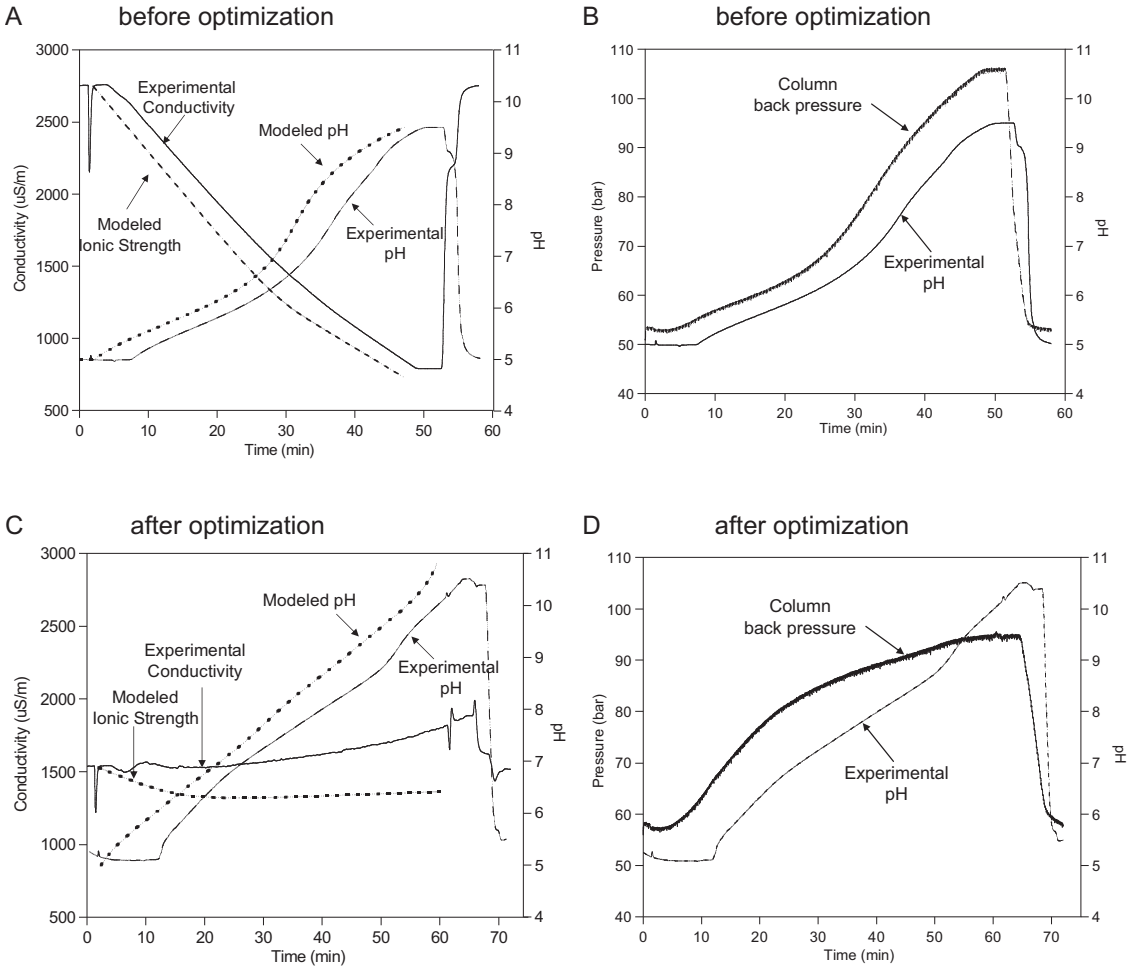

Fig. 2. (A) The ionic strength and pH profiles at column exit and (B) the column back pressure in the pH gradient before optimization; (C) the ionic strength and pH profiles at column exit and (D) the column back pressure in the salt-mediated pH gradient after optimization.

profiles for mAbs with extreme pI values. Low-pI mAbs elute in the low pH region where the buffer components are protonated and the mobile phase has a relatively high ionic strength. Since the pH gradient IEC separation appears to involve a combination of ionic strength-based and pH-based elution mechanisms [17–20], this combination likely leads to poor resolution of the low-pI mAb charge variants. On the other hand, high-pI mAbs typically elute in the high pH region where the buffer components become neutral. Because of the low ionic strength in mobile phase in the high pH region, these high-pI mAb are difficult to elute off of the cation exchange column. In order to confirm that the ionic strength significantly affects the pH-IEC separation and improve the pH-IEC method for mAbs with extreme pI values, we modulated the ionic strength of the pH buffer in the pH-gradient IEC method as discussed below.

3.3. Improving the pH-gradient IEC method by controlling ionic strength

The ionic strength during the course of a pH gradient was modulated in two ways. First, the ionic strength at the low pH region was controlled by using different concentrations of buffers. A series of buffer concentrations were tested to assess their impact on pH gradient IEC as discussed below. Second, the ionic strength at the high pH region was modulated by adding a salt gradient to the pH gradient. The impact of the salt concentration was also investigated. The resulting new method is thus referred to as a "salt-mediated pH-IEC" method.

3.3.1. Buffer concentration

In this work, we propose to use equimolar concentrations of buffer components to form the pH-gradient rather than the mixed ratio used in the reported method [1] for two reasons. First, based on the established model discussed above, a near-linear pH gradient can be obtained by using equimolar concentrations of piperazine, imidazole and Tris (Fig. 2C). The established linear gradient over a broad range of pH would not sacrifice the separation for a given pH region [13]. Second, with a linear pH gradient, we can investigate if the elution pH of a mAb affects the resolution. Based on the yielded information, we may optimize the gradient slope at different pH regions to achieve improved resolution.

Four buffers consisting of equimolar concentrations of piperazine, imidazole and Tris at 1, 2, 4 and 8 mM were investigated. These buffers are referred to as 1, 2, 4 and 8 mM buffers and each was mediated with a linear salt gradient from 0 to 16 mM NaCl. The chromatograms of mAb1 (pI = 6.2) with the four buffers are displayed in Fig. 3A. The resolution between the charge variants evidently depended on the buffer concentration. With the 1 mM buffer, the charge variants were poorly separated. The resolution improved with the 2 mM buffer and peaked with the 4 mM buffer.

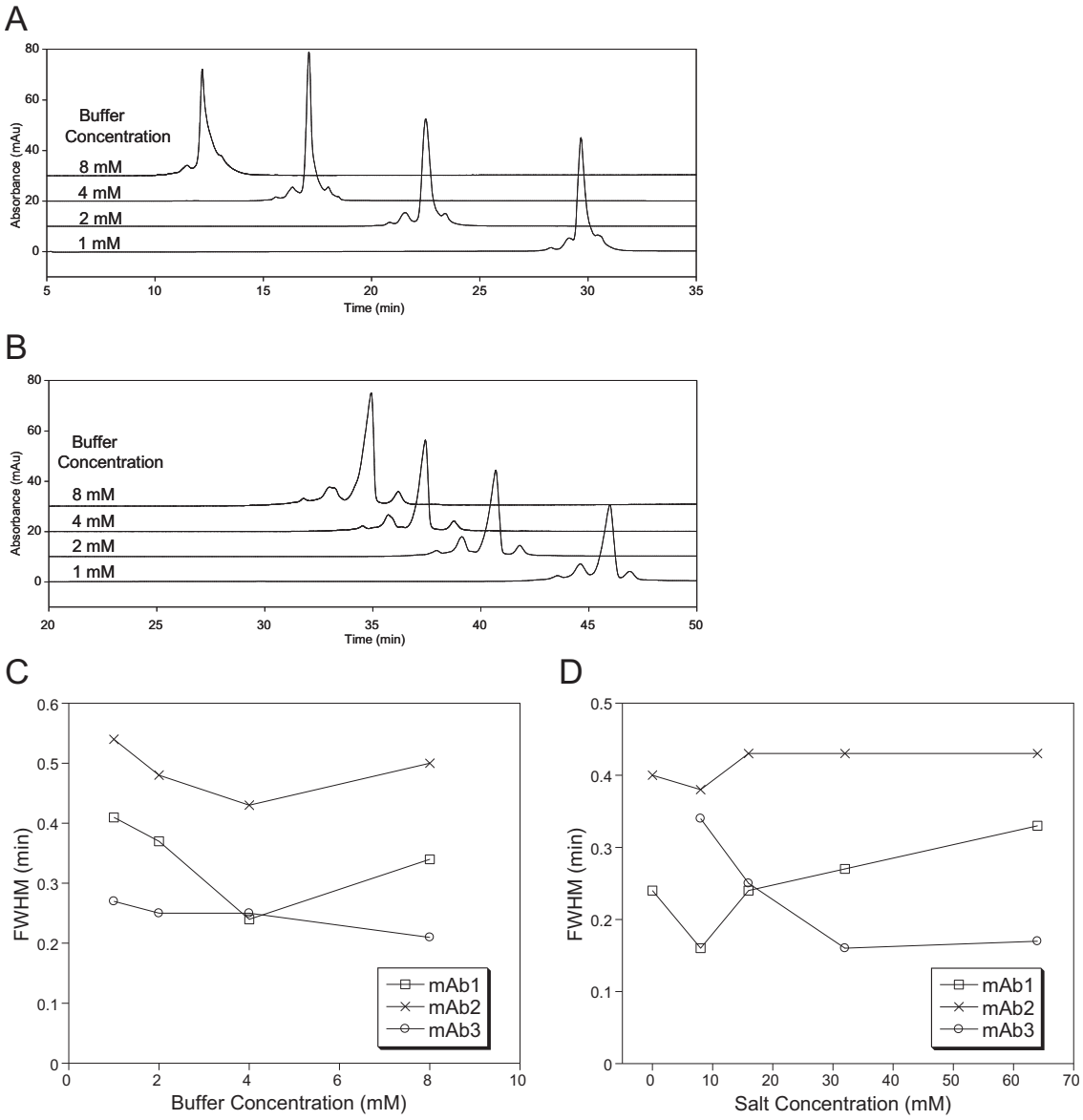

Fig. 3. The salt-mediated pH IEC chromatograms of (A) mAb1 and (B) mAb2 obtained with four buffer concentrations. The full-width at half-maxima (FWHM) of the main peak in the chromatograms of mAbs obtained with different (C) buffer compositions and (D) salt concentrations.

However, the resolution significantly decreased with the 8 mM buffer. On the contrary, the resolutions for mAb2 (pI = 8.2) were less sensitive to the buffer concentration than mAb1 (Fig. 3B). Good resolution for mAb2 (pI = 8.2) was achieved with all four buffers even though the 4 mM buffer offered slightly better resolution than the other three buffers. Based on above visual inspection, the 4 mM buffer appeared to provide the best resolution for mAb1 and mAb2.

To better visualize the effect of buffer concentration on pH-IEC, the full width at half maximum (FWHM) of the main peak of mAbs plotted as a function of buffer concentration is shown in Fig. 3C. The FWHM of the main peak generally correlates with the resolution of pH-IEC in that the lower the FWHM represents the higher resolution. For both mAb1 and mAb2, the FWHM of the main peak with the 4 mM buffer was lowest among the four buffers, suggesting that the 4 mM buffer provided the narrowest peak width, thus good resolution. On the contrary for mAb3 (pI = 9.4), the FWHM of the main peak slightly decreased when the buffer concentration increased from 1 to 8 mM. Thus the 8 mM buffer likely provided the best resolution for mAb3.

The effect of buffer concentration on pH-IEC of mAbs depended on the pI value of a mAb. The mAbs with low (6.2) and mid pI (8.2) values showed optimal separation with the 4 mM buffer, while the mAbs with high pI value (9.4) appeared to prefer higher concentration buffers. This is reasonable since the high pI mAbs strongly bind to the column and thus may require more ionic strength-based elution than low- and mid-pI mAbs to achieve optimal resolution. Since the buffer concentration and conductivity evidently impact the resolution of mAbs in pH-gradient IEC, these should be optimized for each individual mAb whenever high resolution is desired. In this work, we aimed to establish an IEC method that can resolve acidic and basic variants from the main peak for mAbs over a wide pI range. The 4 mM buffer appeared to meet this requirement and thus was chosen for the multi-product salt-mediated pH-IEC method.

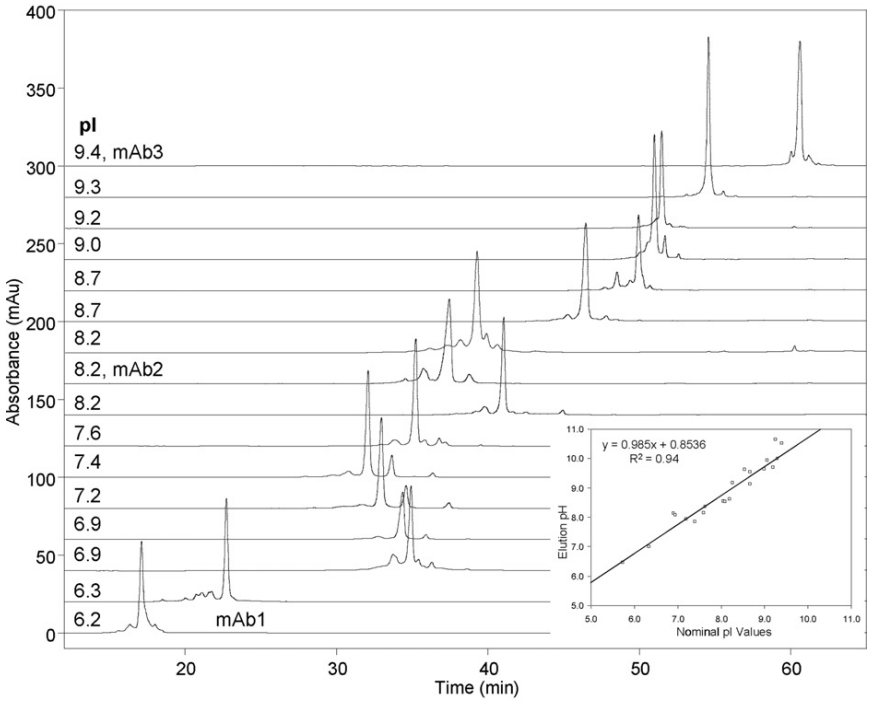

Fig. 4. The charge heterogeneity profiles of 16 mAbs with pI from 6.2 to 9.4 obtained with the salt-mediated pH-IEC method.

3.3.2. Salt concentration

To investigate how the ionic strength affects the pH-IEC separation, five different levels (0, 8, 16, 32 and 64 mM) of sodium chloride were added to the pH gradient (established by the 4 mM buffer) through a linear gradient. mAb1, mAb2 and mAb3 were analyzed in parallel. The FWHM of the main peak of the mAbs were plotted as a function of salt concentration as shown in Fig. 3D. For mAb1, the FWHM of the main peak was highly sensitive to the salt concentration and it reached the minimum with 8 mM NaCl. This suggests that 8 mM NaCl provided the best resolution for mAb1. For mAb2, the FWHM of the main peak was essentially flat across the entire range of salt concentrations, suggesting that the salt concentration did not evidently impact the resolution of mAb2. On the contrary for mAb3, the FWHM of the main peak decreased as the salt concentration increased from 8 to 32 mM and remained unchanged between 32 mM and 64 mM of salt. This suggests that mAb3 required 32 mM of salt to achieve optimal resolution.

The effect of the ionic strength on pH-gradient IEC of mAbs also depended on the mAb's pI. The low pI mAbs showed optimal separation with 8 mM of salt; the high pI mAbs appeared to prefer 32 and 64 mM of salt. Although the mid-pI mAbs showed good resolution with 0–64 mM of salt, it achieved the best resolution with 8 mM of salt. Because of the evident impact on resolution, the salt concentration should be optimized for each individual mAb whenever high resolution is desired. Among the five salt concentrations, the pH-IEC method with a salt gradient of 16 mM NaCl provided acceptable resolution for mAbs with pI values over a broad range from 6.2 to 9.4 and thus it was chosen as the multi-product salt-mediated pH-IEC method in this work.

3.3.3. The optimized salt-mediated pH-IEC method

Based on the above discussion, the optimized salt-mediated pH-IEC method employed 4 mM piperazine, 4 mM imidazole, and 4 mM Tris to establish the pH gradient and was mediated with a linear salt gradient from 0 to 16 mM of NaCl. The pH and conductivity profiles of the method are shown as solid lines in Fig. 2C. For comparison, the modeled pH and ionic strength are shown as dashed lines. The experimental pH at the column exit increased with the retention time in a roughly linear fashion, except for a small concave region at pH from 8.5 to 9.0; the experimentally observed pH profile is mainly comparable with the modeled pH curve except for a delay time of about 6 min, which is largely due to the initial 2 min equilibration and system void volume. The experimentally observed pH curves seem not smooth as the modeled ones, which may reflect that the pH transition time might be slightly different among the three different polyamine buffer components [26]. With the salt mediation, the experimental conductivity of the mobile phase showed just a slight increase during the pH gradient (from 1570 to 1800 μS), but overall remained fairly constant in contrast to the original method (Fig. 2A). Likewise, the modeled ionic strength was essentially constant during the pH gradient. The ionic strength of the amine-based pH gradient was successfully controlled by reducing the buffer concentration and adding a linear salt gradient. As shown in Fig. 2D, when the solution ionic strength was modulated the column back pressure profile became totally differently from that before the optimization (Fig. 2B). The pressure began to plateau when the solution pH went beyond pH 8. It was maintained below 95 bar even when the experimental pH reaches 9.5 (Fig. 2D). This improvement enhanced the robustness of the salt-mediated pH-IEC method. Robustness test of this method is discussed below.

3.4. Profiling the charge heterogeneity of 16 mAbs

To further demonstrate the multi-product capability of the new salt-mediated pH-IEC method, 16 mAbs with pI values from 6.2 to 9.4 were analyzed and their chromatograms are shown in Fig. 4. For both low pI mAb1 (6.2) and high pI mAb3 (9.4), the charge variants were well separated to yield acceptable charge heterogeneity profiles. This is a substantial improvement compared to the reported pH-IEC method (Fig. 1). The charge variants of all 16 mAbs were well separated, indicating that the developed salt-mediated pH-IEC method was capable of profiling the charge

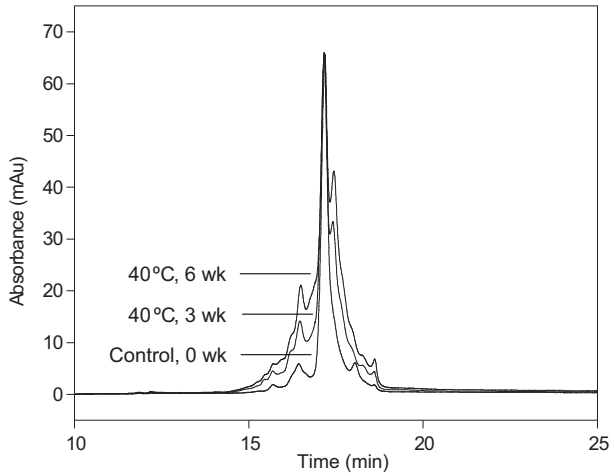

Fig. 5. The chromatograms of native (0w) and thermally stressed mAb1 (1w and 3w at 40 °C) obtained with the salt-mediated pH-IEC method.

heterogeneity of multiple mAb products without any additional method development effort. This improvement is quite significant as the ion-exchange separation of charge variants of those very basic antibodies (pI > 9.0) is often very challenging. With the implementation of the salt-mediated pH gradient IEC, the method development time could be trimmed significantly.

In addition to broader applicability, the salt-mediated pH gradient offered better resolution than the reported pH-IEC method. For mAb2 (pI = 8.2), the salt-mediated pH-IEC method provided a baseline resolution between the charge variants (Fig. 4), a clear improvement in resolution over the previous pH-IEC method (Fig. 1). Although the salt-mediated pH-gradient portion of the method was longer (58 min) than the previous pH-gradient (45 min), the gradient slopes in the two methods were identical (0.1 pH unit/min). The improved resolution by the salt-mediated pH-IEC method was thus not a result of a change in gradient length, but rather from the effect of controlling the ionic strength.

3.5. Monitoring the thermal stability of mAbs

Ion-exchange chromatography is commonly used to assess the batch-to-batch production consistency and stability profile of biopharmaceutical proteins during manufacturing [7]. To demonstrate the ability to monitor protein degradation, the developed salt-mediated pH-IEC method was used to profile the charge heterogeneity of mAb1 after thermal stresses. mAb1 was chosen in this study because it has the lowest retention among the mAbs and its pH-IEC profile was most susceptible to changes in chromatography parameters.

The chromatograms of control and stressed materials of mAb1 are normalized with the main peak (Fig. 5). After the thermal stresses, both acidic and basic variants increased. A new peak also appeared to the right of the main peak for the stressed samples. These profile changes evidently indicate that mAb1 degraded after incubation at 40 °C for 3 and 6 weeks. Likewise, the degradation of mAb2 and mAb3 under thermal stresses was also detected by the salt-mediated pH-IEC method (data not shown).

3.6. Robustness test of the salt-mediated pH-IEC

Because of the complex elution process of the salt-mediated pH-IEC method, it is necessary to ensure its robustness for routine sample testing. As discussed above, we know that the pH buffer composition and the salt concentration affect the retention and resolution of mAbs. Additional studies were conducted to investigate the variability originating from different columns, buffer lots, and instruments when the optimized pH buffer composition and salt concentration were used. Three columns, three buffer lots, and two instruments were tested in four different days. The experimental design is shown Table 1. mAb1 was again chosen in the studies because its pH-IEC profile was most susceptible to changes in chromatography parameters. Four replicate analyses of mAb1 were performed for each experiment and thus a total of 16 chromatograms were obtained.

Table 1
The experimental design for the robustness test of the salt-mediated pH-IEC using mAb1.

	Day 1	Day 2	Day 3	Day 4
Waters 2796	X	X	X	
Dionex U3000				X
Column Lot 1	X			
Column Lot 2		X	X	
Column Lot 3				X
Buffer Lot 1	X	X		
Buffer Lot 2			X	
Buffer Lot 3				X

Table 2
Summary of the robustness data ($n = 16$) of the salt-mediated pH gradient obtained for mAb1.

	Acidic variants	Main peak	Basic variants
Average	12.32	78.99	8.69
Highest	13.20	79.92	9.54
Lowest	10.54	77.91	8.14
STD deviation	0.78	0.56	0.55
% RSD	6.3	0.7	6.4

The chromatograms of mAb1 obtained with different columns and buffer lots were comparable in resolution, but those obtained with different instruments showed slightly different retention times. The difference in the delay volumes expected between instruments accounted for the variation in the retention times observed, but this difference does not impact the performance of the method. The quantitation of the charge variants of mAb1 is summarized in Table 2. For the 16 chromatograms obtained with two different instruments, three columns and three buffer preparations, the quantitation of the charge variants was consistent, indicating that the salt-mediated pH-IEC method was robust at these chromatography conditions.

The salt-mediated pH-IEC method was also robust across a wide range of sample mass loadings on column. As shown in Fig. 6, consistent elution profiles were observed when 5–200 μg of mAb1 was loaded on the column. Although the main peak slightly broadened when the column load was over 50 μg, the quantitation of the charge variants was consistent among all the tested column loads (Table 3).

Table 3
The robustness of the charge heterogeneity of mAb1 at different column loads.

Column load (μg)	Acidic variants	Main peak	Basic variants
5	12.66	79.04	8.30
10	13.04	78.84	8.12
50	13.27	78.14	8.59
100	13.58	77.80	8.62
200	13.55	77.86	8.59
Average	13.22	78.34	8.44
STD deviation	0.38	0.57	0.22
% RSD	2.9	0.7	2.6

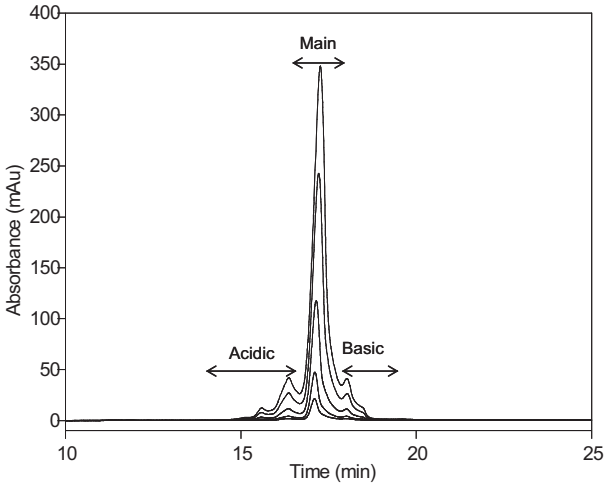

Fig. 6. The chromatograms of mAb1 with 5, 10, 50, 100, and 200 μg of column load obtained with the salt-mediated pH-IEC method.

During the course of the robustness studies, sufficient data was obtained to evaluate most of the variability that is experienced in a typical HPLC experiment. The salt-mediated pH-IEC method provides comparable chromatograms and consistent quantitation results of charge variants for a typical mAb, demonstrating that the method is robust at all chromatography conditions studied here.

4. Conclusion

We have improved the resolution and applicability of the previously reported pH-gradient IEC method [1] by controlling the ionic strength during the course of the pH gradient. We have demonstrated that the ionic strength during the reported pH gradient decreased with increase of solution pH and subsequently reduced the resolution of the separation of the mAbs with high pI values. A mediating salt gradient was added to the pH gradient to modulate the ionic strength. Both the buffer and salt concentrations were shown to impact the resolution of mAbs, and they were both optimized in parallel for several mAbs. The developed salt-mediated pH-IEC method was not only capable of analyzing mAbs over a broad pI range from 6.2 to 9.4, but also offered better resolution than the previous pH-IEC method. The new method was also robust with different HPLC instruments, columns, buffers and injection quantities. Furthermore, the salt-mediated pH-IEC method was able to detect the degradation of mAbs and can thus be used to monitor the stability and the lot-to-lot consistency of mAbs during therapeutic protein manufacturing. Because of its broad applicability and high resolution, the salt-mediated pH-IEC method can be used to assess the charge heterogeneity of most mAbs without time-consuming method development work.

It should be pointed out that the salt-mediated pH-IEC method can be further optimized for each mAb product. First, the pH gradient range can be shortened to achieve faster separation. Since the elution pH showed good correlation with mAb pI, it can be readily estimated (Fig. 4). By using a short pH range (for example two pH units), the separation can be finished in 20 min without changing the gradient slope. Second, the ionic strength in the pH gradient can be further tuned for each mAb to achieve optimal resolution. As discussed above, low-pI mAbs prefer lower ionic strength while high-pI mAbs prefer higher ionic strength. Although the ionic strength can be controlled by changing buffer concentration or modulating with a different concentration of salt, we recommend using constant buffer concentrations of 4 mM and tuning the ionic strength by adjusting the salt concentration. Furthermore, the mediating salt could be combined with the high pH buffer so that the salt gradient and pH gradient could be delivered by a single binary pump.

It is noted that the pH-conductivity hybrid gradient cation-exchange chromatographic methods were reported for analysis of the protein isomers of two human monoclonal antibodies [27] and large-scale purification of a recombinant monoclonal antibody [28]. In the analysis work reported by Kaltenbrunner et al. in 1993 [27], the hybrid gradient was achieved by mixing borate, mannitol and sodium chloride where the solution ionic strength was not controlled as an ascending pH gradient was combined with a descending salt gradient. More importantly, the protein isomers separated by the pH-salt hybride gradient IEC method were differed only in several charged carbohydrate moieties [27], indicating that the formation of borate complexes with the *cis*-diol containing carbohydrate moieties played a major role. However, such *cis*-diol containing carbohydrate moieties can be found on glycated antibodies but not on typical recombinant mAb therapeutics, which was demonstrated by our recent publication [29]. Hence, Kaltenbrunner and coworkers' method may not be applicable for analyzing the charge heterogeneities, which are mainly caused by protein post-translational modifications such as deamidation, of the typical recombinant mAbs. In Zhou and coworkers' report [28], the low conductivity salt gradient was established by replacing sodium chloride with sodium acetate. The acetate salt gradient cation-exchange chromatographic purification of a recombinant mAb from other process related impurities, such as DNA, leached Protein A and protein aggregates, was enhanced by combining it with an ascending pH-gradient in a narrow pH range, from 4.8 to 6.2. Zhou's purification approach is fundamentally different from the ionic strength mediating concept we developed in this work.

Our evaluation in this report gave excellent results for the charge variant separation of recombinant mAbs with a wide range of pI values (6.4–9.4) by controlling the ionic strength. We expect that it represents a platform method that can be quickly implemented in a biotechnology analytical and quality control labs.

Acknowledgements

The authors would like to thank Will McElroy and David Michels for providing the pI values of mAbs. We would also thank Tony Moreno for the helpful discussion about optimizing pH gradient cation-exchange chromatography. We acknowledge Pat Rancatore for critical review of the manuscript.

References

[1] D. Farnan, G.T. Moreno, Anal. Chem. 81 (2009) 8846.
[2] D.J. Barlow, J.M. Thornton, Biopolymers 25 (1986) 1717.
[3] L.W. Dick, D.F. Qiu, K.C. Cheng, J. Chromatogr. B 877 (2009) 3841.
[4] H.C. Liu, G. Gaza-Bulseco, C. Chumsae, Rapid Commun. Mass Spectrom. 22 (2008) 4081.
[5] A.K. Miller, D.M. Hambly, B.A. Kerwin, M.J. Treuheit, H.S. Gadgil, J. Pharm. Sci. 100 (2011) 2543.
[6] W.R. Wang, J. Vlasak, Y.S. Li, P. Pristatsky, Y.L. Fang, T. Pittman, J. Roman, Y. Wang, T. Prueksaritanont, R. Ionescu, Mol. Immunol. 48 (2011) 860.
[7] J. Vlasak, R. Ionescu, Curr. Pharm. Biotechnol. 9 (2008) 468.
[8] X.P.Z. He, A.H. Que, J.J. Mo, Electrophoresis 30 (2009) 714.
[9] Z. Sosic, D. Houde, A. Blum, T. Carlage, Y. Lyubarskaya, Electrophoresis 29 (2008) 4368.
[10] J. Kim, L. Jones, L. Taylor, G. Kannan, F. Jackson, H. Lau, R.F. Latypov, B. Bailey, J. Chromatogr. B 878 (2010) 1973.
[11] G. Teshima, M.X. Li, R. Danishmand, C. Obi, R. To, C. Huang, J. Kung, V. Lahidji, J. Freeberg, L. Thorner, M. Tomic, J. Chromatogr. A 1218 (2010) 2091.
[12] X. Kang, D.D. Frey, J. Chromatogr. A 991 (2003) 117.
[13] L.I. Tsonev, A.G. Hirsh, J. Chromatogr. A 1200 (2008) 166.
[14] A. Nordborg, B. Zhang, X.P.Z. He, E.F. Hilder, P.R. Haddad, J. Sep. Sci. 32 (2009) 2668.
[15] A. Rozhkova, J. Chromatogr. A 1216 (2009) 5989.
[16] J.C. Rea, G.T. Moreno, Y. Lou, D. Farnan, J. Pharm. Biomed. Anal. 54 (2010) 317.
[17] D.J. Anderson, L. Shan, Clin. Chem. 47 (2001) 128.

[18] L. Shan, D.J. Anderson, J. Chromatogr. A 909 (2001) 191.
[19] L. Shan, D.J. Anderson, Anal. Chem. 74 (2002) 5641.
[20] T.M. Pabst, D. Antos, G. Carta, N. Ramasubramanyan, A.K. Hunter, J. Chromatogr. A 1181 (2008) 83.
[21] T. Igawa, H. Tsunoda, T. Tachibana, A. Maeda, F. Mimoto, C. Moriyama, M. Nanami, Y. Sekimori, Y. Nabuchi, Y. Aso, K. Hattori, Protein Eng. Des. Sel. 23 (2010) 385.
[22] J. Wu, T. Huang, Electrophoresis 27 (2006) 3584.
[23] J. Staahlberg, Anal. Chem. 66 (1994) 440.
[24] J. Stahlberg, J. Chromatogr. A 855 (1999) 3.
[25] Product Manual for Propac WCX-10 and Propac SCX-10, 7th ed., Dionex Incorporation, Sunnyvale, CA, 2007.
[26] T. Pabst, G. Carta, J. Chromatogr. A 1142 (2007) 19.
[27] O. Kaltenbrunner, C. Tauer, J. Brunner, A. Jungbauer, J. Chromatogr. 639 (1993) 41.
[28] J.X. Zhou, S. Dermawan, F. Solamo, G. Flynn, R. Stenson, T. Tressel, S. Guhan, J. Chromatogr. A 1175 (2007) 69.
[29] B. Zhang, S. Mathewson, H. Chen, J. Chromatogr. A 1216 (2009) 5676.