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# Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part II: pH gradient approach

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ABSTRACT

The cation exhange pH gradient approach was evaluated for the characterization of 10 model monoclonal antibodies including panitumumab, natalizumab, cetuximab, bevacizumab, trastuzumab, rituximab, palivizumab, adalimumab, denosumab and ofatumumab.

This work shows that retention and resolution can be modelled in cation exchange pH gradient mode, based on only four initial runs (i.e. two gradient times and two mobile phase temperature). Only 6 h were required for a complete method optimization when using a 100 mm × 4.6 mm strong cation exchange column. The accuracy of the predictions was excellent, with an average difference between predicted and experimental retention times of about 1%.

The 10 model antibodies were successfully eluted in both pH and salt gradient modes, proving that both modes of elution can be considered as multi-product charge sensitive separation methods. For most of the compounds, the variants were better resolved in the salt gradient mode and the peak capacities were also higher in the salt gradient approach. These observations confirm that pH gradient approach may be of lower interest than salt gradient cation exchange chromatography for antibody characterization.

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## 1. Introduction

Monoclonal antibodies (mAbs) and related products are the fastest growing class of therapeutic agents [1]. Suitable techniques are requested to qualitatively and quantitatively analyze heterogeneities related to size and charge variants of multimers and aggregates. Common modifications of the primary sequence include N-glycosylation, methionine oxidation, proteolytic fragmentation, and deamidation [2,3].

Cation exchange chromatography (CEX) has been widely used for the separation of mAbs charge variants [4–7] applying a shallow gradient of increasing salt concentration (e.g. sodium chloride) at constant pH. Beside cation-exchange, anion-exchange chromatography (AEX) has also seen application for the separation of the more basic oxidized variants of an intact mAbs [7].

In addition to choosing the appropriate pH of the starting buffer, its ionic strength (salt concentration) should be kept low. The proteins are then eluted by increasing the salt concentration to increase the competition between the buffer ions and proteins for charged groups on the ion exchange (IEX) resin. This mode of separation is considered as the gold standard for proteins characterization, due to its high robustness and good resolving power.

An alternative approach for the separation of charge variants consists in applying a pH gradient, whilst keeping constant the ionic strength [8]. Chromatofocusing (with internal pH gradient) was recognized as the chromatographic analogy to isoelectric focusing (IEF) [9–11] and has been successfully applied for separating protein isoforms, due to its high resolving power and ability to retain the protein native state [12,13]. There are however some limitations to this approach such as the cost of polyampholyte buffers, column regeneration time and the inflexibility in controlling the slope of pH gradient [12,14,15]. Alternatively, the pH gradient can be conducted externally by pre-column mixing of two eluting buffers at different pH values consisting of common buffer species [16]. In contrast to chromatofocusing, the outlet pH gradient in a pH gradient IEX is a result of the superimposition of an external pH-gradient (with respect to time) over an internal column pH-gradient (with respect to the column length) [17]. Therefore, pH gradient IEX generates a well controllable and repeatable pH-gradient over a wide range of pH.

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For pH gradient IEX mode, the use of a mixture of amine buffering species in the high-pH range and a mixture of weak acids in the low-pH range is quite common [14,18,19]. In such a system, maintaining linearity of the pH gradient slope may be somewhat difficult. For the separation of mAb variants, several buffer systems were tested in CEX mode. It was shown that an appropriate mixture of Tris base, piperazine and imidazole provides a linear pH gradient from pH 6 to 9.5 [20]. Triethylamine and diethylamine based buffer systems also offered linear pH gradient in the pH range of 7.5–10.0 [16]. For mass spectrometry (MS) detection, 5 mM ammonium hydroxide in 20% methanol yielded a reasonable pH gradient in a limited pH range [16]. Finally, Zhang et al. applied a salt-mediated improved pH gradient that was used in a wide pH range (between 5 and 10.5) [21]. In their study, a 0.25 mM/min sodium-chloride gradient was performed together with the pH gradient. MAbs possessing isoelectric points (p*I*) between 6.2 and 9.4 were successfully eluted in one generic method by using this salt-mediated pH gradient [21].

One of the benefits of pH gradient based IEX is that the salt concentration can be kept low, yielding less buffer interferences (e.g. on-line or off-line two-dimensional LC). On the other hand, pH gradient based separation using a CEX column was found to be a multiproduct charge sensitive separation method for monoclonal antibodies [8,21].

In this second part of the study, pH gradient CEX was applied to separate mAb charge variants. The impact of gradient steepness and mobile phase temperature on retention, peak capacity and selectivity was studied in details using six selected model mAbs and their variants (i.e. trastuzumab, panitumumab, natalizumab, rituximab, adalimumab and cetuximab). Finally, the possibilities of pH and conventional salt gradient modes were critically evaluated for 10 mAbs possessing p*I* between 6.7 and 9.1.

## 2. Experimental

### 2.1. Chemicals and columns

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). CX-1 pH gradient buffer A (pH 5.6) and CX-1 pH gradient buffer B (pH 10.2) were purchased from Thermo Fisher Scientific AG (Reinach, Switzerland). 1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) solution (BioReagent), 1 M sodium hydroxide (NaOH) solution and sodium chloride (NaCl) (BioChemika) were purchased from Sigma–Aldrich (Buchs, Switzerland).

FDA and EMA approved therapeutic IgG monoclonal antibodies including panitumumab, natalizumab, cetuximab, bevacizumab, trastuzumab, rituximab, palivizumab, adalimumab, denosumab and ofatumumab were kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien en Genevois, France). Papain (from Carica papaya), used for fragmentation of mAbs was obtained from Sigma–Aldrich (Buchs, Switzerland).

YMC BioPro SP-F 100 mm × 4.6 mm, 5 μm non-porous strong cation exchange column was purchased from Stacroma (Reinach, Switzerland).

### 2.2. Equipment and software

All the experiments were performed using a Waters Acquity UPLC™ system equipped with a binary solvent delivery pump, an autosampler and fluorescence detector (FL). The Waters Acquity system included a 5 μl sample loop and a 2 μl FL flow-cell. The loop is directly connected to the injection switching valve (no needle seat capillary). The connection tube between the injector and column inlet was 0.13 mm I.D. and 250 mm long (passive preheating included), and the capillary located between the column and detector was 0.10 mm I.D. and 150 mm long. The overall extra-column volume ($V_{ext}$) is about 14 μl as measured from the injection seat of the auto-sampler to the detector cell. The measured dwell volume is around 100 μl. Data acquisition and instrument control was performed by Empower Pro 2 Software (Waters). Calculation and data transferring was achieved by using Excel templates.

The mobile phase pH was checked and adjusted using a Seven-Multi S40 pH meter (Mettler Toledo, Greifensee, Switzerland).

Method optimization was performed using DryLab® 2000 Plus chromatographic modelling software (Molnar-Institute, Berlin, Germany).

### 2.3. Apparatus and methodology

#### 2.3.1. Mobile phase composition and sample preparation

For the pH gradient CEX separation of mAbs and their fragments, the mobile phase "A" was a 10 times diluted CX-1 pH gradient buffer A (pH 5.6) while the mobile phase "B" was a 10 times diluted CX-1 pH gradient buffer B (pH 10.2) – as described in the protocol provided by the vendor (Thermo Fisher Scientific).

For the salt gradient separations, the mobile phase "A" consisted of 10 mM MES in water, while the mobile phase "B" was 10 mM MES in water containing 1 M NaCl. The pH of both mobile phases was adjusted by adding 1 M NaOH solution to reach the required pH.

The digestion of cetuximab was initiated by addition of papain (diluted to 100 μg/ml with water) to reach a final protein:enzyme ratio of 100:1 (m/m%). The digestion was carried out at 37 °C for 3 h. The final digestion volume was 200 μl and directly injected using low volume insert vials.

#### 2.3.2. Investigation of retention properties of antibodies

Intact antibodies were eluted in pH gradient mode. For studying the retention properties of intact mAbs, six of the 10 available antibodies were selected based on their type (IgG class and isotype) and calculated p*I*, namely panitumumab (huIgG2, p*I* = 6.7), natalizumab (hzIgG4, p*I* = 8.6), cetuximab (chIgG1, p*I* = 8.7), adalimumab (huIgG1, p*I* = 8.8), trastuzumab (hzIgG1, p*I* = 8.8) and rituximab (chIgG1, p*I* = 9.1). Because IgG from different subclasses may have different properties (Fc regions are very close but the structure of the hinge regions may be relatively different), therefore the strength of interactions between the ion-exchange resin and IgG can vary considerably between species and subtypes. Our purpose was to cover the whole p*I* range and to include chimeric (ch), humanized (hz) and human (hu) reference IgG1, IgG2 and IgG4 isotypes, to draw overall and reliable conclusions.

First, the effect of pH gradient steepness on the retention was evaluated. Different gradient times were tested at a given mobile phase temperature. A generic linear gradient, starting from 0% to 100% B (equivalent to a linear pH gradient from 5.6 to 10.2) was applied at a flow rate of 0.6 ml/min for all samples. The gradient time ($t_g$) was varied as 10, 15, 20, 30 and 40 min (at $T$ = 30 °C). The observed apparent retention factors ($k_{app}$) and peak capacity ($P_c$) values were plotted against the gradient time (steepness).

For the investigation of mobile phase temperature, 15 min gradient runs (0–100% B) were carried out using various temperatures between 30 °C and the upper temperature limit of the column (60 °C). The retention properties of intact mAbs and their charge variants were evaluated by plotting the logarithm $k_{app}$ against $1/T$ (Van't Hoff type representation). Peak capacity and resolution were also studied as a function of mobile phase temperature.

#### 2.3.3. Systematic method optimization

A common approach in method development is to simultaneously model the selectivity and/or resolution as a function of temperature and gradient steepness on a selected column

from a limited number of initial runs [22,23]. In this case, initial experiments are those on which the computer-models are based (calculated) to model a few thousand experiments. Then, with the help of resolution maps – which show the critical resolution of the peaks to be separated – the gradient program and column temperature can be rapidly and accurately optimized. This approach was currently applied for the salt gradient CEX separation of antibody variants [24]. In this second part, this procedure was implemented for pH gradient CEX based separations, to evaluate if the same rules for method development can be applied.

Based on the observed effects of the factors on retention and resolution of mAbs peaks, a 4 runs based initial experimental setup is recommended for method optimization in the pH gradient mode. Performing gradient runs with two gradient times (as $t_{g1}$ = 10 min, $t_{g2}$ = 30 min) at two temperature ($T_1$ = 25 °C, $T_2$ = 55 °C) on a 100 mm × 4.6 mm column allowed a reliable optimization of the separation.

The optimization was performed by computer simulation using a DryLab two dimensional model. Cetuximab papain digested samples were injected to build up the DryLab model and study the prediction accuracy error. Cetuximab is a heterogeneous mAb possessing two N-glycosylation sites in the heavy chain and several charge variants including C-terminal lysines and sialic acids [25]. It is therefore a complex example for method development [24].

#### 2.3.4. Generic pH gradient for multiproduct analysis

After studying the retention behavior of a limited number of model antibodies, a generic pH gradient was proposed allowing the elution and separation of all the 10 mAbs within reasonable analysis time (20 min). A pH gradient, starting from pH 5.6 to 10.2 was applied at a flow rate of 0.6 ml/min for all samples. The mobile phase temperature was set at $T$ = 30 °C. Fluorescence detection was carried out at $\lambda_{ex}$ = 280 and $\lambda_{em}$ = 360 nm.

#### 2.3.5. Comparison of pH-gradient and salt-gradient CEX

The generic methods for the 10 mAbs and the optimized separation of cetuximab fragments in both salt and pH gradient modes were compared. The same stationary phase and mobile phase flow rate were employed allowing a fair comparison of the two CEX modes. Peak capacity, selectivity and elution order were studied. The method optimization and final conditions employed for the salt gradient CEX methods have been detailed in the first part of this article series [24].

## 3. Results and discussion

In pH gradient mode, the proteins net charge can be modified during the pH gradient, due to protonation–deprotonation of functional groups. In CEX, the protein is expected to elute at, or close to its p*I*. According to theory, when using pH gradient elution mode and low ionic strength mobile phase, the proteins are focused in narrower bands enabling higher resolution compared to a pH gradient performed at high ionic strength. The width of a protein peak along a linear pH gradient expressed in pH units can be written as follows [9,10,16]:

$$(\Delta \mathrm{pH})^2 \approx \frac{D\left(d\mathrm{pH}/dV\right)}{\varphi\left(dZ/d\mathrm{pH}\right)} \quad (1)$$

where $D$ is the diffusion coefficient of the analyte, $d$pH/$dV$ is the gradient slope, $\varphi$ is the Donnan potential and $dZ$/$d$pH is the change in protein net charge along the pH gradient. Since the Donnan potential depends on the ionic strength, a peak focusing effect is expected at lower ionic strength. In agreement with this expectation, pH gradients at low ionic strength showed better resolution for mAb variants compared to pH gradients performed in high ionic strength medium [16]. However, the mechanism of salt gradient and pH gradient mode is different and therefore hardly comparable.

To achieve an optimal experimental setup for separation or purification of mAbs, the influence of various parameters on separation, such as gradient steepness, temperature or flow rate has to be taken into account. The optimization of mAb separations in CEX mode was performed by the systematic variation of gradient steepness and flow rate and their impact on the peak width and resolution. This approach is known as iso-resolution curve concept and allows developing a stepwise gradient [26].

The work of Snyder and co-workers showed that salt gradient based IEX systems follow non-linear solvent strength (non-LSS) type mechanism [27–29]. In the first part of our study, it was shown that when the net charge of a protein is large (e.g. such as with mAbs), the so called stoichiometric displacement model (SDM) gives virtually linear retention dependence on the gradient span [24].

To the best of our knowledge, the optimization procedure in pH gradient mode CEX has not been yet studied. Here, the impact of gradient steepness and temperature on mAbs retention time and peak capacity was evaluated in a systematic way.

### 3.1. The effect of pH gradient time (gradient steepness) on the retention

For CEX separation of mAb variants, the solutes are eluted in order of increasing binding charge (correlates more or less with the p*I*) and equilibrium constant. It is generally assumed that mAbs elute close to their p*I* in the pH gradient mode [17]. Therefore, the applied pH range clearly determines the proteins that can possibly be eluted. On the other hand, retention times and peak widths depend on the gradient steepness as both are function of $dZ$/$d$pH. Due to the relatively high net charge ($z$ value) of mAbs a small change in pH could lead to significant shift in retention.

The effect of gradient steepness (gradient time) on the retention of intact mAbs and their variants was studied in a systematic way. The gradient time (steepness) was varied as 10, 15, 20, 30 and 40 min (at $T$ = 30 °C). The retention of the six selected mAbs and the variants of trastuzumab, adalimumab and cetuximab showed the same behaviour. Fig. 1 illustrates the effect of gradient time on the apparent retention ($k_{app}$) of intact mAbs and charge variants. The relation between $k_{app}$ and $t_g$ can be accurately described by fitting linear function ($R^2$ > 0.999 for all solutes). On the contrary, the retention behaviour of mAb fragments in RPLC showed a moderate deviation from linear relationship [22]. This behaviour was explained by some possible changes in conformation and contribution of different retention mechanisms (hydrophobic and ion exchange interactions and/or irreversible adsorption) [30]. Surprisingly, in pH gradient CEX mode, an LSS type model perfectly describes the retention behaviour of mAbs. There is no need for logarithmic or polynomial fitting (as it is often applied in RPLC or NPLC modes).

As suggested by our observations, the retention time of intact mAbs can be predicted for any gradient steepness on the basis of only two initial gradient runs (e.g. with $t_{g1}$ = 10 min and $t_{g2}$ = 30 min) in the pH gradient mode.

### 3.2. The effect of mobile phase temperature on retention

The temperature dependence of analyte retention factor in liquid chromatography is generally expressed by the van't Hoff equation. With regular compounds, the van't Hoff plots (log($k$) vs 1/$T$) follow a linear relationship. However, with ionisable compounds and large biomolecules, deviations from linearity were described [31]. Depending on the stability of proteins secondary structure, the molecules unfold to various extents and hence

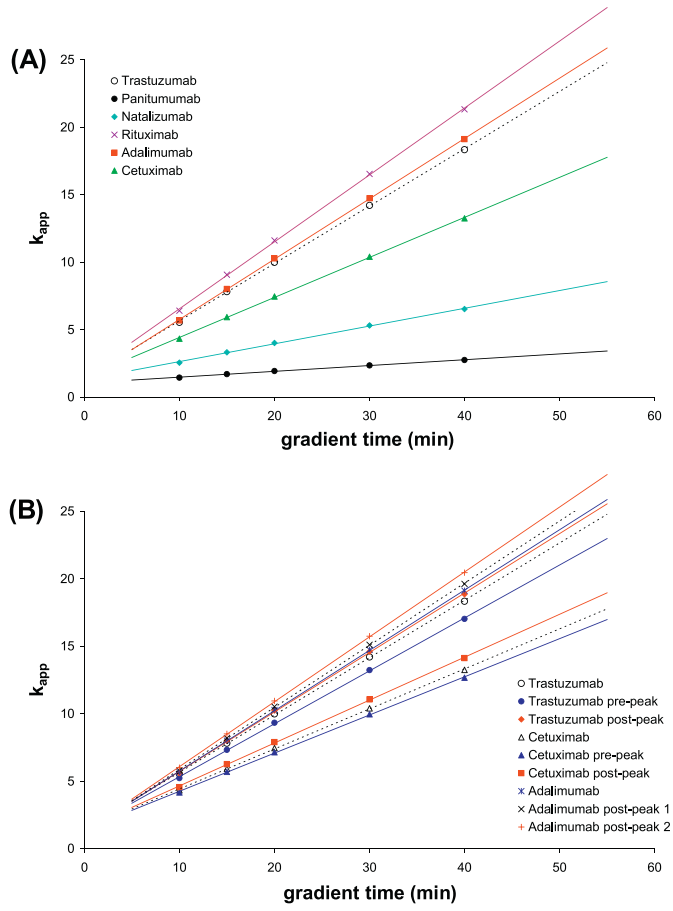



**Fig. 1.** Effect of gradient time (steepness) on the apparent retention factor of native antibodies (A) and antibody variants (B). Column: YMC BioPro SP-F (100 mm × 4.6 mm). Mobile phase "A" CX-1 Buffer A pH 5.6, "B" CX-1 Buffer B pH 10.2. Flow rate: 0.6 ml/min, gradient: 0–100%B in 10, 15, 20, 30 and 40 min, temperature: 30 °C, detection: FL (280–360 nm), injected volume: 2 μl.

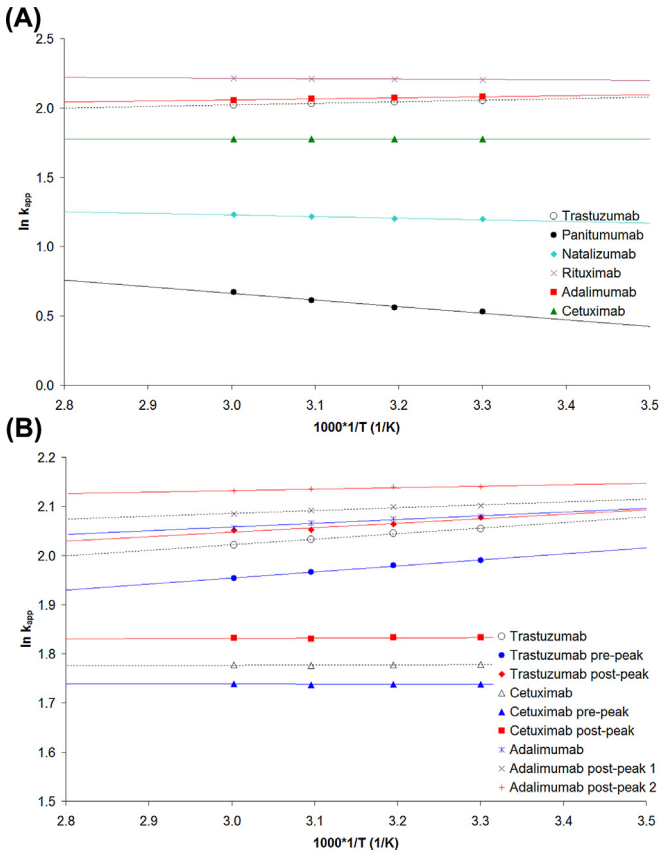



**Fig. 2.** Effect of temperature on the apparent retention factor of native antibodies (A) and antibody variants (B) (van't Hoff type representation). Column: YMC BioPro SP-F (100 mm × 4.6 mm). Mobile phase "A" CX-1 Buffer A pH 5.6, "B" CX-1 Buffer B pH 10.2. Flow rate: 0.6 ml/min, gradient: 0–100%B in 15 min, temperature: 30, 40, 50 and 60 °C, detection: FL. (280–360 nm), injected volume: 2 μl.

interact with the stationary phase with various strengths [32–34]. The effect of mobile phase temperature on protein retention in pH gradient IEX mode has not yet been reported.

Fig. 2 illustrates the obtained van't Hoff type plots. The log($k$) vs 1/$T$ plots show linear behaviour in the investigated temperature range. Similarly to the conclusions drawn in salt gradient based CEX separations, the impact of temperature seems to be less important compared to RP mode [24]. The slope of the curves was indeed significantly lower in IEX vs. RPLC mode. The slope in pH gradient IEX mode was comprised between −0.47 and 0.11 K*$10^3$, while for intact mAbs in RPLC mode the slope is typically around 0.5 and 1.0 K*$10^3$ [22].

Panitumumab (possessing the lowest p$I$) showed the most important retention–temperature dependence. It is probably due to de relatively low number of net charges whilst running the pH gradient between the initial mobile phase pH (5.6) and panitumumab p$I$ (6.7). These low net charges form relatively weak interaction with the stationary phase compared to the other mAbs. Moreover, the retention of panitumumab increases with temperature probably due to a modification of p$I$ with temperature.

Finally, the slopes of log($k$)–1/$T$ curves for related mAbs (e.g. charge variants of a given mAb) are quite similar. Fig. 2B shows these plots for adalimumab, cetuximab and trastuzumab, as representative examples. This suggests that selectivity can hardly be tuned with temperature. However, temperature has significant impact on the peak width (peak capacity) – see Section 3.3 – and has an effect on the overall separation quality (resolution).

### 3.3. Peak capacity

The variation of peak capacity as a function of gradient steepness and temperature was also estimated. The following equation was used to estimate peak capacity based on peak width at half height [35]:

$$P_c = 1 + \frac{t_g}{1.7 \cdot w_{50\%}} \quad (2)$$

A logarithmic relationship perfectly described the evolution of peak capacity with increasing gradient time. Similarly to the observations made in salt-gradient CEX mode with trastuzumab, adalimumab and natalizumab, the peak capacities achieved in pH gradient CEX for the same mAbs and using the same column and gradient steepness were comparable [24]. Peak capacity of $P_c \sim 50$–60 was observed with a 10 min long gradient, while the longest 40 min gradient provided $P_c \sim 100$–120 (Fig. 3A). Since retention also increased with gradient time (Fig. 1), relatively long gradients are required for high resolution separations, albeit the cost of analysis time.

Surprisingly, panitumumab and rituximab eluted with significantly wider peaks than the other mAbs. The peak capacity was comprised between 40 and 75 for panitumumab and only between 20 and 60 for rituximab. This efficiency was clearly lower than the one achievable in salt gradient CEX mode on the same column and using the same gradient steepness [24].

Fig. 3B shows the change in peak capacity as a function of mobile phase temperature at a given gradient steepness. A decrease in peak capacity was observed for half of the mAbs while the other half

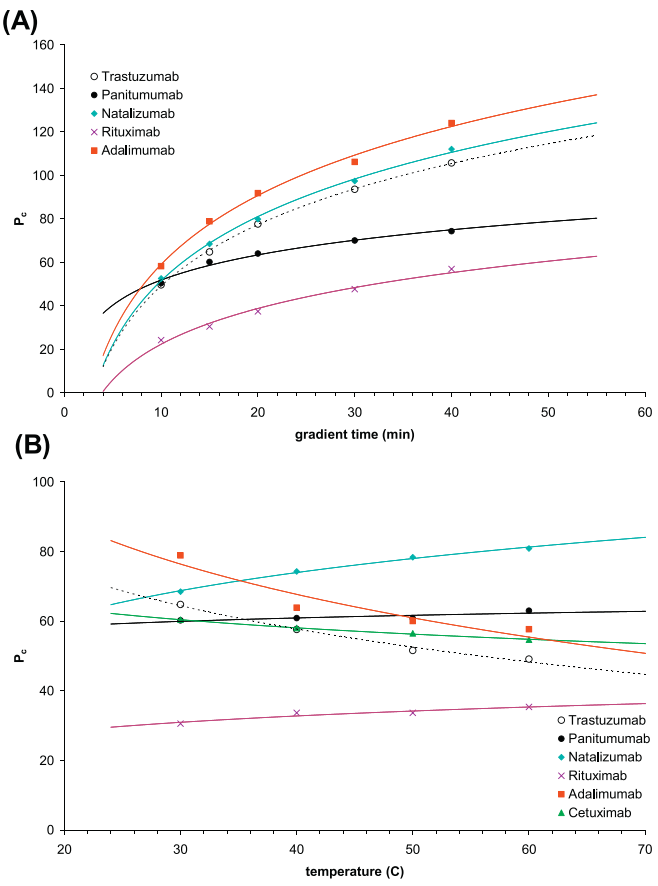



**Fig. 3.** Peak capacity as a function of gradient time (steepness) (A) and temperature (B). Column: YMC BioPro SP-F (100 mm × 4.6 mm). Mobile phase "A" CX-1 Buffer A pH 5.6, "B" CX-1 Buffer B pH 10.2. Flow rate: 0.6 ml/min, detection: FL (280–360 nm), injected volume: 2 μl.

showed the opposite tendency. The most different behaviour was observed for adalimumab and natalizumab. For adalimumab, the peak capacity was reduced from 80 to 60 when increasing the temperature from 30 to 60 °C, while for natalizumab it was enhanced from 70 to 80 in the same temperature range. This observation suggests that temperature may be an important factor in method optimization. Indeed, selectivity does not change significantly with temperature but it can affect resolution through an increase or decrease of peak capacity in pH gradient CEX mode.

For large proteins in CEX mode, it is always hard to predict and explain their band broadening process occurring at different temperatures. On the one hand, due to the changes in mobile phase viscosity and solute diffusion, an increase in efficiency (sharper peak) is expected. On the other hand, temperature can have a strong impact on protein conformation as well. Under certain conditions, the native conformation and/or other intermediate conformations may be present during the analysis. Each of these will interact differently with the stationary phase, resulting in slight variations in retention times of the different species. The existence of the different species at elevated temperature may result in "apparent" peak broadening.

### 3.4. Method development, creating a two dimensional DryLab model for pH gradient CEX

Linear models are generally employed for the simultaneous optimization of two or three variables in liquid chromatography. Polynomial relationship of two variables can be written as:

$$y = b_0 + b_1 x_1 + b_2 x_2 \quad (3)$$

where $y$ is the response (retention time or its transformation), $x_1$ and $x_2$ are the model variables (e.g. $t_g$ and $T$), while $b_0$, $b_1$, $b_2$ are the model coefficients.

As observed with mAbs, the dependence of retention time (or its transformation) on pH, gradient steepness and mobile phase temperature can be described by linear models. This observation suggests that method optimization with gradient steepness and mobile phase temperature as model variables requires the measurement of variable effects at two levels only.

Gradient runs with two gradient times (as $t_{g1}$ = 10 min, $t_{g2}$ = 30 min) at two temperatures ($T_1$ = 25 °C, $T_2$ = 55 °C) on a 100 mm × 4.6 mm column were performed to build up the model. The modelling software implements an interpretive approach, where the retention behaviour is modelled on the basis of experimental runs, and then the retention times, peak widths, selectivity and resolution at other conditions are predicted in a selected experimental domain. This allows calculating the critical resolution, and accordingly, the optimal separation can be found. For this purpose, retention times were transformed into retention factors, and linear models were chosen for both gradient time (steepness) and temperature. This modelling was performed on a rectangular region in the $t_g$–$T$ plane, determined by 2 gradient times (steepness) and 2 temperatures. Hence, this approach requires 4 initial experimental runs for creating the model. Following the execution of the input experimental runs, data (retention times, peak widths and peak tailing values) were imported into DryLab and peak tracking performed. Then, the optimization was carried out on the basis of the created resolution map, in which the smallest value of resolution ($Rs$) of any two critical peaks in the chromatogram was plotted as a function of gradient time and mobile phase temperature.

To establish the accuracy of this 2 dimensional linear model, the predicted and experimentally derived chromatograms (retention times) under the optimal conditions were compared.

### 3.5. Optimization of the separation of Fab and Fc fragments of cetuximab in pH gradient mode

A fast and efficient method optimization process was applied for the determination of variants and degradation products of recombinant cetuximab, using the pH gradient approach in CEX mode. The native mAb was initially digested with papain and our purpose was to separate as many variants of the Fab and Fc fragments as possible, within the shortest achievable analysis time. Fig. 4 shows the obtained chromatograms of the four initial runs. The predicted results are demonstrated in Fig. 5 as a resolution map. Based on the resolution map, a 16 min gradient was found to provide the highest resolution at the mobile phase temperature of $T$ = 25 °C. Then, the predicted optimum condition was set and experimental chromatograms recorded to evaluate the prediction accuracy. Fig. 6 shows the predicted and experimentally observed chromatograms, while Table 1 provides the corresponding retention times.

As shown in Table 1, the predicted retention times were in good agreement with the experimental ones. The average retention time relative errors was systematically under 1.0% (see Table 1), which can be considered as excellent. The highest individual deviation was 1.5%.

In conclusion, this method optimization approach can be considered as reliable and the suggested initial experiments (i.e. 10 and 30 min gradient on a 100 mm long standard bore column at 25 and 55 °C) are suitable for daily routine work. The time spent for method development in this example was around 6 h (2 gradients × 2 temperatures × 3 samples + equilibration time).

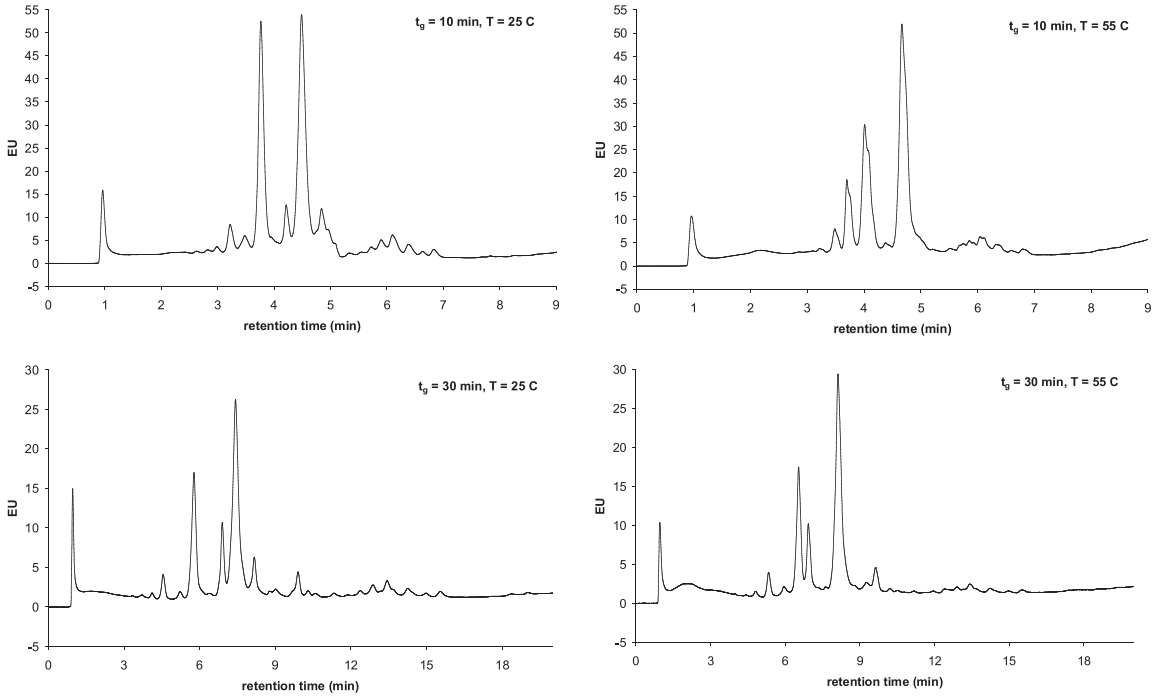



**Fig. 4.** Cetuximab papain digested sample. Column: YMC BioPro SP-F (100 mm × 4.6 mm). Mobile phase "A" CX-1 Buffer A pH 5.6, "B" CX-1 Buffer B pH 10.2. Flow rate: 0.6 ml/min, gradient: 0–80%B, detection: FL (280–360 nm), injected volume: 2 μl. Gradient times: $t_{g1}$ = 10 min, $t_{g2}$ = 30 min, temperatures $T_1$ = 25 °C, $T_2$ = 55 °C.

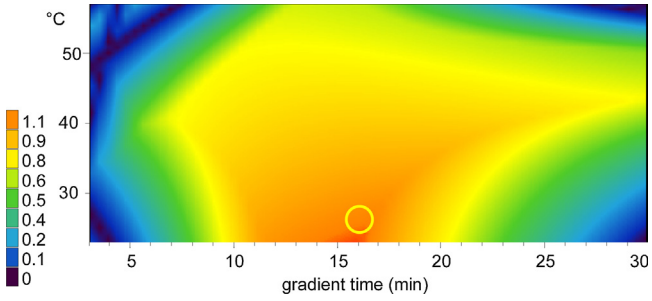



**Fig. 5.** Cetuximab papain digestion resolution map ($t_g$–$T$ model). Column: YMC BioPro SP-F (100 mm × 4.6 mm). Mobile phase "A" CX-1 Buffer A pH 5.6, "B" CX-1 Buffer B pH 10.2. Flow rate: 0.6 ml/min, gradient: 0–80%B, detection: FL (280–360 nm), injected volume: 2 μl. Gradient times: $t_{g1}$ = 10 min, $t_{g2}$ = 30 min, temperatures $T_1$ = 25 °C, $T_2$ = 55 °C.

**Table 1**
Prediction accuracy. Conditions are the same as specified in Fig.7.

| Peak | Retention time | | | |
|---|---|---|---|---|
| | Experimental | Predicted | Difference (min) | Error (%) |
| 1 | 3.47 | 3.42 | 0.05 | 1.37 |
| 2 | 3.79 | 3.74 | 0.05 | 1.34 |
| 3 | 4.18 | 4.14 | 0.04 | 1.01 |
| 4 | 4.72 | 4.68 | 0.04 | 0.88 |
| 5 | 5.20 | 5.12 | 0.08 | 1.50 |
| 6 | 5.92 | 5.97 | −0.05 | −0.89 |
| 7 | 6.57 | 6.48 | 0.09 | 1.33 |
| 8 | 6.92 | 7.00 | −0.08 | −1.09 |
| 9 | 7.39 | 7.48 | −0.10 | −1.27 |
| 10 | 7.76 | 7.82 | −0.06 | −0.73 |
| 11 | 8.31 | 8.39 | −0.08 | −0.99 |
| 12 | 9.03 | 9.13 | −0.10 | −1.10 |
| 13 | 9.85 | 9.98 | −0.13 | −1.30 |
| 14 | 11.09 | 11.14 | −0.05 | −0.47 |
| | Average | | −0.02 | −0.03 |

### 3.6. Generic pH gradient CEX method for various mAbs

The main advantage of pH gradient based separations using a CEX column is described as a multi-product charge sensitive separation method for various mAbs [16,20,21]. In this study, we tried to check this hypothesis by analyzing a mixture of 10 different mAbs (possessing p*I* between 6.7 and 9.1), with a generic pH gradient from pH 5.6 to 10.2.

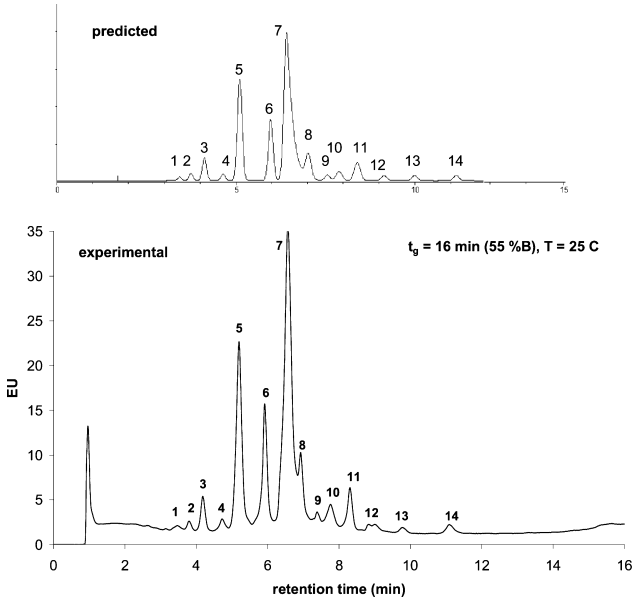



**Fig. 6.** Comparison of predicted and experimental chromatograms. Column: YMC BioPro SP-F (100 mm × 4.6 mm). Mobile phase "A" CX-1 Buffer A pH 5.6, "B" CX-1 Buffer B pH 10.2. Flow rate: 0.6 ml/min, gradient: 0–55%B in 16 min, temperature: 25 °C, detection: FL (280–360 nm), injected volume: 2 μl.

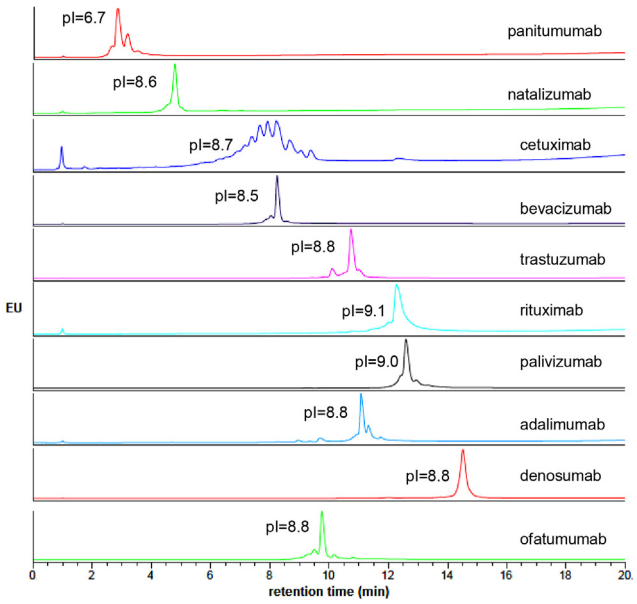



**Fig. 7.** Generic pH gradient. Column: YMC BioPro SP-F (100 mm × 4.6 mm). Mobile phase "A" CX-1 Buffer A pH 5.6, "B" CX-1 Buffer B pH 10.2. Flow rate: 0.6 ml/min, gradient: 0–100%B in 20 min, temperature: 30 °C, detection: FL (280–360 nm), injected volume: 2 μl.

Based on Section 3.5, the pH gradient steepness and mobile phase temperature were varied to find appropriate conditions for these 10 mAbs and their variants.

Fig. 7 shows the obtained chromatograms of 10 intact mAbs, and suggests that pH gradient CEX separation is indeed adequate for multi-product mAb separations. The optimal conditions on a strong cation exchanger resin were found as 20 min long gradient (0–100% B) at 30 °C. On Fig. 7, it can be clearly seen that mAbs do not elute exactly in the order of their p*I*. Theoretically, proteins should elute according to their p*I* in the pH gradient mode. Ahamed et al. observed that basic proteins (p*I* > 8) eluted at pH very close to their p*I*, while acidic proteins (p*I* < 6) eluted at slightly higher pH (0–0.5 pH unit) than their p*I* [17]. On the contrary, neutral proteins (6 < p*I* < 8) eluted at much higher pH than their p*I* and had a tendency to elute at pH ~9 regardless their p*I* [17]. The distribution of charges on the surface of proteins is generally considered as the reason for the minor deviations between the elution pH and p*I*. For neutral proteins, the source of deviation is probably originated from the polypeptidic chain. Indeed the huge deviation between the elution pH and p*I* of neutral proteins originated from the inherent nature of the proteins titration curve [17].

In our example, natalizumab clearly elutes earlier, while denosumab elutes at higher pH than expected. One possible explanation may be the differences in glycosylation profiles of these mAbs. Moreover, some supplementary interactions with the stationary phase can also occur that superposes to the charge-interaction based elution mechanism. Based on these observations and the fact that retention times and p*I* are not perfectly correlated, care should be taken when evaluating the proteins p*I*, based on a pH gradient CEX experiment.

### 3.7. Comparison of generic pH and salt gradient CEX separations

The generic pH gradient method was compared to the recently developed generic salt gradient mode. The conditions of the salt gradient method are detailed in the first part of this article series [24].

For the sake of comparison, the same column (strong cation exchange), instrument, flow rate, gradient steepness, temperature, injected amount and detection mode were applied. The only difference was the mobile phase conditions and the mode of elution. The gradient programs were developed to keep a similar retention range for all the mAbs. The highest retention factor in pH gradient mode was $k_{app}$ = 13.7 while in salt gradient mode it was $k_{app}$ = 14.2. Therefore, these conditions allowed a fair comparison of the two modes.

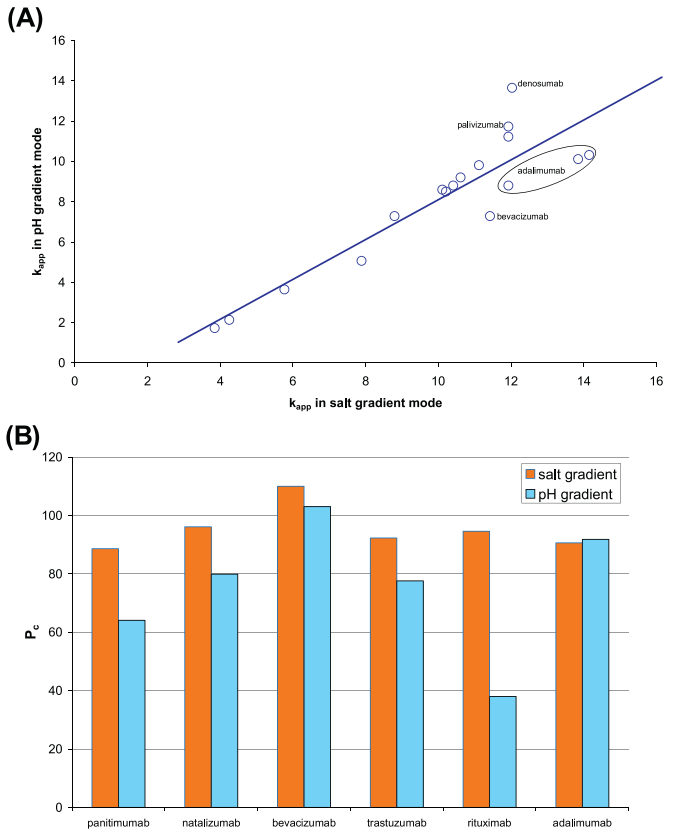



**Fig. 8.** Comparison of retention factors (A) and peak capacity (B) in salt and pH gradient mode cation exchange chromatography. Conditions as defined in the legend of Fig. 7 for the pH gradient, and as defined in Ref. [24] for the generic salt gradient.

First, the retention factors were compared. The $k_{app}$ values observed in the pH gradient mode were plotted against the $k_{app}$ values observed in the salt gradient mode. Fig. 8A shows the observed plot for the 10 intact mAbs and their main variants. As shown, denosumab, palivizumab, bevacizumab and adalimumab show deviation from the linear fitted function. This suggests that the two CEX elution modes offer some alternative selectivity for these particular mAbs. For the six other mAbs, no significant differences in elution order (selectivity) was observed.

Except selectivity and retention, the peak capacities achieved with these two modes were also compared. Fig. 8B shows the peak capacity observed for six representative mAbs, when performing the generic gradient separations. In agreement with our expectations, the salt gradient mode offers higher peak capacity (when applying the same gradient steepness, flow rate and column dimension). In average, a 25% higher efficiency was observed in the salt gradient mode compared to the pH gradient mode. In the case of rituximab, a 2.5 fold higher peak capacity was observed in salt gradient mode. Rituximab eluted in a tailed broad peak in pH gradient mode while it was symmetrical and sharp in the salt gradient mode. For adalimumab, the two modes provide similar efficiency. For most of the mAbs, the variants were better resolved in the salt gradient mode.

To conclude on these two CEX modes, the theoretically expected focusing effect of the pH gradient mode does not bring any gain in resolving power or peak capacity for intact mAbs compared to the salt gradient mode. In addition, the salt gradient mode can also be successfully applied as a multi product generic method for a large variety of intact mAbs [24].

## 4. Conclusion

In this second part, the possibilities offered by CEX pH gradient mode were evaluated for 10 different model mAbs, and systematically compared to CEX salt gradient approach.

First of all, we have evaluated whether the retention can be modelled as a function of gradient steepness and mobile phase temperature in CEX pH gradient mode. Because the retention models were always linear, only four initial experiments (2 gradients times at two temperatures) were required to model the behaviour in CEX pH gradient. Then, only 6 h were required to find out the optimal conditions for the characterization of several mAbs variants.

Next, we also demonstrated that the retention times observed in CEX pH gradient were not systematically proportional to the mAbs p*I*. Thus, the application of this approach for measuring accurate p*I* values should be considered with caution.

Finally, our 10 model mAbs were successfully eluted in both CEX pH gradient and salt gradient, showing that both modes of elution can be considered as multi-product charge sensitive separation methods. These two approaches were also compared in terms of selectivity and peak capacity. Selectivity were quite comparable (except for denosumab, palivizumab, bevacizumab and adalimumab) and for most of the mAbs, the variants were better resolved in the salt gradient mode. The peak capacities were generally improved with the regular salt gradient approach. These observations confirm that pH gradient approach may be of lower interest than salt gradient CEX mode for mAbs characterization.

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