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Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part I: Salt gradient approach

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ABSTRACT

Ion exchange chromatography (IEX) is a historical technique widely used for the detailed characterization of therapeutic proteins and can be considered as a reference and powerful technique for the qualitative and quantitative evaluation of charge variants. When applying salt gradient IEX approach for monoclonal antibodies (mAbs) characterization, this approach is described as time-consuming to develop and product-specific. The goal of this study was to tackle these two bottle-necks.

By modeling the retention of several commercial mAbs and their variants in IEX, we proved that the mobile phase temperature was not relevant for tuning selectivity, while optimal salt gradient program can be easily found based on only two initial gradients of different slopes. Last but not least, the dependence of retention vs. pH being polynomial, three initial runs at different pH were required to optimize mobile phase pH. Finally, only 9 h of initial experiments were necessary to simultaneously optimize salt gradient profile and pH in IEX. The data can then be treated with commercial modeling software to find out the optimal conditions to be used, and accuracy of retention times prediction was excellent (less than 1% variation between predicted and experimental values).

Second, we also proved that generic IEX conditions can be applied for the characterization of mAbs possessing a wide range of p*I*, from 6.7 to 9.1. For this purpose, a strong cation exchange column has to be employed at a pH below 6 and using a proportion of NaCl up to 0.2 M. Under these conditions, all the mAbs were properly eluted from the column. Therefore, salt gradient CEX can be considered as a generic multi-product approach.

1. Introduction

Due to the increasing number of approved monoclonal antibodies (mAb) in the pharmaceutical area and the number of biosimilars potentially entering the market, the need for analytical techniques adapted for their detailed characterization has increased [1]. The intrinsic micro-heterogeneity is of major concern with mAbs and should be critically evaluated because differences in impurities and/or degradation products could lead to serious health implications [2].

In the production of mAbs, the final product often exhibits a number of variations from the expected or desired structure [3]. These alterations may result from either known or novel types of posttranslational modifications or from spontaneous, non-enzymatic protein degradation leading to charge and/or size heterogeneity. Common modifications of the primary sequence include N-glycosylation [4], methionine oxidation [5], proteolytic fragmentation, and deamidation [6,7]. It has been shown that charge variants of therapeutic proteins can have very different bioactivity [8]. The complete characterization of an intact mAb is difficult to achieve; therefore, various enzymes, such as pepsin and papain, are often used to obtain mAb fragments and facilitate the investigation of its micro-heterogeneity [9]. Papain is primarily used to cleave mAbs into three fragments at the heavy chain (HC) hinge region, one crystallizable fraction (Fc) and two identical antigen-binding fraction (Fab) fragments of ~50 kDa each, while

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pepsin generates F(ab′)2 fragments of ~100 kDa. These types of digestion are often called limited proteolysis (LP). The reduction of disulfide bonds is also commonly used to produce two LCs and two HCs.

In general, the identity, heterogeneity, impurity content, and activity of each new batch of mAbs should be thoroughly investigated before release. This examination is achieved using a wide range of analytical methods, including ion exchange chromatography (IEX), reversed-phase liquid chromatography (RPLC), hydrophobic interaction chromatography (HIC), size exclusion chromatography (SEC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), capillary isoelectric focusing (cIEF), capillary zone electrophoresis (CZE), circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR), fluorescence spectrophotometry (FL), and mass spectrometry (MS). The goal of this multi-method strategy is to demonstrate the similarity between production batches of mAb by precisely characterizing the primary, secondary, and tertiary structure of the mAbs [10,11].

Considering the large size of antibodies and the minor structural diversity between the variants, the existence of these variants imposes a great challenge for their chromatographic separation [3]. IEX chromatography is a non-denaturing technique widely used for the separation and isolation of protein charge variants for subsequent characterization. Among the different IEX modes, cation-exchange chromatography (CEX) is one of the best approaches for mAb purification and characterization [12]. Cation exchange chromatography is considered as the gold standard for charge sensitive antibody analysis, however method parameters, such as column type, mobile phase pH, and salt concentration gradient, often need to be optimized for each individual antibody [13].

In the 90s, Moorhouse et al. were among the first to demonstrate the potential of CEX for mAb characterization [14]. Fab and Fc fragments were separated with sufficient resolution and identified with MS. The C-terminal lysine variability of the Fc and the N-terminal glutamine-pyroglutamate variability of the Fab were observed. A more recent study showed the suitability of CEX for studying complex degradation processes involving various IgG1 molecules [15]. This method was particularly useful for characterizing protein variants formed in the presence of salts under accelerated storage conditions. The importance of this assay was further illustrated by characterization of light-induced degradations of mAb formulations. Another study presented the significance of IEX in the analysis of oxidized mAb samples [16]. In addition to cation-exchange, anion-exchange chromatography (AEX) was also applied and found suitable for the separation of the more basic oxidized variants of intact mAbs [16].

In the late 1970s, chromatofocusing (with internal pH gradient) was recognized as the chromatographic analogy to IEF [17–19]. Chromatofocusing has been demonstrated to be useful for separating protein isoforms due to its high resolving power and ability to retain the protein native state [20,21]. There are however some limitations to this technique such as the cost of polyampholyte buffers, column regeneration time and the inflexibility in controlling the slope of pH gradient [20,22,23]. Alternatively, pH gradient can be conducted externally by pre-column mixing of two eluting buffers at different pH values consisting of common buffer species [3]. The externally induced pH gradient has recently been applied for separation of deamidated variants of a mAb, resolving C-terminal lysine isoforms of a mAb after treating with carboxypeptidase B and also for the analysis of charge variants of intact mAbs [6,20,24]. According to the literature, ionic strength based IEX separations (classical salt gradient mode) have excellent resolving power and robustness, but are product specific and time-consuming to develop [24]. On the other hand, pH gradient based separation using a CEX column is described as a multiproduct charge sensitive separation method for monoclonal antibodies [3,24,25].

The high complexity of the analytical works related to mAbs requires new, more simple and generic ways of judging the quality and the variability of the quality of mAb products. This is one of the major requirements for HPLC methods according to Quality by Design (QbD) principles. We can expect a great step forward the simplification of HPLC methods to a higher degree of flexibility in life science, especially in pharmaceutical development work. For this purpose, computer based chromatographic models can explain complex details much faster and helps in judging the quality of mAb products.

In this contribution, the possibilities and limitations of the classical salt gradient approach is studied and discussed. The applicability of salt gradients as a generic multi-product method is presented for 10 therapeutic mAbs, approved both by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) and possessing a wide range of isoelectric points (p*I*) between 6.7 and 9.1. Up to now, the method development procedure in ion exchange chromatography was empirical and time consuming. In our work, the impact of mobile phase pH, gradient steepness and 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). Based on the observed effects on resolution, a six runs based initial experimental setup was suggested for successful method optimization in the salt gradient mode. Performing gradient runs with two gradient times and three mobile phase pH on a 100 × 4.6 mm column allowed a fast and reliable optimization of the separation. This optimization was performed by computer simulation using DryLab modeling software and a custom made model.

2. Experimental

2.1. Chemicals and columns

Water was obtained with a Milli-Q Purification System from Millipore (Bedford, MA, USA). 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 × 4.6 mm, 5 μm non-porous strong cation exchange column was purchased from Stagroma (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 adjusted using a SevenMulti S40 pH meter (Mettler Toledo, Greifensee, Switzerland).

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

2.3. Apparatus and methodology

2.3.1. Mobile phase composition and sample preparation

For the gradient separation of mAbs and their fragments, 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 0.1 M NaOH solution to reach the required pH (pH = 5.6, 5.7, 6.0, 6.3, 6.4 and 6.6).

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 salt gradient mode. For studying the retention properties of intact mAbs, 6 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). 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 as well in order to draw overall and reliable conclusions.

First, the effect of salt gradient steepness on the retention was evaluated. Different gradient times were tested at a given mobile phase temperature and pH. A generic linear gradient, starting from 0% to 20% B (equivalent with 0–0.2 M NaCl gradient) 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 and at pH 6.3). 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 were carried out at pH 6.3 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.

The impact of mobile phase pH in salt gradient mode was finally evaluated by performing 15 min long gradients at T = 30 °C and varying the mobile phase pH between 5.7 and 6.6. The commonly applied mobile phase pH in salt gradient CEX separation of mAbs is between 6 and 6.5, therefore our selected range represents well the conditions of real-life separations [14,26,27]. Again, the k_{app}, P_c and resolution were studied as a function of mobile phase pH.

2.3.3. Systematic method optimization

Initial basic runs for multifactorial experimental designs were already suggested in the 90s for reversed phase liquid chromatographic method optimization [28]. A general approach consists in modeling simultaneously the effect of the most important factors e.g. gradient steepness and temperature on selectivity with a previously selected column [29,30]. Then, with the help of resolution maps generated by modeling software – which show the critical resolution of the peaks to be separated [31] – the selected variables can be rapidly and efficiently optimized. This approach was currently applied for the reversed phase separation of antibody variants [32]. In this study, this procedure was implemented for IEX salt gradient based separations.

Based on the observed effects of the factors on retention and resolution of mAbs peaks, a six runs based initial experimental setup was suggested for method optimization in the salt gradient mode. It was indeed found that the impact of temperature on selectivity and resolution was not significant. Performing gradient runs with two gradient times (as t_{g1} = 10 min, t_{g2} = 30 min) and three pH (as pH_1 5.6, pH_2 6.0, pH_3 6.4) on a 100 × 4.6 mm column allowed a reliable optimization of the separation. The optimization was performed by computer simulation using DryLab and a custom made 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 [33]. It is therefore a particular complex example for method development.

2.3.4. Generic salt gradient for multiproduct analysis

After studying the retention behavior of antibodies, a generic salt gradient was proposed that allowed the elution and separation of all the ten mAbs within reasonable analysis time (20 min). A linear NaCl gradient, starting from 0% to 20% B was applied at a flow rate of 0.6 ml/min for all samples. The mobile phase temperature was set at T = 30 °C and pH was equal to 5.7). Fluorescence detection was carried out at λ_{ex} = 280 and λ_{em} = 360 nm.

3. Results and discussion

To achieve an optimal separation of mAbs, the influence of various parameters on separation, such as pH, gradient steepness, temperature or flow rate has to be taken into account. The classical IEX mode employs a linear salt gradient. In previous studies, isocratic experiments were used to determine the salt concentration dependence of the protein retention. Then, the gradient retention times were calculated from the established salt dependence model using mathematical functions describing the gradient profile [34–36].

The work of Snyder and co-workers showed that IEX systems follow non-linear solvent strength (LSS) type mechanism [37,38]. Consequently, solute-specific correction factors are required to use LSS model for retention predictions, thereby limiting the applicability of the LSS model. The non-linearity of LSS model was assessed by comparing the elution data to the stoichiometric displacement model (SDM) commonly used in IEX [39]. The retention factor (k) can be written in the following way according to the SDM model:

$$\log\ k = \log K - z \log C \quad (1)$$

where K is the distribution constant, z is associated with the protein net charge or number of binding sites (effective charge) and C is the salt concentration (that determines the ionic strength). The non-linearity of Eq. (1) is most pronounced for small values of z [38]. If $z > 6$, an LSS type model may provide reliable data for retention factor (retention time).

Beside the LSS and SDM, several other models were developed for IEX such as the slab [40–42], mechanistic [39] or steric mass action models (SMA) [43]. Recently, Schmidt et al. extended the SDM to describe the retention behavior of a mAb in linear salt and pH gradient elution in CEX [44]. Protein retention in IEX was also predicted by structure based models [45,46].

In addition to salt gradient profile (steepness), the mobile phase pH and flow rate were also found to be critical parameters for the IEX separation (purification) of mAbs. To obtain sufficient binding of the proteins to the cation exchange resin, the pH should be set

at least 1 pH unit below the p*I* value [26]. Usually, lower pH provides more positive charge to the protein and increases retention. From a kinetic point of view, lower flow rates were found to offer higher efficiency for monoclonal antibodies in CEX [47]. Therefore, reduction of flow rate could be a solution to improve resolution of charge variants.

3.1. The effect of salt gradient time (gradient steepness) on mAbs retention in CEX

For CEX analysis of mAbs, the gradient elution mode is preferred in practice. The solutes are eluted in order of increasing binding charge (correlates more or less with the p*I*) and equilibrium constant. The retention of mAbs in salt gradient mode is strongly dependent on the salt concentration (gradient steepness) – due to the relatively high z value – and a small change could lead to significant shift in the retention. For this reason, isocratic conditions are impractical, and gradient elution is mandatory in real-life mAb separations.

For linear salt gradient in IEX, the salt concentration varies with time during the gradient, therefore Eq. (1) can be written as:

$$\log\ k = \log K - z \log\left(C_0 + \frac{\Delta C}{t_g}\right) \tag{2}$$

where C_0 is the salt concentration at the beginning of the gradient and ΔC is the change in C during the gradient. Similarly to RPLC, the LSS model describes the analyte retention as a function of the volume fraction (Φ) of the B solvent. For gradient elution mode, the following general equation can be written:

$$\log\ k^* = \log k_w - S\phi^* \tag{3}$$

where k^* is the median value of k during gradient elution when the band has reached the column mid-point, k_w is the value of k in pure water, S is a constant for a given compound (slope of the curve described in Eq. (3)) and Φ^* is the corresponding value of Φ. It is practical to show the dependence of k^* on the gradient time (t_g). For this purpose, the following equation can be derived [43,44,48]:

$$k^* = \frac{t_g}{1.15 t_0 \Delta\phi S} \tag{4}$$

where t_0 is the column dead time. For practical reasons, modeling software such as DryLab generally deal with transformed variables of k or k^* to $\log(k)$ or $\log(k^*)$ to build mathematical models. On the basis of Eqs. (3) and (4), $\log(k^*)$ should follow a linear model when plotted against the logarithm of gradient time (which is related to the gradient steepness) in case of "regular" samples. For linear salt gradient in IEX, a similar equation for k^* can be derived [30]:

$$k^* = \frac{t_g}{1.15[t_0 |z| \log(C_f/C_0)]} \tag{5}$$

where C_f is the concentration of the counter-ion at the end of the gradient program. Please note that both RPLC and IEX separations vary with gradient conditions in a similar way. However, because of the differences in the dependence of k on the mobile phase composition C in IEX (log–log relationship) versus RPLC (log–linear relationship), the LSS model is theoretically not applicable for IEX. Nevertheless, as shown in Eqs. (2) and (5), the higher the z, the lower the deviation from non-linearity is. Therefore, the LSS approach may be applicable for large proteins (mAbs) possessing an important number of charges. Consequently, the linear salt gradient IEX separation of molecules with $z \geq 3$ can be described semi-quantitatively by the LSS model [30,37,38].

The effect of gradient steepness (gradient time) on the retention of intact mAbs and their variants was practically investigated. The gradient time (steepness) was varied as 10, 15, 20, 30 and 40 min (at T = 30 °C and at pH 6.3). The retention of the six selected mAbs and the variants of trastuzumab, adalimumab and cetuximab showed the same behavior. Fig. 1 illustrates the effect of gradient time on the apparent retention (k_{app}) of intact mAbs and charge variants, as representative examples. The relationship between k_{app} and t_g can be perfectly described by fitting a linear function ($R^2 > 0.999$ for all solutes). This linear type behavior can be expected for large proteins possessing several charges, but surprisingly it seems to be even more linear in IEX than in RPLC. As shown in [32], the retention behavior of mAb fragments showed a slight deviation from linear relationship in RPLC. The experimental points in $\log(k_{app})$ vs. $\log(t_g)$ representation followed a slightly concave curvature, while it is not the case in IEX. In IEX salt gradient mode, it can be concluded that a LSS type model perfectly describes the retention behavior of mAbs. There is no need for logarithmic or polynomial fitting (as it is often the case in RPLC mode).

The results suggest that only two gradient runs are required (e.g. with t_{g1} = 10 min and t_{g2} = 30 min) for the optimization of a salt gradient. Then, the retention times can be predicted for any gradient program, due to the linear behavior.

3.2. The effect of mobile phase temperature on mAbs retention in CEX

The effect of temperature on retention factor (k) can generally be expressed in liquid chromatography with the van't Hoff equation:

$$\log\ k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \log\beta \tag{6}$$

where ΔH represents the enthalpy change associated with the transfer of the solute between phases, ΔS the corresponding entropy change, R the molar gas constant, T the absolute temperature in Kelvin and β the phase ratio of the column. When $\log(k)$ is plotted against $1/T$, the enthalpy is given by the slope of the curve.

With regular compounds, these van't Hoff plots follow a linear relationship. However, a quadratic dependence of $\log(k)$ versus $1/T$ over a wide range of temperature was noticed by different authors using silica-based as well as non silica-based stationary phases [49]. The effect of temperature on the retention of partially ionized compounds which exist in two forms (i.e. molecular and ionized forms) can also be well described with Eq. (6). However, both enthalpy and entropy are expected to be different for the two forms and as a result, both H and S can vary with temperature when both forms are present to a significant extent [49]. With large biomolecules, the effect of temperature on retention sometimes becomes more complex. Depending on the stability of the secondary structure, the molecules unfold to various extents and hence interact with the stationary phase with various strengths [50]. Due to the different conformation-dependent responses of proteins at elevated temperatures, the change in retention can be difficult to assess [51,52]. In RPLC separation of proteins, temperature is a useful parameter for adjusting selectivity. However, for IEX separations of proteins, the impact of temperature was not reported in the literature. Data on the distribution coefficients as a function of mobile phase temperature were only reported for amino acids in IEX mode [53].

Fig. 2 illustrates the obtained van't Hoff type plots. Three conclusions can be drawn. First, the $\log(k)$ vs. $1/T$ shows linear behavior in the investigated temperature range. Second, the slope of the curves is much lower than in RPLC mode. Indeed, the slopes in IEX salt gradient mode were comprised between −0.13 and $0.14K \times 10^3$, while for intact mAbs in RPLC mode, the slopes of van't Hoff curves are typically around $0.5–1.0K \times 10^3$ [32]. This suggests that the effect of temperature on the retention properties of mAbs is less important in IEX mode than in RPLC mode. Finally, the slope of $\log(k)$ vs. $1/T$ curves for related mAbs (e.g. charge variants of a given mAb) is practically the same. This means that selectivity cannot be tuned by the temperature, as illustrated in Fig. 2B with the plots of

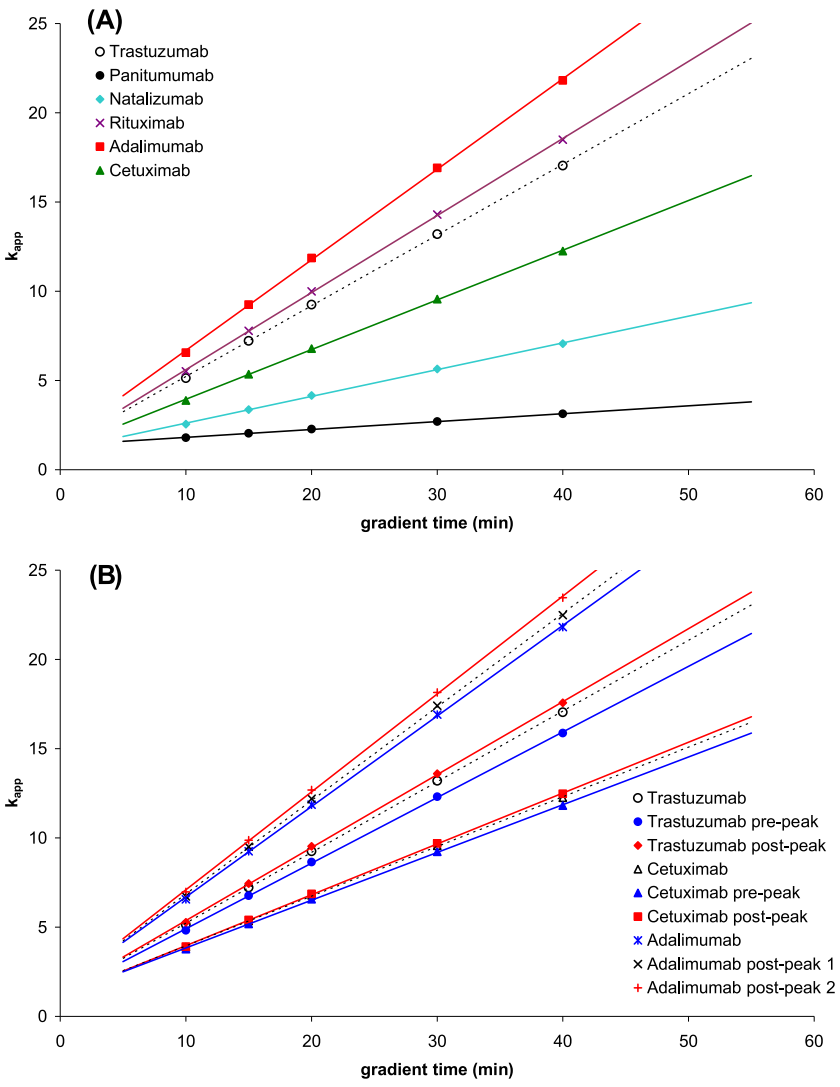

Fig. 1. Effect of gradient time (steepness) on the apparent retention factor of native antibodies (A) and antibody variants (B). Column: YMC BioPro SP-F (100 × 4.6 mm). Mobile phase: "A", 10 mM MES pH 6.3; "B", 10 mM MES pH 6.3 + 1 M NaCl. Flow rate: 0.6 ml/min; gradient: 0–20% B in 10, 15, 20, 30 and 40 min; temperature: 30 °C, detection: FL (280–360 nm); injected volume: 2 μl.

adalimumab, cetuximab and trastuzumab. However, temperature has an impact on the peak width (peak capacity) – see in Section 3.4 – and therefore could modify the quality of the separation.

3.3. The effect of mobile phase pH on mAbs retention in CEX

One of the primary variables for varying IEX retention is the mobile phase pH [54]. Indeed, retention is strongly affected by the ionization state of a compound. In CEX mode, lower pH increases the number of positive charge on the mAb and increases the retention.

Fig. 3 shows the dependency of apparent retention factor of native mAbs on mobile phase pH. MAbs possessing p*I* of at least 8.6 show linear relationships in the studied pH range (5.7–6.6) and the slopes of the apparent *k* vs. pH functions were very similar. However, panitumumab with p*I* = 6.7 behaves in a different way. At mobile phase pH beyond ~6.2, a clear deviation from linear relationship was observed. Close to the p*I*, the retention decreases drastically, but a second order polynomial function can appropriately fit the observed data. The curve intersects the *x*-axis at around pH ~ 6.65, this value is very close to the calculated p*I* of panitumumab. At pH equal to p*I*, no retention is expected. Please note, that panitumumab is a special case as it possesses an extremely low p*I*, compared to other mAbs (p*I* between 8 and 9). Therefore, the commonly used pH in salt gradient mode is between 6 and 6.8 and ensures appropriate retention in CEX mode.

From the retention modeling of view, all the mAbs have to be considered. Chromatographic optimization software commonly use second order polynomial models to describe *k* vs. pH dependence in RPLC mode, based on three initial runs. On the basis of the results obtained with intact mAbs, this approach is probably also appropriate for the modeling of mAbs retention in salt gradient CEX mode.

3.4. Peak capacity

Peak capacity as a function of gradient time and temperature was also estimated. Peak capacity is a measure of the separation power that includes the entire chromatographic space together with the variability of the peak width over the chromatogram. The general expression for peak capacity (P_c) in liquid chromatography, assumes unit resolution between the successively eluted peaks [55]. In this study, peak capacities were experimentally determined from the gradient time (t_g) and the average measured peak width at 50% height ($w_{50\%}$). The following equation was used to estimate the

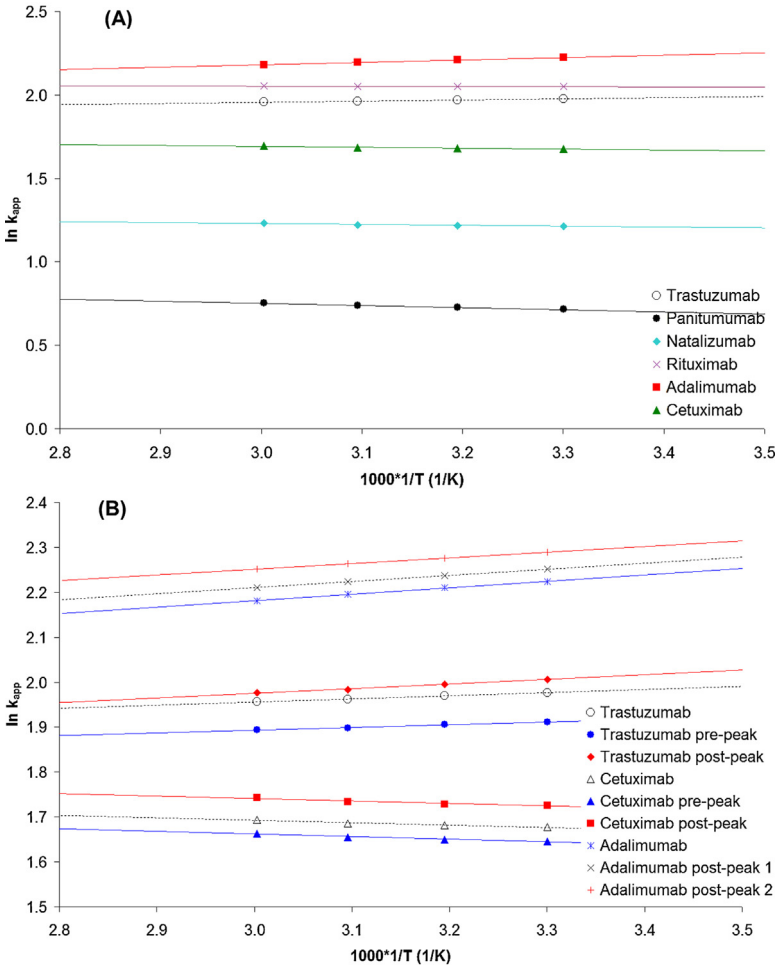

Fig. 2. Effect of temperature on the apparent retention factor of native antibodies (A) and antibody variants (B) (van't Hoff type representation). Column: YMC BioPro SP-F (100 × 4.6 mm). Mobile phase: "A", 10 mM MES pH 6.3; "B", 10 mM MES pH 6.3 + 1 M NaCl. Flow rate: 0.6 ml/min; gradient: 0–20% B in 15 min; temperature: 30, 40, 50 and 60 °C; detection: FL (280–360 nm); injected volume: 2 μl.

peak capacity based on peak width at half height, corresponding to a resolution of $R_s = 1$ between consecutive peaks:

$$P_c = 1 + \frac{t_g}{1.7 \cdot w_{50\%}} \quad (7)$$

To avoid the imprecision associated with the measurement of peak widths around baseline for mAbs which often contain closely related variants (i.e. heterogeneous sample), the peak width was measured at half height in this study.

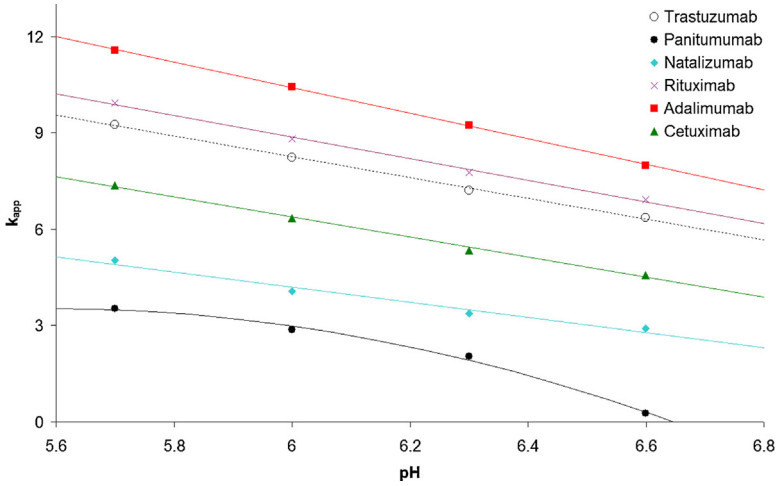

Fig. 3. Effect of mobile phase pH on the apparent retention factor of native antibodies. Column: YMC BioPro SP-F (100 × 4.6 mm). Mobile phase: "A", 10 mM MES; "B", 10 mM MES + 1 M NaCl (pH was set at 5.7, 6.0, 6.3 and 6.6). Flow rate: 0.6 ml/min; gradient: 0–20% B in 15 min; temperature: 30 °C; detection: FL (280–360 nm); injected volume: 2 μl.

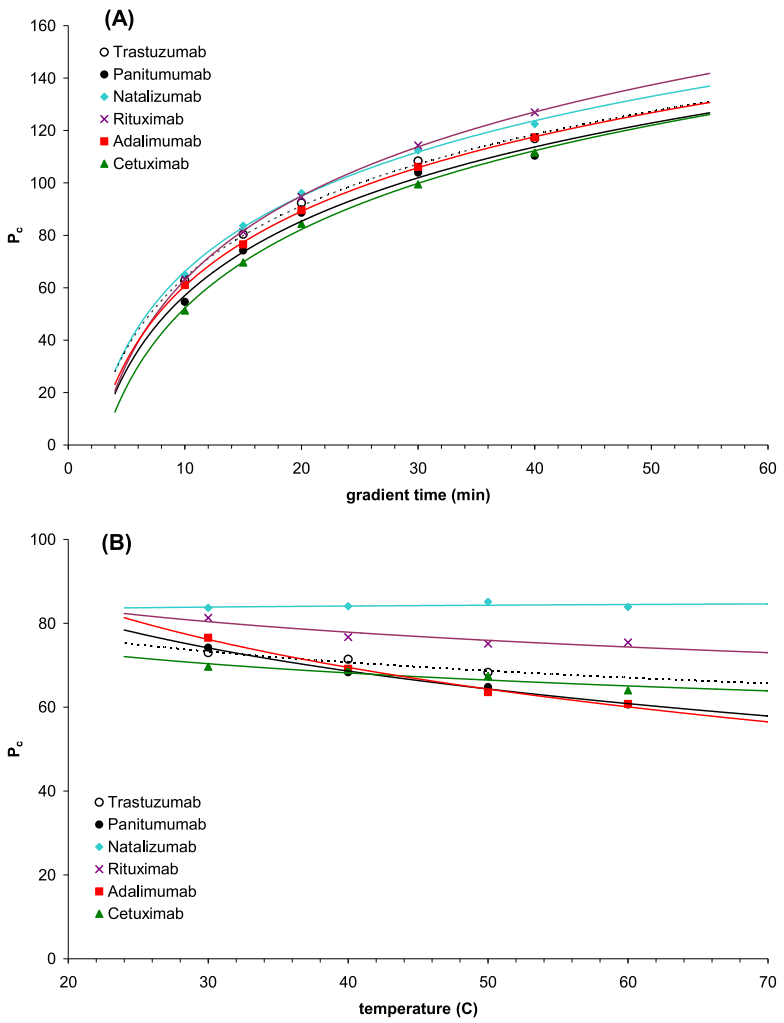

Fig. 4. Peak capacity as a function of gradient time (steepness) (A) and temperature (B). Column: YMC BioPro SP-F (100 × 4.6 mm). Mobile phase: "A", 10 mM MES pH 6.3; "B", 10 mM MES pH 6.3 + 1 M NaCl. Flow rate: 0.6 ml/min; detection: FL (280–360 nm); injected volume: 2 μl.

As shown in Fig. 4A, peak capacity (P_c) of ~50–60 was observed with 10 min long gradient while the longest 40 min gradient provided $P_c \sim 110$–130.

Fig. 4B shows the change in peak capacity as a function of mobile phase temperature. A slight decrease in peak capacity was observed for most mAbs (except natalizumab). The peak capacity for adalimumab changed from 75 down to 60 when increasing the temperature from 30 to 60 °C. This observation suggests that lower temperature is more favorable for the IEX separations of mAbs. Because selectivity does not change significantly with temperature in IEX and since peak capacity is somewhat higher at lower temperature, better resolution is expected under such conditions. This is opposite to what is commonly observed in RPLC. Indeed, when analyzing mAbs under RPLC conditions, a temperature of at least 70–80 °C is required to have sharp symmetrical peaks and appropriate recovery [56]. The improvement of peak shape in RPLC is related to the improvement of the mass transfer process through the porous particles. In the case of IEX separation, a non-porous hydrophilic polymer bead is used, therefore there is no trans-particle mass transfer process. Only eddy dispersion, longitudinal diffusion and external film mass transfer contribute to band broadening. The two latter may be affected by mobile phase temperature; and increase in the film mass transfer and longitudinal diffusion is expected. In our study, the impact of longitudinal diffusion is probably not negligible, since we do not have trans-particle mass transfer resistance. Maybe, this enhanced longitudinal diffusion can explain the slight decrease in peak capacity when increasing mobile phase temperature. Other possible explanations could be the change of p*I* and mobile phase pH with temperature and on-column aggregation at elevated temperature.

3.5. *Creating a two dimensional DryLab model for CEX*

Optimization software packages generally employ linear models for the simultaneous optimization of two or three variables. The polynomial equation for two variables can be written as:

$$y = b_0 + b_1 x_1 + b_2 x_2 \quad (8)$$

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 already discussed it is better to use quadratic models with mAbs, to have adequate prediction accuracy of retention times as a function of mobile phase pH. The general quadratic model for two variables can be written as:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + b_{12} x_1 x_2 \quad (9)$$

In this study, DryLab was used for further method optimization and determination of the unknown coefficients of the model. The software implements an interpretive approach, where the retention behavior is modeled using experimental information

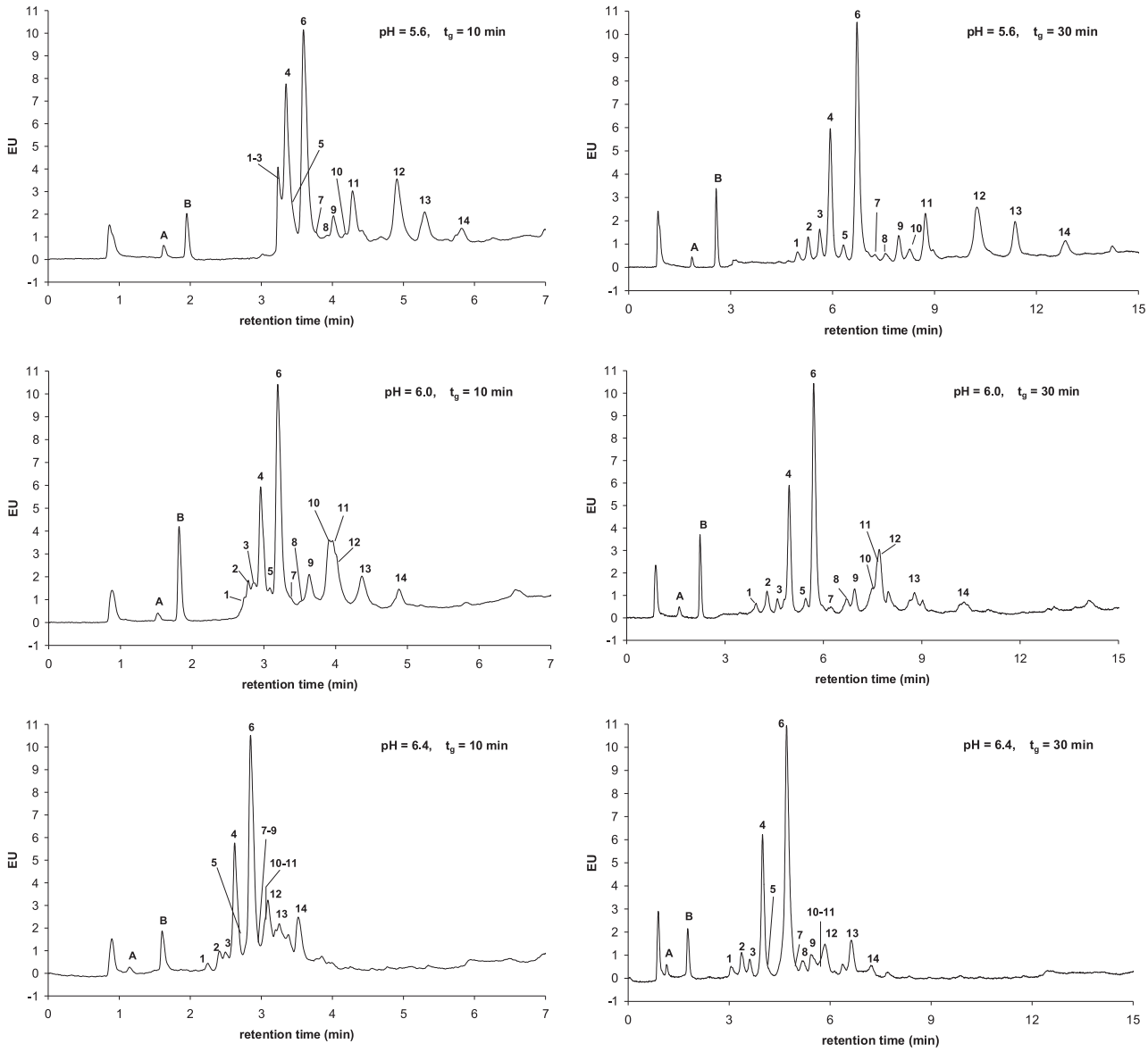

Fig. 5. Cetuximab papain digested sample. Column: YMC BioPro SP-F (100 × 4.6 mm). Mobile phase: "A", 10 mM MES; "B", 10 mM MES + 1 M NaCl. Flow rate: 0.6 ml/min; gradient: 0–20% B; temperature: 30 °C; detection: FL (280–360 nm); injected volume: 2 μl. Gradient times: t_g1 = 10 min, t_{g2} = 30 min, pH_1 5.6, pH_2 6.0, pH_3 6.4.

from initial runs, and the retention times at other conditions are predicted in a selected experimental domain. This allows calculating the critical resolution, and accordingly, the optimal separation can be found [57].

As shown in Sections 3.2 and 3.4, temperature is not an important variable. It does not influence significantly the retention and selectivity. On the contrary, the salt gradient steepness and mobile phase pH appear as the most important factors to adjust selectivity and resolution. Regarding temperature, it is beneficial to work at low temperature to guarantee the highest peak capacity. In our optimization, gradient time and pH were selected as model variables, while temperature was kept constant at 30 °C.

A new two dimensional mode was created in DryLab software. Retention times were transformed to retention factors, and quadratic and linear models were chosen for pH and gradient time (steepness), respectively. The modeling takes place in a rectangular region in the t_g–pH plane determined by three pH and two gradient times (steepness) values. Then, the model requires measuring the effects of the variables at three different pH levels and two gradient times. Hence, this approach necessitates six initial experimental runs for creating the model. Gradient times were set to t_{g1} = 10 min and t_{g2} = 30 min while pH was varied as pH_1 5.6, pH_2 6.0 and pH_3 6.4. Following the execution of the input experimental runs, the figures of merit (i.e. retention times, peak widths and peak tailing values) were imported into DryLab and peak tracking was performed. Peak tracking was performed on the basis of peak areas. It has to be mentioned that in some cases, slight tendencies were observed in peak areas as the digestion process cannot be stopped completely. Therefore in the case of digested mAb samples the peak tracking process maybe more complex than for common small molecules. Next, the optimization was carried out on the basis of the created resolution map. In the resolution map, the smallest resolution (R_s) value of any two critical peaks in the chromatogram was plotted as a function of two simultaneously varied experimental parameters.

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

3.6. Optimization of the separation of Fab and Fc fragments of cetuximab

Separation of the Fc and Fab domains has facilitated investigation of the micro-heterogeneity of human mAbs (confirmation of chemical and post-translational modifications such as N-terminal cyclization, oxidation, deamidation, and C-terminal processed lysine residues [58,59]). The present example describes a fast and efficient method development applied for the determination of charge variants of a recombinant mAb (cetuximab), using salt gradient approach in CEX mode. The native mAb was digested with papain and the aim of the method development was to separate as many variants of the Fab and Fc fragments as possible, within the shortest achievable analysis time. The two initial gradients with different slopes were carried out at three pH values. Fig. 5 shows the chromatograms of the six initial runs.

The corresponding resolution map is presented in Fig. 6. As shown, a 17 min gradient was found to provide the highest resolution when the mobile phase pH is ~5.6. The predicted optimum condition was set and experimental chromatograms recorded. Fig. 7 shows the predicted and experimental chromatograms.

To evaluate the accuracy of this approach (with 10 and 30 min initial gradient runs) applied for 100 × 4.6 mm column, the predicted and experimental chromatograms (retention times) were compared (Table 1). The predicted retention times were in good agreement with the experimental ones; the average retention time relative errors was ~1.0% (see Table 1), which can be considered as excellent.

To conclude, this method optimization approach can be considered as reliable and the suggested initial experiments (10 and 30 min gradient on a 100 mm long standard bore column at pH 5.6, 6.0 and 6.4) can be applied in daily routine work, resulting in time saving. The time spent for method development in this example was approximately 9 h (2 gradient times × 3 pH × 3 samples + equilibration), and then the predicted method was experimentally verified.

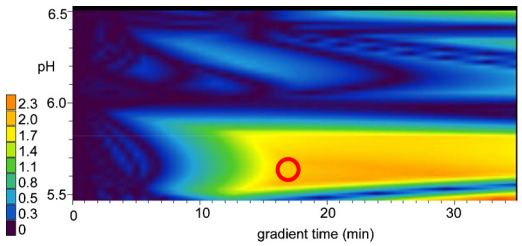

Fig. 6. Cetuximab papain digestion resolution map (t_g–pH model). Column: YMC BioPro SP-F (100 × 4.6 mm). Mobile phase: "A", 10 mM MES; "B", 10 mM MES + 1 M NaCl. Flow rate: 0.6 ml/min; gradient: 0–20% B; temperature: 30 °C; detection: FL (280–360 nm); injected volume: 2 μl. Gradient times: t_{g1} = 10 min, t_{g2} = 30 min, pH_1 5.6, pH_2 6.0, pH_3 6.4.

However please note that for more complex samples, the optimum conditions for high resolution separations can be shifted to the lower pH and longer gradient time ranges. Therefore for high resolution separations an extended model might be useful.

3.7. Generic salt gradient CEX method for various mAbs

The main criticism of salt gradient IEX separations is that it is product specific and time-consuming to develop [24]. On the contrary, pH gradient based separation using a CEX column is described as a multi-product charge sensitive separation method for mAb samples [3,24,25]. In this study, we wanted to prove that salt gradient separation is also suitable for multi product separations (mAbs possessing various p*I*), provided that using appropriate conditions.

Similarly to Section 3.6, the gradient steepness and mobile phase pH were systematically varied to obtain a suitable separation of 10 mAbs and their variants. In addition, the impact of the salt

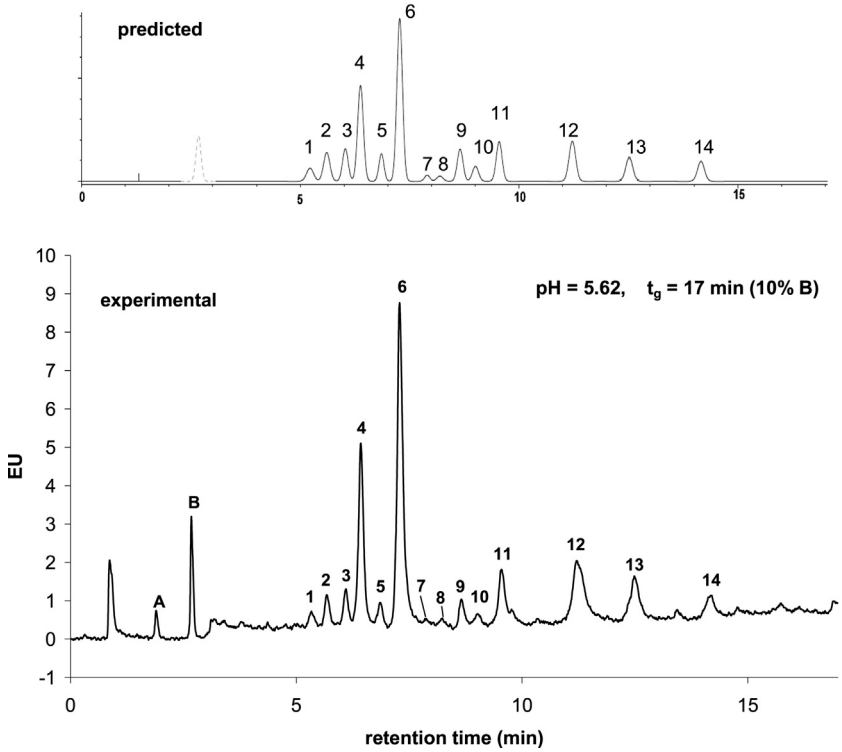

Fig. 7. Comparison of predicted and experimental chromatograms. Column: YMC BioPro SP-F (100 × 4.6 mm). Mobile phase: "A", 10 mM MES; "B", 10 mM MES + 1 M NaCl. Flow rate: 0.6 ml/min; gradient: 0–10% B; temperature: 30 °C; detection: FL (280–360 nm); injected volume: 2 μl. Gradient times: t_g = 17 min, pH 5.62.

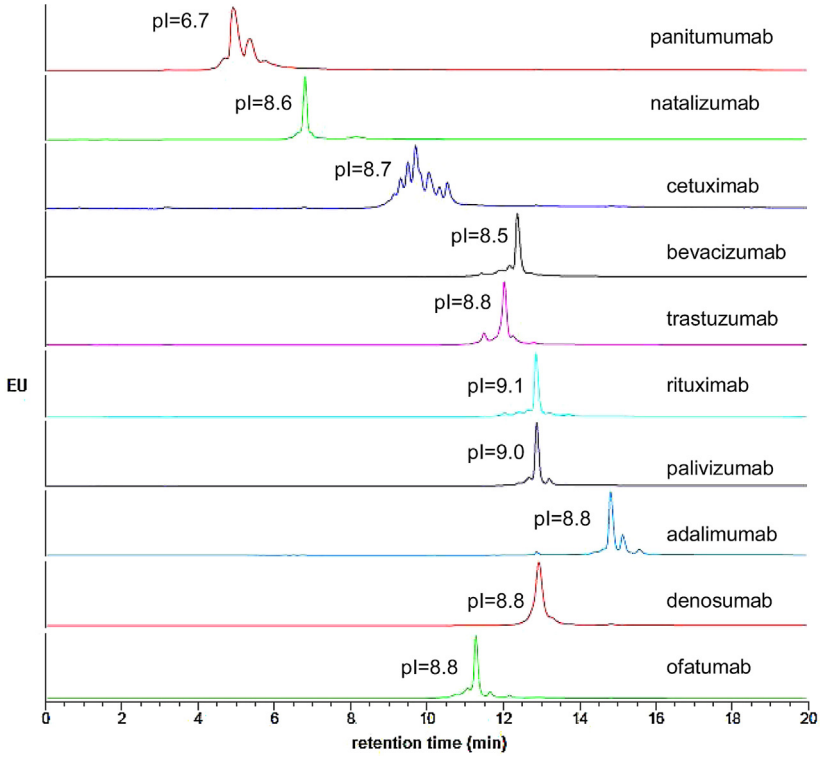

Fig. 8. Generic salt gradient. Column: YMC BioPro SP-F (100 × 4.6 mm). Mobile phase: "A", 10 mM MES pH 5.7; "B", 10 mM MES pH 5.7 + 1 M NaCl. Flow rate: 0.6 ml/min; gradient: 0–20% B in 20 min; temperature: 30 °C; detection: FL (280–360 nm); injected volume: 2 μl.

fraction was also studied and it was found that 0.2 M NaCl was sufficient to elute mAbs possessing the highest p*I* (~9). On the other hand, to ensure a sufficient retention of mAbs with low p*I* (~6.5), the mobile phase pH was kept under 6 (e.g. pH 5.7).

Fig. 8 shows the obtained chromatograms of 10 intact mAbs, and suggests that salt gradient CEX separation may also be adequate for multi-product mAb separations. The optimal conditions on a strong cation exchanger resin were found as 20 min long gradient (0–0.2 M NaCl) at pH 5.7 and at a mobile phase temperature of $T = 30\,^{\circ}C$. If mAbs with lower or higher p*I* are also included in the production line, the conditions can be adjusted accordingly. This example clearly shows that ionic strength based CEX separation of mAbs is not product specific, and generic conditions can easily be found for several different types of mAbs.

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

Peak	Retention time (min)			
	Experimental	Predicted	Difference	Error (%)
B	2.68	2.67	0.01	0.26
1	5.33	5.26	0.07	1.41
2	5.68	5.60	0.08	1.34
3	6.10	6.03	0.07	1.16
4	6.43	6.38	0.05	0.77
5	6.86	6.85	0.01	0.09
6	7.29	7.27	0.02	0.23
7	7.88	7.90	−0.02	−0.24
8	8.24	8.19	0.04	0.55
9	8.66	8.65	0.01	0.07
10	9.02	9.00	0.02	0.18
11	9.55	9.55	0.00	−0.04
12	11.21	11.22	−0.01	−0.11
13	12.49	12.49	0.00	−0.01
14	14.19	14.16	0.03	0.24
Average			0.02	0.39

4. Conclusion

There are two main criticisms when applying salt gradient IEX for the characterization of mAbs. First of all, it is generally long and tedious to develop a CEX method and secondly, the conditions are not enough generic (product specific conditions). The goal of this study was to tackle these two limitations.

In a first instance, the retention models when varying mobile phase temperature, pH and gradient steepness were assessed under CEX conditions, thanks to therapeutic reference mAbs and their charge variants. It appears that temperature was not a relevant parameter for tuning selectivity and should be kept at 30 °C, to achieve high resolving power. Because the relationship between apparent retention factors and gradient time – in IEX mode – can be described with a linear function, only two initial gradient runs of different slopes are required for optimizing the salt gradient program. Finally, second order polynomial models (i.e. based on three initial runs) are required to describe *k* vs. pH dependence, for the modeling of mAbs retention in salt gradient CEX mode. Based on these observations, we demonstrated that the development of a CEX method can be performed rapidly, in an automated way thanks to a HPLC modeling software, using two gradient times and three mobile phase pH (e.g. 10 and 30 min gradient on a 100 mm long standard bore column at pH 5.6, 6.0 and 6.4). Such a procedure can be applied routinely and the time spent for method development would be only 9 h. At the end, the differences between experimental and predicted retention times were lower than 1%, making this approach highly accurate.

Secondly, we have also demonstrated that generic salt gradient CEX conditions can be applied for the characterization of 10 mAbs possessing p*I* between 6.7 and 9.1. A proportion of 0.2 M NaCl was sufficient to elute the most basic mAbs from the strong cation exchange column, while a pH lower than 6 was appropriate to sufficiently retain the mAbs with lowest p*I*. This example shows that salt gradient CEX can be considered as a multi-product approach

and generic conditions can be applied for the characterization of various mAbs.

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