Tuning selectivity in cation-exchange chromatography applied for monoclonal antibody separations, part 1: Alternative mobile phases and fine tuning of the separation

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Cation exchange chromatography (CEX) of therapeutic monoclonal antibodies is generally performed with either salt gradient (MES buffer+NaCl) or using commercial pH gradient buffer. The goal of this study was to find out some alternative buffer systems for CEX separation of mAbs, which may offer alternative selectivity, while maintaining similar peak shapes. Among the new buffers that were tested, (N-morpholino)ethanesulfonic acid (MES) / 1,3-diamino-2-propanol (DAP), and citric acid / 2-(cyclohexylamino)ethanesulfonic acid (CHES) systems were particularly promising, especially when combining them with a moderate salt gradient of NaCl. This two buffer system provides an equivalent or slightly better separation than the standard, mobile phases for therapeutic mAbs.

It was also demonstrated that working with salt-mediated pH gradients, allows to extend the possibilities in method development, since the concentration of salt in the mobile phase has a significant impact on selectivity. Using HPLC modeling software (Drylab), it was possible to successfully develop CEX methods for authentic mAb samples within only 6 h, by optimizing the gradient steepness and salt concentration in the B eluent.

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\section*{1. Introduction}
Monoclonal antibodies (mAb) belong to the class of therapeutic proteins, and have become key treatment [1]. Their main areas of applications are within the field of oncology, as well as infectious and immune diseases [2]. The high number of variants, formed during the production of mAbs, and the wide variety of chemical modifications due to posttranslational modifications (PTM) (e.g. oxidation, deamidation, glycosylation, glycation, fragmentation), can influence their biological activity. Therefore, proper analytical methods are required to determine their structures and understand their behavior [3,4]. Numerous chromatographic approaches are available for the analytical characterization of mAbs, including reverse-phase liquid chromatography (RPLC), ion-exchange chromatography (IEX), size-exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC) and hydrophilic interaction chromatography (HILIC) [5–7]. In addition to IEX methods, electrophoretic techniques, such as capillary zone electrophoresis (CZE) and imaged capillary isoelectric focusing (icIEF) are often applied to separate mAb charge variants [8,9].

Cation-exchange liquid chromatography (CEX) is a commonly used, non-denaturing method for the identification and quantification of acidic and basic mAb variants, depending on their accessible surface charges [10]. Besides icIEF, CEX is considered as another gold standard strategy to analyze protein charge variants. Depending on the strength of the interaction between the stationary phase and the protein, weak or strong cation-exchanger resins can be used, including carboxylic or sulfonate groups at the surface, respectively [11]. The retention properties, selectivity and resolution of five different state-of-the-art CEX stationary phases were recently investigated for mAb separations [12].

In CEX, two approaches are available to elute the ionic compounds from the column: (i) weakening the ionic interactions between the analyte and the stationary phase by increasing the salt

\begin{itemize}
  \item [(i)]
\end{itemize}
concentration (salt-gradient mode) or (ii) changing the pH of the eluent as a function of time at constant ionic strength and therefore the charge of the proteins (pH-gradient mode). The two different gradient modes and their combination were previously studied by several research groups.

The generic salt-gradient mode can be optimized by adjusting the nature of the stationary phase, the mobile phase pH and the gradient profile. The steepness, range and shape of the applied gradient are the main parameters to optimize mAb separations, but are scarcely studied in CEX. Farjami and coworkers validated a salt gradient, chromatographic method for cetuximab charge variants using different salt concentrations, salt gradient steepness, mobile phase pHs, flow rates and temperatures [13]. Another study showed the impact of eluent pH, gradient steepness and temperature on retention, selectivity and peak capacity, while Joshi and coworkers investigated a fast, non-linear, salt gradient CEX method for mAb separations [14]. Sodium chloride was mostly used as salt additive.

Alternatively, the mobile phase pH can be increased to elute the mAb charge variants. The buffer system used to perform a linear pH-gradient generally contains a weak acid and a weak basic (mostly amines) buffer in a relatively narrow pH range [15,16]. The greatest advantage of these buffers is their low concentration, which makes the approach potentially mass spectrometry (MS) friendly. The reference buffer systems consist of Tris base, piperazine and imidazole, providing mostly linear pH responses between pH 6 and 9.5, and are now widely used and commercially available [17,18]. More recently, Füssl et al. reported the application of a buffer system including acetic acid, ammonium bicarbonate and ammonium hydroxide [19]. Using such a volatile buffer at low concentrations, enabled the performance of direct IEX-MS separation for mAbs. In addition, the applied pH range could be extended (5.3 ≤ pH ≤ 10.2). This method has recently been applied successfully for the comprehensive characterization of adalimumab [20]. Talebi and coworkers studied the charge heterogeneity profile of three different IgG2bs by comparing a mixture of triethanolamine (TEA) and diethanolamine (DEA) and ammonium hydroxide [21].

A pH gradient mode was also applied in the anion-exchange mode, as well. As example, amine buffering species having $pK_a$ values in regular intervals (piperidine 11.1; piperazine 9.7, 5.3; triethanolamine 7.7; bis-tris propane 9.0, 6.8 and N-methyl piperazine 4.7) were used as mobile phase buffers [22]. Such a system was used to separate a wide range of proteins possessing pI between 3.9 and 11.

Very recently, salt and pH gradient elution modes were compared, in order to develop an optimal separation for the mAb, standard reference material from NIST, on a strong cation exchange phase [23]. A separation using the pH gradient approach was found to outperform salt gradient separation. The developed pH gradient method was transformed into an ultra-fast separation method by using a 50 x 2.1 mm column.

Combining the salt and pH gradient methods is known as salt-mediated pH-gradient approach and can be suitable for improving the separation and extending the range of mAbs (wide range of pI) that can be simultaneously analyzed [24]. For most buffer systems, providing a linear pH response, as function of pH, the ionic strength in mobile phases A and B could be different. For mobile phases containing multiple polyamine buffers – which are often used for mAb separations, such as the commercially available pH gradient buffer from Thermo (CX-1), the total ionic strength needs to be reduced, so as to increase the pH in a linear fashion. For the mobile phase with high buffer concentration, the ionic strength is higher at low pH values, leading to insufficient retention of acidic mAbs on a cation-exchanger column. However, it is important to keep in mind that the basic mAbs may not have an optimal elution profile at high mobile phase pH. This can be because the relatively low, ionic strength of the mobile phase, may broaden the peaks, thus decreasing resolution (due to the lack of band compression driven by the counter ions). Therefore, to modulate the ionic strength and develop a proper gradient, an additional salt gradient was suggested to compensate for the lower ionic strength in mobile phase B [24]. An optimized salt-mediated pH gradient method can finally be applied to mAbs, over a broader pI range from 6.2 to 9.4. This offered better resolution for mAbs possessing high pI values (7.3 < pI < 9.0). Zhang et al. optimized the buffer pH and salt concentrations simultaneously, and achieved a wide range pH method, which provided high resolution for various mAbs [24]. In a fundamental study, 20 mM sodium citrate, 20 mM sodium dihydrogen phosphate, 20 mM Tris and 20 mM glycine were used as buffer components [25]. Different amounts of sodium chloride were added to tune the retention using the same gradient slope. By using such a buffer system, a pH gradient from 4 to 11 could be performed.

The purpose of the present work (which is the first part of a series of study) was to find some new, simple and cheap buffer systems, which can be applied to perform salt-mediated pH gradients and may offer alternative selectivity for the separation of mAbs in cation–exchange chromatography compared to the commonly used mobile phases. Only two component systems (acid and base pairs) were considered. The pH responses, peak shape, selectivity and elution window have been systematically studied for various buffer systems, using therapeutic mAbs as model compounds. The possibilities of retention modeling and method development, were also evaluated and a practical method development workflow was proposed based on only six initial experiments. The second part of this series focus on new stationary phases (efficiency and selectivity) and on the use of small columns in CEX.

2. Experimental

2.1. Equipment and software

The experiments were performed on a Waters Acquity UPLC™ I-Class system, consisting of a binary solvent delivery pump, an auto-sampler with flow-through-needle (FTN) injection and a fluorescence detector (FL) with a 2 μL flow-cell. The inner diameter of the tube between the injector and the column was 0.13 mm (active preheater included) and 0.10 mm between the column and the detector. The overall extra-column volume was around 8 μL and the dwell volume was equal to 100 μL. Data acquisition and instrument control was performed by Empower Pro 3 software (Waters). Calculation and data transfer was achieved by using Excel templates.

The mobile pH was checked and adjusted using a Seven- Multi S40 pH meter (Mettler Toledo, Greifensee, Switzerland). Retention modeling and method optimization were performed by DryLab® 4 chromatographic modeling software (Molnar-Institute, Berlin, Germany). The pH of the various buffer systems was initially evaluated with BATE pH Calculator 1.0.3.15 (ChemBuddy).

2.2. Chemicals, samples and column

Gradient grade water and piperazine were purchased from Fisher Scientific (Dublin, Ireland), 2- (N-morpholino)ethanesulfonic acid (MES), dithiothreitol (DTT), 1,3-diamino-2-propanol (DAP), citric acid, sodium chloride (NaCl), 2-(cyclohexylamino)ethanesulfonic acid (CHES), imidazole, Tris(hydroxymethyl)aminomethane hydrochloride (TRIS–HCl), ammonium hydroxide solution (28% NH₃ in H₂O) and ammonium acetate were purchased from Sigma-Aldrich (Buchs, Switzerland). Sodium hydroxide (NaOH) was purchased from VWR Scientific (Radnor, PA, USA), acetic acid from Biosolve (Dieuze, France), CX-1 buffer from Thermo Fisher Scientific (Waltham, MA, USA) and IdeS enzyme (FabRICATOR®, art. A0-FR1-050) from Genovis AB (Lund, Sweden).
Therapeutic IgG monoclonal antibodies, including bevacizumab, dalotuzumab, daratumumab, obinutuzumab, panitumumab, per-
tuzumab, rituximab, trastuzumab and cetuximab were obtained as European Union pharmaceutical-grade drug products from their respective manufacturers.

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

2.3. Apparatus and methodology

2.3.1. Sample preparation

Monoclonal antibodies were diluted in water to get 1 mg/ml solutions. Intact bevacizumab, daratumumab, panitumumab, per-
tuzumab and rituximab were individually injected to study their retention behavior in different buffer systems. A mixture of obin-
utuzumab, daratumumab and rituximab (1 mg/ml) was prepared and eluted with MES – DAP system, at various gradient steepness, temperatures and with different amounts of NaCl. For method optimization, cetuximab and daratumumab were digested with IdeS to get F(ab’)2 and Fc subunits. Digestion was initiated by addition of 10 mM Tris to mAb solution. The digestion was carried out at 38 °C for 30 min [26]. The final volume of the samples was 100 μL, and these were directly injected using low volume insert vials.

2.3.2. Mobile phase preparation

For pH-gradient separations, mobile phases were prepared by dissolving 10, 20 and 50 mM of MES, 1,3-diamino-2-propanol (DAP), citric acid, CHES, 10 mM piperezine and imidazole in water. The pH dependence of the eluent composition was measured by mixing acidic and basic eluent pairs in the range from 0 to 100%, in 10% intervals. The studied eluent pairs were: (1) MES/piperazine, (2) citric acid/DAP. (3) MES/DAP, (4) citric acid/imidazole, (5) MES/imidazole and (6) citric acid/CHES.

An ammonium acetate based eluent was also studied as a candidate as an MS friendly mobile phase. A 50 mM solution was prepared and the pH was set to 6 by adding acetic acid (as mobile phase A) and to 10 by adding ammonium hydroxide (as mobile phase B).

Classical salt-gradient was performed with a 10 mM MES (pH = 6) solution as eluent A and with 0.4 M NaCl added to 10 mM MES as eluent B. For the so-called pH gradient measurements, the commercially available CX-1 pH gradient buffer (Termo) was used and run from 0 to 100% B (corresponds to pH response from 5.6 to 10.2). Based on the safety data sheet of CX-1 buffer, it contains four zwitterionic buffer salts (2-(N-morpholino)ethanesulfonic acid, 3-(N-morpholino) propane sulfonic acid, N-Tris(hydroxymethyl) methyl-3-amino propane sulfonic acid and 3-(cyclohexylamino)-2-hydroxy-1-propane sulfonic acid) and in addition NaCl and NaOH.

For salt-mediated pH-gradients, the same mobile phases were used as for the pH gradients but different amounts of NaCl (20, 60, 100 and 150 mM) were added to eluent B.

2.3.3. Studying retention properties and selecting the most promising mobile phases

Intact mAbs and a mAb mixture were injected, so as to study the retention behavior and peak shapes of mAbs possessing a wide pI range. During the scouting runs, the peak shape and elution window were studied with 6 different buffer systems (see Section 2.3.2.), in both “pure pH gradient” mode and “salt-mediated pH gradient” modes. In the latter case, 150 mM NaCl was added to mobile phase B to perform the salt–mediated gradients. During this scouting process, a generic linear gradient was run from 0 to 100% B in 10 min, at 0.8 ml/min flow rate and 25 °C. Fluorescence detection was carried out at λex = 280 and λem = 350 nm.

In a second step, the MES/DAP system was selected as a good alternative buffer system for mAbs cation-exchange separations and was further investigated. The impact of (i) salt concentration (cS) in mobile phase B, (ii) gradient steepness (gradient time, Tg) and (iii) mobile phase temperature (T) were studied on the retention of intact mAbs (i.e., obinutuzumab, daratumumab and rituximab). The following values were set for the three selected variables, Tg = 8, 12, 16, 20 and 24 min, cS = 0.20, 60, 100 and 150 mM, T = 25, 30, 35, 40 and 45 °C. Mobile phase A consisted of 90% 20 mM MES and 10% 20 mM DAP, while mobile phase B consisted of 30% 20 mM MES and 70% 20 mM DAP, providing a pH gradient from 5.6 to 9.9. Please note that only the practically useful ranges have been studied. Based on our experiments, most of the intact mAbs and their subunits can be eluted with these conditions. Then, retention time vs. gradient time, retention time vs. salt concentration and retention time vs. reciprocal temperature (van’t Hoff) plots were constructed.

2.3.4. Retention modeling and method optimization

Based on the preliminary experiments, Tg and cS were selected as the two most important method variables, in order to tune selectivity and optimize resolution of mAb separations in salt-mediated pH gradient mode. Subunit separations of cetuximab and daratumumab have been optimized on the basis of 6 initial runs. Within these 6 runs, the Tg was studied at 2 levels (Tg1 = 8 min and Tg2 = 24 min) while cS at 3 levels (cS1 = 20 mM, cS2 = 60 mM and cS3 = 100 mM). Then, resolution maps were built and the two variables were simultaneously optimized. Then optimal conditions have been set and experimentally verified (the accuracy of retention time prediction was estimated). For all the measurements, flow rate was set at 0.8 ml/min, while temperature was set at 25 °C. The injection volume was 1 and 2 μL for daratumumab and cetuximab, respectively.

3. Results and discussion

3.1. pH Responses of various mobile phase systems

When performing a pH gradient in CEX, the net charge of all mAbs is changing during the elution, as functional groups are deprotonated. In the interest of applying mobile phase conditions for proteins possessing a wide range of pI, the gradient has to embrace a wide pH range. Our idea was to identify some simple, alternative buffer systems, consisting of only two compounds and providing a sufficiently wide pH range. Therefore, some preliminary calculations were done to evaluate the pH ranges and the responses of various combinations of common acids and bases. Then, the most promising buffer systems were prepared, and their pH was measured by 10% steps from 0 to 100% base content. The experimental pH responses of the 20 mM buffer systems and 50 mM ammonium acetate are shown in Fig. 1.

MES/piperazine: MES is often used in CEX, but mostly, in order to assist salt gradient separations and to stabilize the mobile phase pH around 6. As far as we know, it has not been applied for pH gradients, even if it can be considered as a good candidate for the most acidic buffer (pK= 6.27). On the other hand, piperezine (pK= 9.73) is often used as the buffering component to perform pH gradients, and therefore, it was interesting to try this combination. With this buffer system, a pH range from 4.5 to 11 was realized. However, the pH response was clearly not linear, and showed a steep intermediate section between pH 6.5 and 9.5 (corresponding to 40 and 60% piperezine, respectively).

MES/imidazole: Imidazole has an amphoter characteristic (pK= 7.0 and T4.5). Imidazole is commonly used for the CEX separations, as a mobile phase additive. The commercial CX-1 buffer (Thermo) also contains it. Nevertheless, a MES/imidazole combination has not yet
been applied. This system gives a perfect linear response in $5 \leq \text{pH} \leq 8$, which can be useful mostly for the less basic mAbs.

Citric acid/imidazole: Imidazole might also be combined with other common acids - such as citric acid, which is a trifunctional organic acid, with $pK_a$ values of 3.13, 4.76 and 6.40 - to extend the pH range. In this way, the pH response range was broadened ($2.5 \leq \text{pH} \leq 9.4$). Considering the pH range, this may provide sufficient retention for the most acidic mAbs (e.g. panitumumab) and other acidic fusion proteins (e.g. abatacept). This system shows a continuous, convex pH response as a function of composition.

Citric acid/CHES: CHES ($pK_a = 9.3$) is also frequently utilized for ion-exchange and electrophoretic separations of proteins, as a buffer component. The citric acid/CHES system showed a very similar trend as did citric acid-imidazole. When titrating CHES with sodium hydroxide, the pH range could be extended ($2.5 \leq \text{pH} \leq 10$).

Citric acid/DAP: DAP is scarcely used, especially for protein separations. However, it could improve CEX separations, as it is a bidentate diamine with $pK_a$ of 7.9 and 9.7, and thus would provide an appropriate buffer capacity in the range of $7 \leq \text{pH} \leq 10.5$. Combining it with citric acid should cover a wide range of applicable pH, with proper buffer capacity ($\text{pH} = 2.5-11$). And, therefore it can be considered as a good candidate for the preparation of a universal buffer for CEX. In practice, we found a slightly sigmoidal shape when looking at the evolution of pH as function of buffer composition, with an inflexion point at pH 7.

MES/DAP: For common mAb separations, a narrower pH range may be sufficient, therefore we tried to replace citric acid by MES. This MES-DAP system gave a slightly concave response in the range of $4.4 \leq \text{pH} \leq 11$.

Ammonium acetate: Finally, ammonium acetate was tried as a volatile buffer, since it has recently been applied for CEX separations of mAbs coupled directly to MS [27]. A sigmoidal response was observed, with an inflexion point at pH 7.

3.2. Comparison of elution properties of mAbs using various mobile phase systems

Preliminary runs were performed with the seven buffer systems (at 10, 20 and 50 mM), so as to investigate the peak shapes of intact daratumumab ($pI = 8.3$), bevacizumab ($pI = 8.3$), panitumumab ($pI = 6.8$), pertuzumab ($pI = 9.0$) and rituximab ($pI = 9.4$). Among the studied buffer systems, only MES/DAP, citric acid/CHES and ammonium acetate gave acceptable peak shapes. With the other mobile phases, often split or distorted peaks were observed for various mAbs. In some cases, mAbs eluted at the very end of the gradient program despite their $pI$ was much lower than the pH of final mobile phase composition (suggesting not appropriate buffer capacity). (In the supplementary material, SM1 shows some chromatograms obtained with ammonium-acetate gradients.)

Then, depending on the pH responses of the selected buffers, different mixtures of acidic and basic eluents were prepared, so as to get a nearly linear pH response. Accordingly, for the MES/DAP system, 20 mM MES and 20 mM DAP in $80:20$ ratio was set as eluent A ($\text{pH} = 6.2$) and $20:80$ as eluent B ($\text{pH} = 10.2$). For the citric acid/CHES system, the two compounds were mixed in a $30:70$ ratio for eluent A ($\text{pH} = 4.6$), while eluent B was $100\%$ CHES ($\text{pH} = 10$, adjusted with NaOH). For the ammonium acetate system, eluent A was $50\%$ ammonium acetate and the pH was set to $\text{pH} = 5.5$ by adding acetic acid. At the same time, eluent B was $50\%$ ammonium acetate and the pH was set to $\text{pH} = 10.1$ using ammonium hydroxide.

Fig. 2 shows some representative chromatograms of bevacizumab obtained with MES/DAP, citric acid/CHES, an ammonium acetate pH gradient buffer system, and also reference conditions (standard salt gradient with NaCl and pH gradient (CX-1 buffer)). The same conclusions could be drawn for all studied mAbs, and therefore, only the data obtained with bevacizumab were reported. The MES/DAP, citric acid/CHES and ammonium acetate buffer systems were also evaluated in the salt-mediated pH gradient mode, by adding 150 mM NaCl into eluent B.

As shown in Fig. 2, the two common buffers (classical salt gradient with 10 Mm MES + NaCl and Thermo CX-1 pH gradient) resulted in similar retention and peak width, but the selectivity of acidic variants (pre-peaks) was slightly different. The MES/DAP pH gradient provided somewhat higher retention and broader peaks, but also better selectivity, as some basic variants could be separated (not the case with the classical buffer systems). The addition of NaCl into the MES/DAP system, strongly improved the peak shape (better focusing effect), and slightly decreased retention, which is expected. A very similar separation quality could be achieved with the salt-mediated MES/DAP system as with the classical salt and pH gradients. Therefore, this can be considered as a good alternative to the standard buffers. The citric acid/CHES system provided very high retention and insufficient selectivity. Increasing its molality (from 20 to 50 mM) resulted in more reasonable retention, but with

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**Fig. 1.** Measured pH responses of various buffer systems as a function of base content.
poor selectivity (no pre- and post-peaks were separated). However, the addition of NaCl into the citric acid/CHES buffer system (already at 20 mM buffer concentration), enabled one to separate the acidic and basic variants. It suggests that the salt-mediated citric acid/CHES system could be another alternative mobile phase for mAbs CEX separations. Finally, with ammonium acetate, neither in pH gradient mode nor in salt-mediated mode, was it possible to separate acidic and basic mAb variants from the main form.

Next, the selectivity and elution window of the most promising, three alternative buffer systems ((1) MES/DAP true pH gradient, (2) MES/DAP salt-mediated gradient and (3) citric acid/CHES salt-mediated gradient), were evaluated. These were, also compared to the standard salt gradient and to the standard commercial pH buffer, for five model therapeutic mAbs, having different physicochemical properties (Fig. 3). When considering the pI of the mAbs, the different mAb products eluted in the order of their increasing pI with the three systems, namely with the (i) CX-1 buffer, the (ii) MES/DAP pH gradient, and with the (iii) citric acid/CHES salt-mediated pH gradient. Among these three systems, the MES/DAP pH gradient provided the largest selectivity and widest elution window. This behavior is quite logical, as this buffer system provides the only “true” pH gradient among all the tested buffer systems. It is indeed important to notice that the commercial pH gradient buffer (Thermo CX-1) – which is promoted and known as “the” mobile phase to perform pH gradient CEX separations – does not perform a “true” pH gradient, despite its pH response being linear, since it also contains NaCl and NaOH, both acting as counter ions in the CEX mode.

In our previous study, we have seen that the elution order of mAbs did not always, strictly follow their pH [18]. However please note, that the definition of pI is not obvious in this manner. In the mentioned previous study, pI values were determined either from the amino acid sequence or on the basis of pIIEF measurements (applying denaturing conditions). Therefore, in CEX – where non-denaturing conditions are applied and the native form is analysed – the retention may not correlate with those pI values. Hence, using the term “apparent pI” is more correct. In addition, we have to also keep in mind that at a mobile phase pH which is equivalent with the pI, the protein may still carry charges (either positive or negative) even if the net charge is zero. Thus, both repulsive and attractive interactions may occur with the stationary phase at pI which can decrease or increase the retention, respectively. However, there is an interest in having a method, which enables the user to have an idea about at least the relative pI of mAbs. Such CEX method clearly needs to apply a pH gradient to continuously change the charge state of proteins. The MES/DAP buffer system seems to be a good candidate for this purpose. Though, it also worth mentioning that the distribution of charges on the surface of proteins is generally considered as a reason for the deviations between the elution pH and pI in ion-exchange chromatography [22]. An additional benefit of the MES/DAP system, is that it provided the highest selectivity for panitumumab variants, which is typically a challenging sample in CEX, since it is one of the most acidic commercial mAbs (pI 6.8).

On the other hand, Fig. 3 demonstrates that the five mobile phase systems studied, may result in different selectivities and elution orders. Therefore, they can be considered as good alternatives for a set of conditions to be tested, during the initial method screening. Depending on the mAb nature, both the new MES/DAP and citric acid/CHES systems can provide better selectivity than commonly used buffers. As an example, the basic variants of rituximab were better separated with these two buffers in the salt-mediated gradient mode.

To conclude, the two suggested buffer systems (MES/DAP and citric acid/CHES) are good alternative buffers for mAbs CEX separations. They both provide sharp peaks and a changing selectivity, when compared to the common salt gradient or to the CX-1 gradient.

3.3. Studying the retention behavior with a salt-mediated pH gradient

When comparing Fig. 3 C and D (corresponding to only pH gradient and to a salt-mediated pH gradient, using MES/DAP), it is obvious that the addition of salt into a pH gradient system, drastically changes the selectivity and elution order. Thus, there is a valid interest to perform a salt-mediated pH gradient, as it may modify the range of selectivity and broaden the experimental design space. And, much larger spaces can be mapped to tune the selectivity and resolution. The probability to find an appropriate separation, should significantly increase compared to a single salt-
or pH gradient separation. It was, therefore interesting to study the retention behaviors of mAbs in salt-mediated pH gradients, in order to determine the most important method variables for method development.

First, the impact of gradient steepness (inversely proportional to $t_G$) was studied. Retention times were measured for intact mAbs (i.e. daratumumab, obinutuzumab and rituximab), at various gradient times, as well as different temperatures and salt concentrations. Note that during the gradients (0–100% B), the mobile phase pH changed from 5.6 to 9.9 (see Section 2.3.3 for details). Whatever the temperature and salt concentration in mobile phase B, we always found a linear relationship between retention time and $t_G$ (when performing linear gradient profile). An example observed at $T = 25\,^\circ\text{C}$ and with 20 mM NaCl in mobile phase B is shown in supplementary material (SM 2A). The trend suggests the validity of a common, linear solvent strength (LSS) like behavior (sometimes called exponential model). A similar behavior was previously found, but only for salt- and pH gradient CEX separations [11,18,28].

Van't Hoff type plots were prepared to study the effects of temperature on retention (in the supplementary material, SM 2B corresponds to $t_G = 24\,\text{min}$ and 20 mM NaCl). Again, a linear relationship was found, however the applied temperature range was quite narrow (IEX separations are mostly performed within a limited temperature range, to avoid protein denaturation). At the same time, temperature had no significant impact on either the selectivity or the peak width.

When plotting retention times against the mobile phase salt concentrations, at a fixed $t_G$, a hyperbolic trend was observed (in the supplementary material, SM 2C, corresponding to $t_G = 16\,\text{min}$ and $T = 25\,^\circ\text{C}$). This hyperbolic trend is expected, and it often occurs in IEX (also in normal phase LC and HILIC). It is often referred as the adsorption model or log-log model [29,30]. The salt concentration in mobile phase B, had a huge impact on the retention, and it could even modify the elution order of mAbs. Probably, the greater the effect of salt concentration on retention, the higher the charge of a mAb is under the given conditions. In the end, we found that salt concentration and gradient steepness were the two most important parameters for tuning retention and selectivity.

Fig. 4 is an example of the impact of salt concentration on selectivity and elution order for intact, therapeutic mAbs ($t_G = 24\,\text{min}$, $T = 25\,^\circ\text{C}$, $c_{s1} = 20\,\text{mM}$, $c_{s2} = 60\,\text{mM}$ and $c_{s3} = 100\,\text{mM}$). At a low salt concentration, daratumumab eluted first (peak 1), followed by obinutuzumab (peak 2), while at higher salt concentrations, their elution order was reversed.

Finally, the possibility of retention modeling was studied. Some detailed design spaces were built for the three intact mAbs, based on 3 $t_G \times 3\,T \times 4\,c_s$ experiments (corresponding to 36 conditions). We again saw that temperature had no significant effect on selectivity/resolution, and therefore, it should be kept at a constant, low value (e.g. at $25\,^\circ\text{C}$), so as to maintain non-denaturing conditions. We found that at a given temperature, a limited number of experiments (e.g. 6 runs: 2 $t_G \times 3\,c_s$), was sufficient to model retention with appropriate accuracy (average error in retention time prediction lower or equal to 5%).

To conclude on salt-mediated pH gradients, the benefit of combining pH and salt gradients in one method is to extend the possibilities of changing selectivity and to gain in peak focusing effects. In this combined mode, the elution is based on both salt displacement and on changing the charge state of the analytes. The benefit of this combined elution mode is further discussed in the second part of the article, especially for more basic species when

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(FIG. 3. Cation-exchange chromatograms of intact mAbs with five different mobile phase systems (classical salt gradient (A), commercial pH gradient buffer from Thermo (B), MES/DAP pH gradient (C), MES/DAP salt-mediated pH gradient (D) and citric acid/CHES salt-mediated pH gradient (E)). Exact mobile phase compositions and corresponding pH ranges are detailed in Section 3.2.1. Injected mAbs: bevacizumab ($pI = 8.3$), daratumumab ($pI = 8.3$), pertuzumab ($pI = 9.0$), rituximab ($pI = 9.4$) and panitumumab ($pI = 6.8$). (Column, flow rate, gradient program, temperature, injected volume and detection, as described in the caption of FIG. 2).
the additional salt gradient indeed has huge impact on band broadening.

3.4. Method optimization and accuracy of retention modeling

A general method development approach, usually consists in modeling simultaneously the effects of the most important factors on selectivity, with a previously selected column. Then, a so-called resolution map can be generated by using common retention models (e.g. LSS). The model parameters can be derived from a few initial experiments, using a fitting procedure. Finally, with the help of a resolution map, the critical resolution of the peaks to be separated can be quickly, virtually optimized and experimentally checked.

When working with a conventional 100 × 4.6 mm CEX column, in a salt-mediated pH gradient mode, performing two linear gradients (i.e. tG = 8 and 24 min) at three salt concentration levels (i.e. cS1 = 20 mM, cS2 = 60 mM and cS3 = 100 mM) allows one to build up reliable retention models. With these generic conditions, most therapeutic mAbs and their subunits can be eluted, and appropriate retention is to be expected, even for the most acidic mAb species (e.g. panitumumab). Using this setup, the elution order of the mAbs is expected to follow their pl. In addition, thanks to the moderate (mediated) salt gradient, further peak focusing may occur, thus sharper peaks are expected, for both acidic and basic mAbs (which is not always the case for “true” pH gradients [24]). It also worth mentioning that, by introducing salt concentration as a method variable, the design space of method optimization can be significantly extended, and therefore the probability to find the optimum conditions should increase.

Our suggested procedure was applied to separate F(ab′)2 and sFc subunits - and the corresponding charge variants - of daratumumab and cetuximab. For these experiments, mobile phase A consisted of 90% 20 mM MES and 10% 20 mM DAP, while B consisted of 30% 20 mM MES and 70% 20 mM DAP, providing a pH gradient from 5.6 to 9.9.

3.4.1. Separation of daratumumab subunits and their variants

A two dimensional resolution map was built on the basis of the initial six runs (Fig. 5). On the resolution map, the warm (red) colors indicate the conditions where high resolution can be obtained, while the darker colors (blue), correspond to co-elution or poor resolution. It can be seen that there are two promising regions in the entire design space, the first one at 60 mM salt concentration and long gradient times (tG > 30 min) (WS1). A second one occurs at 30 mM salt concentration and shorter gradient times (10 min ≤ tG ≤ 15 min) (WS2).

Two gradient programs were tested in WS1. For gradient A (SM 3A), cS = 60 mM and tG = 35 min, were considered. However, as no peak eluted after 15 min, the rear part of the gradient program was eliminated to save time (while maintaining the same gradient steepness). Therefore, the following gradient program was applied: 0-57%B in 20 min. For the second gradient program, the purpose was to stretch the elution window (extend the retention space between the first and last eluting peaks). Hence, both the initial and final mobile phase compositions were changed to perform a shallow gradient. The applied gradient was set at 17-38%B in 10 min (SM 3 B). Finally, to check the reliability of the model, a salt concentration of cS = 30 mM was also tested (different from the concentrations used for the initial model runs). In this WS2 space, a fast gradient of 0-78%B in 10 min was performed (SM 3 C). The accuracy of retention time prediction was tested for the three proposed conditions. The two main peaks (sFc and F(ab′)2), three sFc pre-peaks and two other small peaks, between the main peaks, were considered. The average error of retention time prediction was systematically lower than 5%. This deviation can probably be explained because the pH response of the mobile phase system was not perfectly linear. Fig. 6 shows an experimental chromatogram of partially digested daratumumab, obtained under optimized conditions (corresponding to WS2). This example demonstrates the benefit of salt-mediated pH gradient, as compounds eluted in symmetrical and very sharp peaks. The practical peak capacity was Pc = 83 (considering the elution window of tG-tb), which is quite high and close to the efficiency of RPLC conditions. This application proves the applicability of the salt-mediated MES/DAP buffer system for typical, mAb separations.

3.4.2. Separation of cetuximab subunits and their variants

A two dimensional resolution map was again built based on six initial runs for cetuximab (Fig. 7). The map suggests that a low salt concentration is beneficial for this separation, as it provides the fastest analysis (shortest tG). It is also worth mentioning that narrow peaks were obtained at high salt concentrations, but selectivity
increasing the initial %B and decreasing final %B content, the elution window could be stretched. However, at an initial B composition higher than 15%, no further gain was observed. When decreasing the final %B below 70%, the selectivity between the last eluting peaks decreased. The best separation was achieved when performing a 15–70 %B gradient in 8 min. Fig. 8 shows the experimentally derived chromatogram, now offering a suitable separation for the 6 main species, as well as some additional minor variants. In this case, the average error of retention time prediction was around 5%.

4. Conclusions

The main purpose of this work was to find some alternatives to current, CEX buffer systems, which might offer new selectivity, when compared to standard salt gradients and commercial pH gradient buffers from Thermo, and provide appropriate peak shape and efficiency – possibly at lower costs. Various, two components buffer systems were tested. Among them, the MES/DAP and citric acid/CHES systems, allowed the separations of mAb charge variants (with similar or slightly better efficiency than the standard mobile phases), especially when combining them with a moderate salt gradient. In the CEX mode, the MES/DAP system was suggested to be applied in the range of 5 ≤ pH ≤ 10, and it provides a nearly linear pH response. The citric acid/CHES system may be useful in a lower pH range (3 ≤ pH ≤ 9), but the non-linearity of the pH response needs to be critically considered.

Fig. 5. Two dimensional, resolution map (tG x cG) obtained for daratumumab subunit separation. Column: YMC BioPro SP-F (100 × 4.6 mm, 5 μm), flow rate: 0.8 ml/min, mobile phase system: MES/DAP pH gradient + NaCl salt gradient, temperature: 25 °C, injected volume: 1 μl, detection: FL (280–350 nm). Promising working spaces (WS) were indicated as WS1 and WS2 (corresponds to optimal conditions).

Fig. 6. Experimental chromatogram of digested daratumumab, obtained under optimized conditions (corresponding to WS2, conditions as specified in Fig. 7 C). Mobile phase A: 90% 20 mM MES + 10% 20 mM DAP, mobile phase B: 30% 20 mM MES + 70% 20 mM DAP + 30 mM NaCl. Simultaneously dropped significantly compared to low salt concentration conditions. Therefore, various initial and final mobile phase compositions were tested at cG = 20 mM and at tG = 8 min, in silico, and the most promising conditions were experimentally verified. Only the six most intense (main) peaks, observed for digested cetuximab, were considered during the optimization process. By
The prediction of concentration gradients, in the gradient measured basis, were indeed the gradient steepness and salt concentrations in the B eluent. Then, combining the LSS and adsorption models, enables one to predict retention in a large design space, and this was found to be beneficial for tuning selectivity. The parameters of the model can be obtained by using simple fitting to a few experimental data. For this purpose, retention times need to be measured at two, different gradient steepness intensities (or tG) levels, while salt concentration (cS) needs to be studied at three levels. At the end, tG and cS can be simultaneously optimized on the basis of only six initial runs (2 tG x 3 cS). By using such a retention model, the predicted and experimentally verified retention times were in good agreement. However, if the pH response of the buffer system is not linearly proportional to pH (e.g. citric acid/CHES system), then the tG should be measured at three, different levels, which should improve the model and increase the prediction accuracy (non-linear model for tG). In such a case, the method development requires nine initial experiments (3 tG x 3 cS). We suggest column re-equilibration time which corresponds to about five column volumes in order to improve the repeatability of the measurements.

To perform a generic, salt-mediated pH gradient, a good starting point is to set cS at 20, 60 and 100 mM in mobile phase B. This salt concentration (typically NaCl), should allow elution of most of the therapeutic mAbs in CEX mode, when simultaneously performing a pH gradient.

Our proposed buffer system (salt-mediated pH gradient) and method development approach, have been successfully applied to the subunit analysis of daratumumab and cetuximab. The time spent for method development was only about 6 h (i.e. 8 and 24 min. gradients, at three salt concentrations, equilibration, washing, calculation and experimental verification of the optimum).

It worth mentioning that recovery of acidic and basic species in CEX may depend on the applied conditions (e.g. mobile phase additive, pH or stationary phase). A following study is planned to be performed to better understand protein recovery in CEX.

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Appendix A. Supplementary data

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References


