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# Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range

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ABSTRACT

Recombinant antibodies with high isoelectric point are frequent since most of them are constructed from the same framework. Classically, cation exchange chromatography is used as a standard method for the determination of antibody charge heterogeneity. In contrast, in this study highly linear pH gradients were achieved by keeping the buffering capacity over the length of the gradient constant. The buffering compounds were selected to be unretained on the column and their respective concentration was adjusted in the start and end buffer of the pH gradient to achieve constant buffering capacity. This helps conserve linearity and stability of the gradient. The method allows quantification of charge variant distribution and the determination of chromatographic isoelectric point. To demonstrate the effectiveness of this novel method, a ProPac WCX-10 column was used to separate isoforms of trastuzumab biosimilar antibodies. Effects of pH gradient linearity and of varying the analytical amount of sample on the separation are shown.



## 1. Introduction

Cation exchange (CEX) chromatography is a standard method for the determination of charge heterogeneity of recombinant antibodies with high isoelectric point [1]. NaCl gradient elution is the preferred method at the moment, but requires optimization for each macromolecule analyzed [2]. Linear pH gradient elution has been shown to be a reliable alternative [2–4]. However, to create a linear pH gradient the following conditions must be met: the buffering capacity of the buffers used has to be constant over the pH range used and the buffer substances should not interact with the stationary phase. Kröner and Hubbuch [5] have applied these principles to obtain highly linear pH gradients over a wide pH range for proteomic applications. Such principles can be applied to create highly linear gradients for the elution of immunoglobulin G (IgG) on cation exchange columns.

Monoclonal antibodies (mAbs) are a constantly growing class of biopharmaceuticals, owing to their ability to bind antigens with high specificity and to elicit receptor functions [6,7]. MAbs, which are most often IgG, are already approved for the treatment of a variety of diseases, such as various cancers [8] and multiple sclerosis [9], showcasing their ability to be adapted for a plethora of uses.

A series of recombinant IgG molecules are based on the same framework, which has an isoelectric point (pI) around 9. Thus a lot of medically and commercially relevant antibodies present an isoelectric point in the alkaline range. Moreover, besides purity [10] charge heterogeneity is often considered as one critical quality attribute, due to several reasons, such as serum half-life, effector functions, solubility and stability, which may all be connected to the surface charge of the molecule [11–13].

Many of the modifications affecting IgG could influence the pI of the protein, e.g. sialylation of the glycan, oxidation of methionine residues, N-terminal lysine processing, and isomerization of aspartic acid [14–17]. Some of these modifications also influence the effector functions and serum half-life of IgG and as such have to be closely monitored to ensure safety and efficacy of the final product [18].

CEX, as mentioned before, is a robust method for the characterization of the charge heterogeneity of IgG, exploiting small binding differences of the charge variants to the stationary phase under salt gradient elution. However, when using a salt gradient for elution, the pH of the equilibration buffer strongly influences binding, generally necessitating optimization of the pH and the NaCl concentration of the equilibration and elution buffers for each mAb product [2]. A different mode of ion exchange gradient chromatography utilizes pH gradients to affect the surface charge of the

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**Table 1**
$pK_a$ values of the selected buffer substances at room temperature.

| Substance | $pK_a$ |
|---|---|
| MOPSO | 6.90 |
| HEPES | 7.48 |
| Bicine | 8.26 |
| CAPSO | 9.60 |
| CAPS | 10.40 |

protein to modify the binding to the stationary phase. Using this strategy, many different recombinant antibodies have been analyzed using the same unmodified method and buffers, as shown by Farnan et al. [2]. Previous pH gradient methods, such as those used by Zhang et al. [4], Farnan and Moreno [2], Rea et al. [19] and Tsonev and Hirsh [20], used buffer systems that were either interacting with the stationary phase or with large variations in buffering capacity resulting in non-linear pH gradients. As pointed out earlier, this deviation from pH gradient linearity results in lowered resolution. In contrast, the current work presents highly linear pH gradients for the analysis of mAb.

## 2. Material and methods

### 2.1. Buffer systems for pH gradients

The buffer compounds used for the generation of the pH gradients were3-morpholino-2-hydroxypropanesulfonic acid (MOPSO), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), *N*,*N*-bis(2-hydroxyethyl)glycine (bicine), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), all Sigma Aldrich (St. Louis, MO, USA). Sodium chloride (Merck, Darmstadt, Germany) was used to adjust the conductivity of the buffers to the same level. Sodium hydroxide was obtained from Merck (Darmstadt, Germany).

Buffer systems were designed for two different pH ranges, covering either pH 8.0 to pH 10.5 or pH 7.0 to pH 10.5. Acidic or zwitterionic buffer substances were selected with $pK_a$ values evenly distributed across the desired pH range. For the pH 8.0 to 10.5 gradient, the following buffers were selected: HEPES, bicine, CAPSO, CAPS and for the pH 7.0 to 10.5 gradient, HEPES was replaced by MOPSO. The $pK_a$ values of the buffers used can be found in Table 1.

The buffer composition was optimized by keeping the buffer capacity constant over the whole buffered range. The constancy of the buffer capacity was quantified by calculating the difference between the minimum and the maximum buffer capacity over the covered pH range. By varying the concentration of buffers in both buffer A and B, instead of using one stock buffer, the buffering capacity was further improved. The average buffer capacity was set at 5 mmol/l to keep the ionic strength low. The conductivity of buffer A was adjusted to the conductivity of buffer B with a 1 M solution of NaCl.

Five buffer systems, listed in Table 2, were tested on an ÄKTA Explorer 100 system with a Dionex ProPac WCX-10 column (Thermo Fisher, Sunnyvale, CA, USA). This column consists of a pellicular cation-exchange layer grafted to a non-porous support particle, resulting in high mass transfer rates [11] and is considered as the gold standard for mAb charge heterogeneity analysis [18]. The length of the gradient was 10 column volumes. Each buffer system was used to run the experiment five times. The linearity was quantified by calculating the coefficient of correlation. The details of the buffer systems used can be found in Table 2.

### 2.2. Gradient elution of IgG

An Agilent 1100 HPLC system was used with a Dionex ProPac WCX-10, 4 × 250, column to elute IgG under linear pH and salt gradients. The sample IgG used was a Trastuzumab biosimilar antibody produced in CHO cells and purified using protein A chromatography [21]. The samples were buffer exchanged into the running buffer A and diluted to a concentration of 1 mg/ml for all experiments. A volume of 25 μl was injected for all experiments, unless stated otherwise.

The chromatograms of the pH gradient were blank corrected, by subtracting a blank run, due to the difference in absorbance of the buffers at high and low pH. The IgG was eluted in 10 CV linear gradients and the absorbance was measured at 280 nm. The chromatograms were then integrated to calculate the relative distributions of the main peak, the acidic variants (eluting before the main peak) and the basic variants (eluting after the main peak). The peaks were also fitted using the exponentially modified Gaussian function [22] to calculate first moments and second central moments.

### 2.3. Isoelectric focusing

Immobilized pH gradient (IPG)-polyacrylamide-gels (size: 125 × 260 × 1 mm) were cast on a GELbond-PAG-film backing (GE Healthcare), polymerized, washed and dried following a procedure previously described by Westermaier et al. [23]. The desired pH gradient from 8.0 to 10.0 was obtained by graphic interpolation from a recipe previously published by Görg et al. [24].

Directly before use, the gel was cut into 3 mm strips, which were rehydrated for 2 h in a solution containing 6 M Urea, 2% Tergitol, 2% Pharmalyte 3–10, 10% Glycerol and 16% Isopropanol.

The rehydrated strips were put onto the IPGphor (GE Healthcare) using the manifold. The electrodes were positioned on the acidic and basic ends of the strips with wetted paper wicks between gel and electrode to ensure good contact. The samples were applied by cup loading under a covering layer of paraffin and electrophoresis was performed overnight (at 150 V for 1 h, followed by 300 V for 1 h, a gradient step to 3000 V for 30 min, 3000 V for 18 h, and 3500 V for 5 min). After electrophoresis, the strips were stained with Coomassie G250.

**Table 2**
Composition of the five buffer systems used.

| pH 8.0 to 10.5 buffers | | HEPES | Bicine | CAPSO | CAPS |
|---|---|---|---|---|---|
| System 1 | Buffer A [mM] | 5.5 | 4.2 | 9.5 | 0.8 |
| | Buffer B [mM] | 0.0 | 10.5 | 2.5 | 7.0 |
| System 2 | Buffer A & B [mM] | 5.0 | 5.0 | 5.0 | 5.0 |
| System 3 | Buffer A [mM] | 20.0 | 0.0 | 0.0 | 0.0 |
| | Buffer B [mM] | 0.0 | 0.0 | 0.0 | 20.0 |
| pH 7.0 to 10.5 buffers | | MOPSO | Bicine | CAPSO | CAPS |
| System 4 | Buffer A [mM] | 7.1 | 5.3 | 14.9 | 0.7 |
| | Buffer B [mM] | 14.6 | 4.9 | 1.4 | 7.1 |
| System 5 | Buffer A & B [mM] | 7.0 | 7.0 | 7.0 | 7.0 |

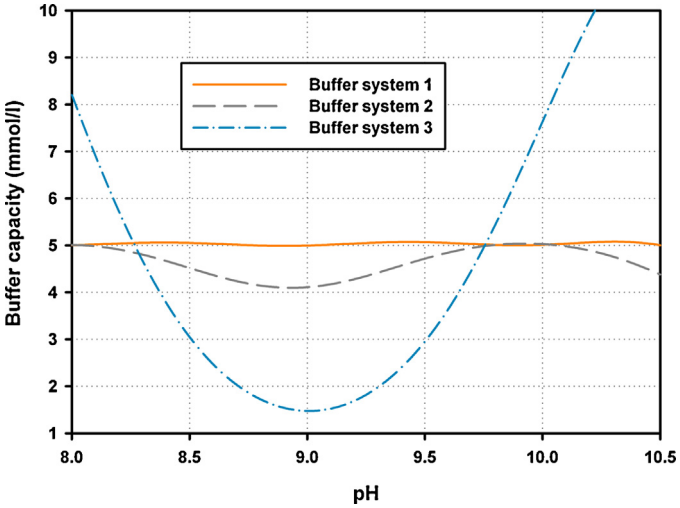


**Fig. 1.** Buffer capacity of buffer system 1 to 3. By varying the concentrations in buffer A and B of buffer system 1, the buffer capacity can be kept constant in the working range from pH 8 to 10.5.

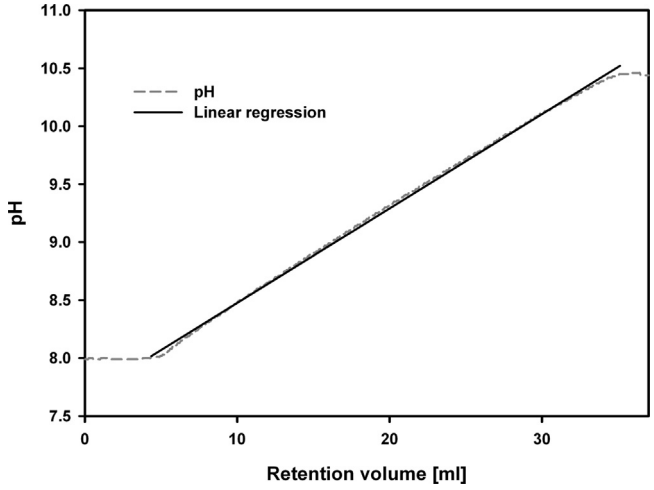


**Fig. 2.** Comparison of experimental pH gradient and the linear regression. The experimentally determined pH gradient, of buffer system 1, is shown as a gray dashed line and the linear regression used to calculate the coefficient of correlation is shown as a solid trace.

## 3. Results

### 3.1. Linearity of pH gradient

Eq. (1) was used in order to generate a linear gradient with a constant buffer capacity over the entire pH.

$$\beta = \frac{dB^+}{d\mathrm{pH}} = \ln(10) \times \frac{[H^+] + C_A K_A [H^+]}{K_A + [H^+]^2 + [OH^-]} \quad (1)$$

where $\beta$ is the buffer capacity, $dB^+$ is the infinitesimal amount of added base, $d$pH is the resulting change in the pH, $C_A$ is the concentration of buffering weak acid and $K_A$ is the dissociation constant of the weak acid. We assumed thermodynamically ideal conditions and concentrations were used instead of activities, because of the dilute solutions. The buffer capacity of the optimized buffer system (buffer system 1), and the non-optimized buffer systems 2 and 3 are shown in Fig. 1. For the optimized system, the capacity fluctuated less than 0.1 mM for a 5 mM buffer over the entire pH range, while the buffer capacity of buffer system 2 and 3 were less stable. Buffer systems 1 to 5 were then used to create a linear pH gradient on a weak cation exchange stationary phase. Systems 1 to 3 cover pH 8.0 to 10.5 and systems 4 and 5 cover pH 7.0 to 10.5. Systems 1 and 4 were optimized by changing the amount of buffering component in buffer A and B. Systems 2 and 5 were optimized by using buffer components with $p$K$_a$ values covering the pH range. System 3 uses one buffer component for the start pH and another for the end pH. A clear increase in linearity is observed for each optimization of the buffering capacity.

The resulting pH gradient from buffer system 1 is given as an example in Fig. 2. The largest deviations from linearity can be seen at the beginning and the end of the gradient. The coefficient of correlation is equal to 0.9809 if a linear regression is performed on only the first 10% of the gradient, and 0.9901 for the last 10%. The part of the gradient in between those chosen beginning and end points, i.e. the remaining 80%, show a much higher coefficient of correlation of 0.9995. These initial deviations from linearity can be attributable to non-ideal pumps and gradient mixers [25]. Table 3

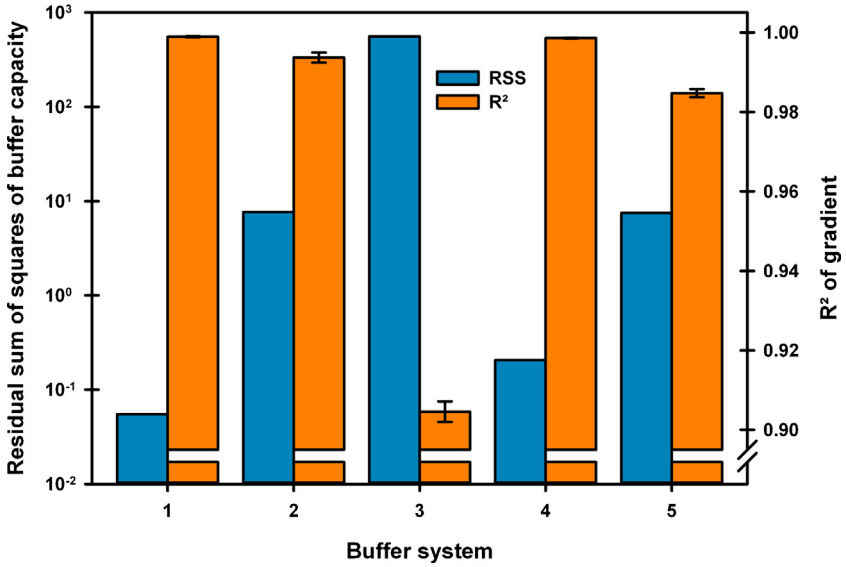


**Fig. 3.** Maximum relative deviation in buffering capacity. The dark grey bars represent the maximum relative deviation in buffering capacity of the buffer system and the light grey bars represent the linearity of the pH gradient. The dependence of the gradient linearity on the buffer capacity stability can be seen.

**Table 3**
Summary of the maximum relative deviation in buffering capacity Δ β, the residual sum of squares, the coefficient of correlation (R) and the standard deviation (s.d.) of $R^2$ for the five buffer systems.

| | $\Delta\beta$ (%) | RSS | $R^2$ | s.d. |
|---|---|---|---|---|
| pH 8.0 to 10.5 buffers | | | | |
| System 1 | 98.2 | 0.055 | 0.99894 | 0.00014 |
| System 2 | 81.3 | 7.7 | 0.99368 | 0.00130 |
| System 3 | 12.6 | 560 | 0.90455 | 0.00257 |
| pH 7.0 to 10.5 buffers | | | | |
| System 4 | 96.5 | 0.21 | 0.99860 | 0.00009 |
| System 5 | 68.3 | 7.5 | 0.98473 | 0.00104 |

and Fig. 3 show a clear relationship between buffering capacity and linearity of the pH gradient.

From these results shown in Table 3 and Fig. 3, it is clear that the stability of the buffering capacity determines the linearity of the pH gradient. For buffer systems 2 and 5, the same buffer compounds were used as in buffer system 1 and 4 but their concentrations were not adjusted. Because the p$K_a$ values of those buffer compounds cover the whole pH range of the gradient and are at least 0.7 pH units apart, the resulting buffer capacity is relatively stable. This is an easy way to create mostly linear gradients, especially for narrow gradients up to three pH units.

To obtain highly linear pH gradients, the deviation from linearity of the buffering capacity can be kept minimal by varying the buffer concentrations in both buffers A and B as illustrated for example in buffer systems 1 and 4.

### 3.2. Linearity of pH gradient and chromatographic performance

A Trastuzumab biosimilar antibody was used to demonstrate the effect of linearity of pH gradient on chromatographic specificities such as resolution and peak capacity. The buffer systems described in Section 3.1 were used for pH gradient elution of 25 μg of recombinant antibody. A NaCl gradient was also performed in parallel for comparison. Each experiment was performed in replicates of 5. The resulting chromatograms were fitted by exponentially modified Gaussian function [22] and the peak capacity and the resolution between adjacent peaks were calculated. Due to IgG consisting of a large number of charge variants, the chromatogram contains many peaks. In order to perform the analysis of the data, only the 4 representative peaks, which could be identified easily in all 6 experiments, were evaluated. The chosen peaks are labeled from 1 to 4 in Fig. 4, which shows one of the chromatograms obtained with buffer system 1. Isoelectric focusing performed with the same protein gave similar results in regards to isoelectric point and number of major peaks.

The resolution and the peak capacity both increased with the linearity of the pH gradient. Fig. 5 and Table 4 show the peak capacity and the resolution obtained by elution with all5 buffer systems and with a salt gradient used as a reference in parallel. A large difference between the salt gradient and the highly linear pH gradients can be seen. The non-linear pH gradient obtained with buffer system 3, shows peak capacity and resolution values comparable to the ones from the salt gradient.

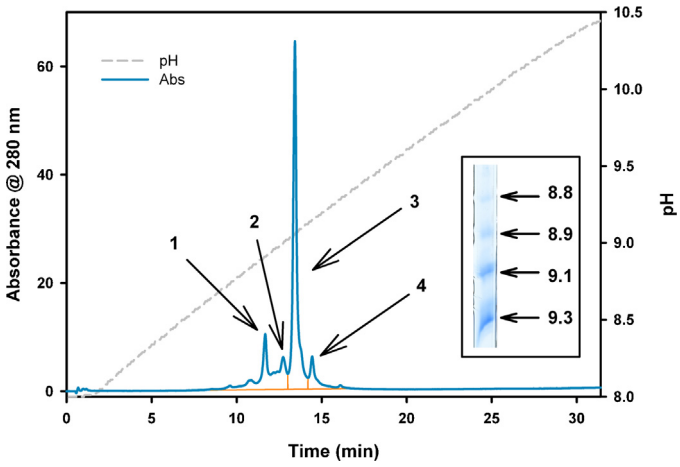


**Fig. 4.** Representative chromatogram from the experiments outlined in Section 3.2. An amount of 25 μg Trastuzumab biosimilar antibody were injected to a WCX-10 column and eluted using a linear gradient over 10 column volumes with buffer system 1. The black solid trace shows the chromatogram obtained by measuring the absorbance at 280 nm. The gray dashed trace gives the pH gradient, which had to be measured independently, due to the lack of online pH monitoring on the HPLC system. It can be seen that the IgG is a mixture of many isoforms, all eluting between pH 8.6 and 9.3. To analyze and compare all seven experiments, only four representative peaks, marked with arrows, were chosen for analysis. The calculated chromatographic pI values for the labeled peaks are 8.85, 8.94, 8.99 and 9.07, respectively. These values correspond to the pI values determined by IPG IEF, shown in the inset. The boundaries for integration are shown under the peaks.

**Table 5**
Determination of the isoform composition with different buffer systems. In these experiments, a salt gradient has been used for comparison in parallel to the five buffer sytems studied.

| Buffer | Acidic isoforms (%) | CV (%) | Main isoform (%) | CV (%) | Basic isoform (%) | CV (%) |
|---|---|---|---|---|---|---|
| 1 | 30 | 4 | 61 | 3 | 9 | 5 |
| 2 | 33 | 1 | 59 | 1 | 8 | 4 |
| 3 | 35 | 2 | 55 | 1 | 9 | 7 |
| 4 | 31 | 2 | 59 | 2 | 10 | 7 |
| 5 | 36 | 1 | 55 | 1 | 10 | 7 |
| Salt | 33 | 3 | 55 | 2 | 12 | 3 |

The chromatograms were also integrated fully, by perpendicular drop method [26], and the peaks assigned to acidic, main or basic isoforms as previously reported for mAb [18]. Fig. 5(C) and Table 5 show the distribution determined by all six buffer systems used, which are all in agreement about the composition (>50% main peak, ~35% acidic variants and ~10% basic variants). A trend towards higher main peak content can be seen for buffer systems 1, 2 and 4, which is due to the better resolution and the better separation from the acidic peaks.

**Table 4**
Summary of the results of the pH gradient elution experiments. The peak capacity and the resolution between all 4 representative peaks are given with the coefficient of variation (CV) from 5 experiments. In these experiments, a salt gradient has been used for comparison in parallel to the 5 buffer sytems used in this study.

| Buffer | Peak capacity | CV (%) | Resolution | Peak 1 & 2 | CV (%) | Peak 2 & 3 | CV (%) | Peak 3 & 4 | CV (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 4.9 | 0.5 | | 1.86 | 0.5 | 1.21 | 0.3 | 1.79 | 0.6 |
| 2 | 4.3 | 5.1 | | 1.63 | 6.6 | 1.12 | 3.9 | 1.54 | 5.3 |
| 3 | 2.9 | 6.3 | | 1.12 | 12.3 | 0.85 | 2.9 | 0.90 | 2.3 |
| 4 | 4.5 | 0.4 | | 1.86 | 0.8 | 1.09 | 0.2 | 1.57 | 0.6 |
| 5 | 3.9 | 0.6 | | 1.55 | 1.2 | 0.94 | 0.8 | 1.37 | 0.2 |
| Salt | 2.9 | 0.6 | | 1.06 | 1.0 | 0.85 | 0.4 | 1.03 | 0.6 |

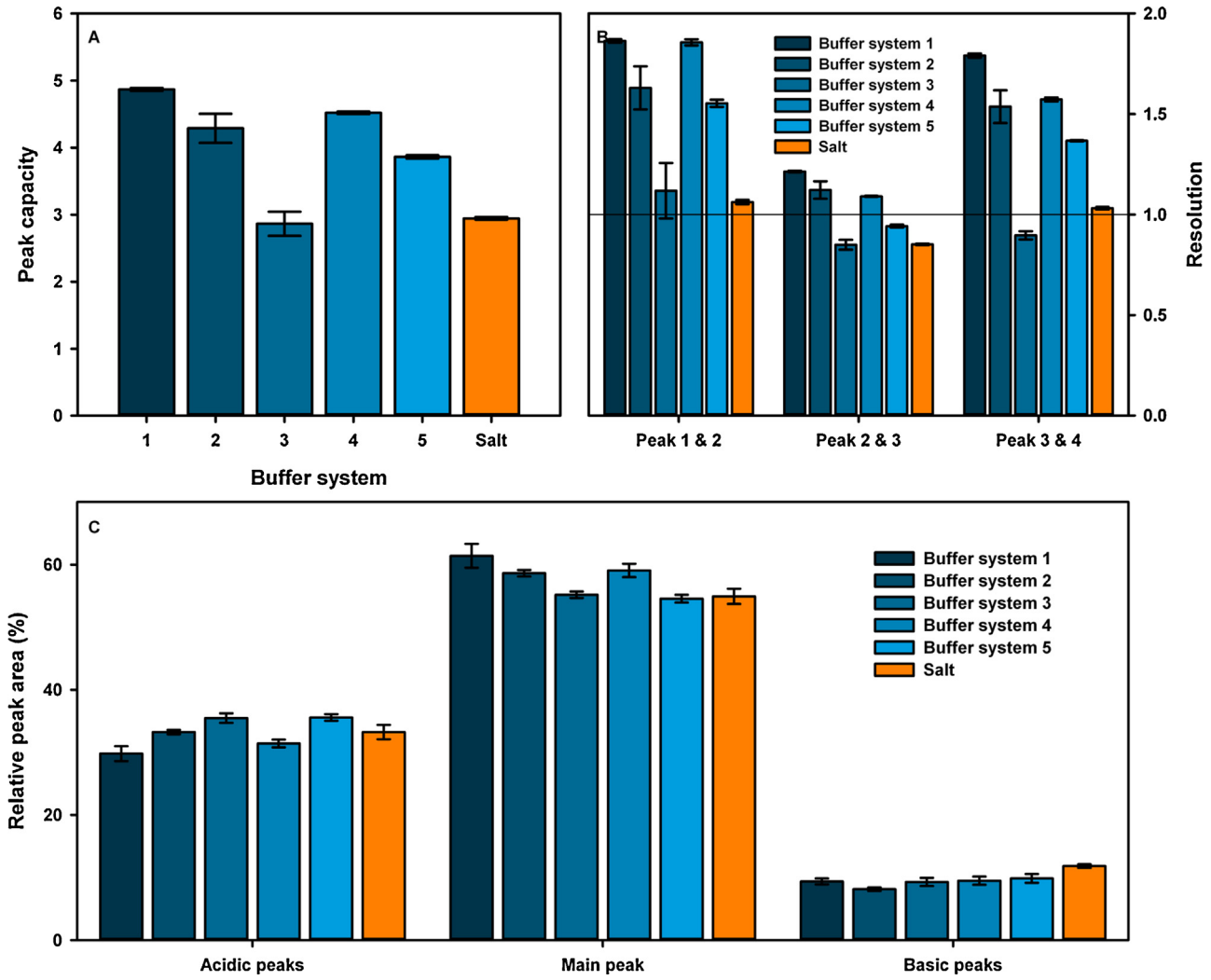


**Fig. 5.** The dependence of the peak capacity (A) and the chromatographic resolution (B) on the linearity of the pH gradient. Buffer systems with a gradient linearity of $R^2 > 0.98$ (buffer 1, 2, 4, 5) show a peak capacity between 4 and 5, while the salt gradient and the buffer system 3 show a peak capacity lower than 3. The resolution between the 4 peaks behaves similarly. Panel (C) shows the relative distribution of charge variants determined by integration of the chromatogram.

**Table 6**
Influence of the injection amount on the peak capacity and the resolution of the pH gradient elution.

| Injection amount [μg] | Peak capacity | CV (%) | Resolution: | Peak 1 & 2 | CV (%) | Peak 2 & 3 | CV (%) | Peak 3 & 4 | CV (%) |
|---|---|---|---|---|---|---|---|---|---|
| 10 | 3.9 | 3.0 | | 1.5 | 1.4 | 1.0 | 0.8 | 1.5 | 0.9 |
| 25 | 4.9 | 2.3 | | 1.9 | 0.9 | 1.2 | 0.4 | 1.8 | 1.0 |
| 50 | 4.8 | 0.6 | | 1.8 | 0.5 | 1.2 | 0.2 | 1.8 | 0.1 |

### 3.3. Influence of sample amount

To test the robustness of an optimized buffer system, the effect of sample amount on the chromatographic key values was determined by injecting 10, 25 or 50 μg of IgG and eluting it with the buffer system 1. The column supplier recommends an injection amount of 25 μg. Very similar peak capacity, resolution and isoform distribution for the 25 and 50 μg injections were observed (Fig. 6, Tables 6 and 7). The 10 μg injection shows lowered values for peak capacity and resolution as well as higher standard deviations in isoform distribution. Nevertheless, the isoform distribution varies little with the injection amount.

## 4. Discussion

Kröner et al. have shown that by keeping the buffering capacity constant over the length of the gradient, the resulting pH gradient will be linear [5]. The value that was being used for optimization

**Table 7**
Influence of the injection amount on the determination of the relative isoform distribution.

| Injection amount [μg] | Acidic isoforms (%) | CV (%) | Main isoform (%) | CV (%) | Basic isoform (%) | CV (%) |
|---|---|---|---|---|---|---|
| 10 | 30 | 7 | 59 | 5 | 11 | 13 |
| 25 | 30 | 4 | 61 | 3 | 9 | 5 |
| 50 | 32 | 0 | 60 | 0 | 8 | 6 |

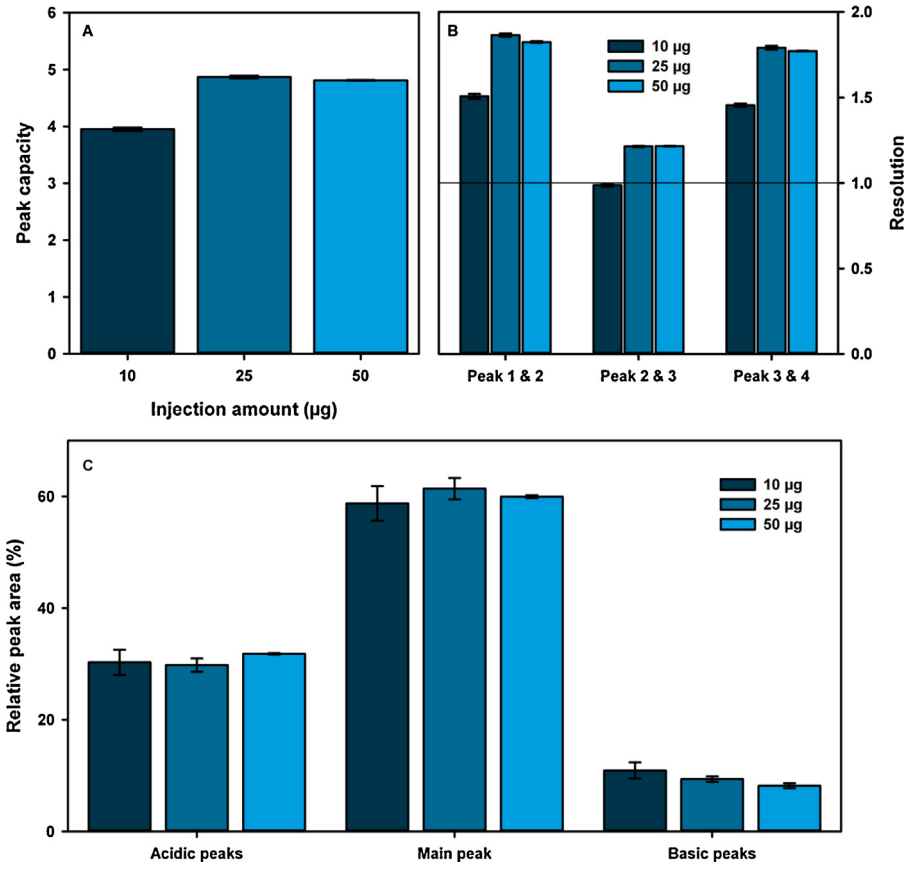


**Fig. 6.** Influence of the injection amount on the peak capacity (A) and the resolution (B) of the separation. A decline in separation performance can be seen for injections below the recommended 25 µg. Panel (C) shows the influence of the injection amount on the isoform distribution determination. Minor differences can be seen in the acidic peaks between all three injection values. The main and basic peak values for the 10 µg injection do not agree with the 25 and 50 µg injections.

was the ratio of minimum to maximum buffer capacity over the pH range. This variable turned out to be a more suitable metric for optimization in the relatively narrow pH gradients covered here, compared to the metrics used by Kröner et al. [5] or the residual sum of squares used by Giaffreda et al. [27] for the optimization of the immobilized IEF pH gradient recipes.

Kaltenbrunner et al. [3] have used pH gradient elution for the characterization of mAb in 1993, but their method relied on the chemical reaction of mannitol with borate. This limits the customization possibilities of such a gradient. Zhang et al. [4], Farnan and Moreno [2], Rea et al. [19] and Tsonev and Hirsh [20] are all using cationic buffering species on a cation exchange stationary phase. This means that the buffering species themselves are interacting with the functional groups, resulting in partial retention and therefore deviation of the pH gradient from the optimum. In the case of the works of Tsonev and Hirsh [20], they are using algorithms to correct for the deviations. With the method presented in this work, such corrections are not necessary. Kang et al. [28] and Ng et al. [29] used induced pH gradients for heterogeneity analysis of antibodies, but the method is limited to proteins with a neutral or acidic pI. Nordborg et al. [30] used buffering compounds with p$K_a$ values across the pH range to create linear pH gradients, but did not optimize the buffering capacity of their buffers. Rozhkova [31] used phosphate as the only buffering compound in very low concentrations, leading to unstable buffering capacity in the pH range used and losing the ability to adjust the pH range of the pH gradient.

Kröner and Hubbuch [5] chose buffering components that carry the same charge as the immobilized ligands and therefore do not interact with the stationary phase. Their pH gradients are very wide range for proteomics applications and are based on one stock buffer that gets titrated to the pH values spanning the pH gradient. By using a narrower gradient in the present work, we were able to vary the buffer composition in both buffers, which further resulted in a more even distribution of the buffering capacity.

As shown in Fig. 5, the measured isoform distribution changes depending on the method used. The trend towards larger relative amounts of main peak in methods with higher peak capacity stems simply from the better resolution and the more accurate integration of the chromatograms. These differences have to be considered for technology transfer or the characterization of biosimilars, but should not be seen as a disadvantage of the method presented here.

The determination of isoforms is independent of the injected amount of sample, as shown in Fig. 6. The quantification of minor isoforms, next to more abundant isoforms is possible, which speaks for the robustness of the whole method.

Isoelectric focusing (IEF) and capillary electrophoresis (CE) methods are popular methods for the evaluation of charge heterogeneity of mAbs [1,32]. Capillary IEF (cIEF) is a high resolution method for the determination of the pI and the intrinsic protein net charge. Thus, this method can be considered orthogonal to linear pH gradient elution cation exchange chromatography which determines the protein surface charge only. CIEF does have a very high resolution and requires low sample amounts. However, problems are encountered with cIEF like the use of ampholytes, which can lead to reduced robustness of the method due to batch to batch variations; the ampholyte mix and the electrode stabilizers have to be optimized for the pH range [33]. CIEF in immobilized gradients have also been reported [34] and would alleviate the problems associated with the use of ampholytes. Nevertheless, IEF and CE

methods require their own instruments and know-how, while linear pH gradient CEX can be easily adopted as a method in a lab where other chromatographic methods are already in place.

## 5. Conclusions

In this study, we proposed a novel method for the characterization of mAb charge variants. The new method requires optimized buffer systems for the creation of a highly linear pH gradient. The buffer systems described here are able to quantify the general distribution of acidic, basic and the main charge variants in IgG samples. For accurate quantification of each charge variant, further complementary analysis of the peaks with other method, e.g. mass spectrometry, is required to confirm that each peak corresponds to only one specific charge variant.

Furthermore, it was shown that the linearity of the pH gradient is correlated with the constancy of the buffer capacity over the pH range. The linearity of the pH gradient is correlated with the resolution and peak capacity of the pH gradient elution. The peak capacity improves from 2.9 to 4.9 when eluting with a highly linear pH gradient instead of a linear salt gradient.

The advantage of pH gradient methods over conventional salt gradient elution methods is their broad applicability for a variety of IgG, without the need for optimization. This is the main difference over salt gradient elution, in which the initial and final salt concentrations have to be adjusted for each IgG. The buffers presented in this work can cover pH ranges from 7 to 10.5 and so should be useful to elute most IgG.

The separation problem at hand, i.e. the separation of IgG charge variants, is a difficult one. IgG consists of many charge variants, sometimes with only minor differences in surface charge distribution. Even with high performance stationary phases and improved elution methods, base line separation of all isoforms is not possible at the moment. The method presented in this work offers another step towards improved characterization of monoclonal IgG.

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