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Systematic generation of buffer systems for pH gradient ion exchange chromatography and their application

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ABSTRACT

pH gradient protein separations are widely used techniques in the field of protein analytics, of which isoelectric focusing is the most well known application. The chromatographic variant, based on the formation of pH gradients in ion exchange columns is only rarely applied due to the difficulties to form controllable, linear pH gradients over a broad pH range. This work describes a method for the systematic generation of buffer compositions with linear titration curves, resulting in well controllable pH gradients. To generate buffer compositions with linear titration curves an in silico method was successfully developed. With this tool, buffer compositions for pH gradient ion exchange chromatography with pH ranges spanning up to 7.5 pH units were established and successfully validated. Subsequently, the buffer systems were used to characterize the elution behavior of 22 different model proteins in cation and anion exchange pH gradient chromatography. The results of both chromatographic modes as well as isoelectric focusing were compared to describe differences in between the methods.

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1. Introduction

pH gradient based separations of proteins for analytical purposes are among the most used techniques in protein analytics, e.g. isoelectric focusing or 2D-gelelectrophoresis. The chromatographic variant, pH gradient ion exchange chromatography (IEC), has been valued as an analytical separation technique in the field of proteomics [1] and as a screening tool for the selection of pH-related parameters in industrial bioseparation process development [2]. In addition, pH gradient ion exchange chromatography showed its potential as an excellent analytical tool in the routine analysis of monoclonal antibody charge variants [3] as well as PEGylation variants [4]. Another study on the differences between pH gradient and salt gradient IEC showed that pH gradient IEC might provide a higher chromatographic resolution [5].

Analogous to conventional IEC, protein binding is charge mediated in pH gradient IEC. In pH gradient IEC proteins are eluted by changing the pH of the mobile phase gradually and thus titrating the interaction of the protein with the resin. The fact that an interaction with a charged surface is involved suggests a fundamental difference between classical isoelectric focusing and pH gradient IEC, which could cause a deviation between the pI and the elution-pH of proteins.

There are two main ways of generating pH gradients in IEC: an internal and an external method. For the internal method, a weak ion exchange resin with an intrinsic buffer capacity is used. The column is first equilibrated with the application buffer. The elution is done by titrating the intrinsic buffer capacity with the running buffer to generate the outlet pH gradient. The internal method is mostly referred to as chromatofocusing [6]. For the external method, the pH gradient is formed before entering the column by gradually mixing the running buffer with its titrant. The focus of this work is on the externally generated pH gradients, which are regularly run on strong ion exchange resins.

The major challenge in running pH gradients in IEC is the controllability of the pH gradient. Additionally, it is preferable to keep the ionic strength of the buffer system low, in order to reduce its influence on the proteins retention behavior. One approach is to use ampholytes, providing a high buffer capacity covering a broad pH range. However the usage of ampholytes has major disadvantages, being their lot-to-lot variability, their interaction with the proteins and chromatographic adsorbers, as well as the fact that they are difficult to remove from solution. Another approach is to use multiple, equally concentrated buffer substances with equally spaced pKa values in the chosen pH range [7]. The latter leads to reproducible, linear pH gradients covering a broad pH range on the corresponding chromatographic resin, but still with relatively high ionic strength and an inconstant buffer capacity. To lower the ionic strength of the buffer composition, a further approach uses a very similar buffer chemistry with lower concentrated buffer substances, compensating gradient non-linearities with a software-enabled, algorithmic

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control of the gradient-mixing [8], resulting in linear pH gradients with uneven buffer capacity, but reduced ionic strength.

This means that so far there is no approach delivering a simple, fast, systematic way to generate buffer compositions for controllable pH gradients in every chosen pH range with minimized and known ionic strength. To generate controllable pH gradients, the buffer capacity of the buffer has to be kept constant, resulting in a buffer composition with a linear titration curve, which can then be controlled by the liquid chromatography system to generate the pH gradient. Other publications focusing on a similar problematic, the in silico optimization of buffer compositions for isoelectric focusing, as well as the simulation of the resulting pH gradient, have proven to be successful for the specific case presented [9–12].

In this work, the idea of optimizing a given buffer composition's capacity, predicting its titration curve and the course of the ionic strength throughout the gradient was adapted for the purpose to generate buffer systems for pH gradient IEC. The established in silico buffer optimization tool was used to generate buffer compositions for long range pH gradient IEC. To demonstrate their applicability, the optimized buffer compositions were applied for the formation of linear pH gradients and validated for the characterization of protein elution behavior in pH gradient IEC. Subsequently, the methods were used for an extensive study of the elution behavior of 22 model proteins on differently charged chromatographic resins. A comparison of the proteins elution-pH values of the two different chromatographic modes with literature values on the pIs of the proteins was made to gain better understanding of the differences between the methods, as well as the electrostatics of the single proteins.

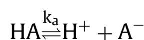
2. Theory

For the generation of controllable pH gradients, the titration curve of the applied buffer system has to be linear, which results from an even buffer capacity throughout the pH range. The buffer capacity, the titration curve and the ionic strength from single buffer substances as well as mixtures can be calculated. By varying the buffer substances and their single concentrations, buffer mixtures can be optimized to achieve linear titration curves. The theoretical background, the derivation of the needed equations and the optimization procedure are described in the following section.

2.1. Calculations

For chromatographic operations, most used buffer substances are mono-, di- or tribasic/-protic, while higher degrees are only rarely used. Therefore, this work is only focusing on these substances. In the following part, the derivation of the necessary equations is shown for a weak monoprotic substance.

The dissociation of a weak monoprotic acid



$$HA \overset{k_a}{\rightleftharpoons} H^+ + A^-$$

concludes to the following mass balance, whereby C_A is the total acid concentration.

$$C_A = [HA] + [A^-], \text{ while } [HA] = \frac{[A^-][H^+]}{k_a}$$

Combining both results in the following term for $[A^-]$:

$$[A^-] = C_A \frac{k_a}{[H^+] + k_a}$$

The electroneutrality condition defines the following charge balance for the dissolved acid

$$[H^+] = [A^-] + [OH^-]$$

Adding a strong, monobasic substance changes the charge balance to

$$[B^+] + [H^+] = [A^-] + [OH^-]$$

Including the ionic product of water

$$[H^+][OH^-] = k_w$$

the charge balance can be written as

$$[B^+] + [H^+] = [A^-] + \frac{[k_w]}{[H^+]}$$

combining the mass balance and the charge balance leads to

$$[B^+] = C_A \frac{k_a}{[H^+] + k_a} + \frac{k_w}{[H^+]} - [H^+] \tag{1}$$

from which the titration curve of the monoprotic acid can be calculated iteratively. To calculate the buffer capacity B, the equation has to be differentiated with respect to $[H^+]$, while remembering that

$$pH = -\log_{10}[H^+] = -\frac{1}{\ln(10)}\ln([H^+])$$

the buffer capacity is

$$B = \frac{dB^+}{dpH} = \ln(10)(C_A \frac{k_a[H^+]}{([H^+] + k_a)^2} + \underbrace{\frac{k_w}{[H^+]} + [H^+])}_{B(w)} \tag{2}$$

which includes the intrinsic buffer capacity of water (B(w)). To calculate the ionic strength of a buffering solution at different pH values, the single ionic species have to be summed up, independent of their charge. The general formula for the ionic strength of a solution including n different ionic species is

$$I = \frac{1}{2}\sum_{i=1}^{n} C_i Z_i^2$$

C is the molecular concentration of ions; *z* is the charge number and in case of a monoprotic acid the equation is

$$I = \frac{1}{2}\left(\underbrace{\frac{C_A k_a}{[H^+] + k_a}}_{[A^-]} + \underbrace{\frac{k_w}{[H^+]}}_{[OH^-]} + [H^+] + [B^+]\right) \tag{3}$$

The derived Eqs. (1)–(3) can be used to compute the course of the titration curve, the buffer capacity and the resulting ionic strength of a single substance. To calculate the values for di- and tribasic/-protic buffer substances the corresponding equations can be derived in the same way, the results are:

2.1.1. Diprotic substances

The titration of a diprotic substance

$$[B^+] = C_A \left(\frac{k_{a1}[H^+] + 2k_{a1}k_{a2}}{[H^+]^2 + k_{a1}[H^+] + k_{a1}k_{a2}}\right) + \frac{k_w}{[H^+]} - [H^+] \tag{4}$$

The buffer capacity of a diprotic substance

$$B = \ln(10) * \left(C_A \left(\frac{k_{a1}[H^+]^3 + 4k_{a1}k_{a2}[H^+]^2 + k_{a1}^2 k_{a2}[H^+]}{([H^+]^2 + k_{a1}[H^+] + k_{a1}k_{a2})^2} \right) + \frac{k_w}{[H^+]} + [H^+] \right) \quad (5)$$

The ionic strength of a diprotic substance

$$I = \frac{1}{2} \left(C_A \frac{k_{a1}[H^+] + 4k_{a1}k_{a2}}{[H^+]^2 + k_{a1}[H^+] + k_{a1}k_{a2}} + \frac{k_w}{[H^+]} + [H^+] + [B^+] \right) \quad (6)$$

2.1.2. Triprotic substances

The titration of a triprotic substance

$$[B^+] = C_A \left(\frac{k_{a1}[H^+]^2 + 2k_{a1}k_{a2}[H^+] + 3k_{a1}k_{a2}k_{a3}}{[H^+]^3 + k_{a1}[H^+]^2 + k_{a1}k_{a2}[H^+] + k_{a1}k_{a2}k_{a3}} \right) + \frac{k_w}{[H^+]} - [H^+] \quad (7)$$

The buffer capacity of a triprotic substance

$$B = \ln(10) \left(C_A * \left(\frac{k_{a1}[H^+]^5 + 4k_{a1}k_{a2}[H^+]^4 + k_{a1}^2 k_{a2}[H^+]^3 + 9k_{a1}k_{a2}k_{a3}[H^+]^3}{([H^+]^3 + k_{a1}[H^+]^2 + k_{a1}k_{a2}[H^+] + k_{a1}k_{a2}k_{a3})^2} + \frac{4k_{a1}^2 k_{a2}k_{a3}[H^+]^2 + k_{a1}^2 k_{a2}^2 k_{a3}[H^+]}{([H^+]^3 + k_{a1}[H^+]^2 + k_{a1}k_{a2}[H^+] + k_{a1}k_{a2}k_{a3})^2} \right) + \frac{k_w}{[H^+]} + [H^+] \right) \quad (8)$$

The ionic strength of a triprotic substance

$$I = \frac{1}{2} \left(C_A \frac{k_{a1}[H^+]^2 + 4k_{a1}k_{a2}[H^+] + 9k_{a1}k_{a2}k_{a3}}{[H^+]^3 + k_{a1}[H^+]^2 + k_{a1}k_{a2}[H^+] + k_{a1}k_{a2}k_{a3}} + \frac{k_w}{[H^+]} + [H^+] + [B^+] \right) \quad (9)$$

With the derived Eqs. (1)–(9), the titration curve, the buffer capacity and the ionic strength of mono-, di-, and triprotic substances can be calculated. To calculate the values for basic substances k_a has to be substituted by $k_b = k_w/k_a$ and $[H^+]$ by $[OH^-]$, with $[OH^-] = 10^{-14}/[H^+]$.

For the computation of the buffer capacity, the titration curve, or the ionic strength of buffer compositions consisting of multiple single substances, the values for the single substances are calculated and summed up. For example, the buffer capacity of m monoprotic, d diprotic and t triprotic acids is a sum of the single substances buffer capacity and the intrinsic buffer capacity of water.

$$B_{sum} = \sum_{i=1}^{m} B_i + \sum_{j=1}^{d} B_j + \sum_{k=1}^{t} B_k + \ln(10) \left(\frac{k_w}{[H^+]} + [H^+] \right)$$

The corresponding equations can also be formulated for the computation of the titration curve and the ionic strength. Combining all the equations in the computing environment MATLAB (MathWorks, Natick, MA, USA), made it possible to calculate the values for any mixture of mono-, di- and triprotic/-basic substances.

2.2. Optimization

The key parameter for buffer compositions with a linear titration curve is an even buffer capacity throughout the chosen pH range. To cover broad pH ranges, multiple buffer substances have to be chosen with pK_a values covering the chosen pH range. The buffer capacity can be kept constant throughout the pH range by choosing buffer substances with equally distributed pK_a values [9,7]. Another approach is to vary the concentrations of the single buffer substances in the mixture. Due to the lack of compatible buffer substances with a broad variety of pKa values, a buffer composition with evenly distributed pK_a values for a linear pH gradient with a broad pH range is difficult to achieve. A combination of the right choice of commercially available buffer substances with optimized concentrations leads to buffer compositions with constant buffer capacities resulting in linear titration curves.

The deviation of a buffer composition's buffer capacity from its mean buffer capacity $\overline{B}$, throughout a chosen pH range is formulated in the following equation:

$$\Delta B_{pH_{min-max}} = |B_{pH_{min-max}} - B|$$

$B_{pH_{min-max}}$ is the buffer compositions summed buffer capacity B_{sum} in the chosen pH range. To keep the buffer capacity as constant as possible, the deviation $\Delta B_{pH_{min-max}}$ has to be as small as possible, therefore the target function for the optimization of buffer compositions is:

$$\Delta B_{pH_{min-max}} \rightarrow \min$$

To optimize buffer compositions the MATLAB-based calculation of a mixture's buffer capacity was enabled to calculate the target function $\Delta B_{pH_{min-max}}$. Further on, the concentrations of the single buffer substances were defined as variables. By varying the single buffer concentrations the minimum of the target function $\Delta B_{pH_{min-max}}$ can be determined. The non-linear least square algorithm was used to minimize the target function.

Fig. 1 describes the MATLAB-based optimization procedure graphically. For the optimization of a buffer composition, the single pK_a values of the chosen buffer substances, the chosen mean buffer capacity $\overline{B}$, as well as the chosen pH range have to be entered. After minimizing the target function by varying the single buffer concentrations, the titration curve, the buffer capacity and the course of the ionic strength are calculated. This tool enables the generation of buffer compositions with a linear titration curve in every pH range with a chosen mean buffer capacity $\overline{B}$. The only major requirement that has to be fulfilled is the availability of suitable buffer substances for the chosen pH range.

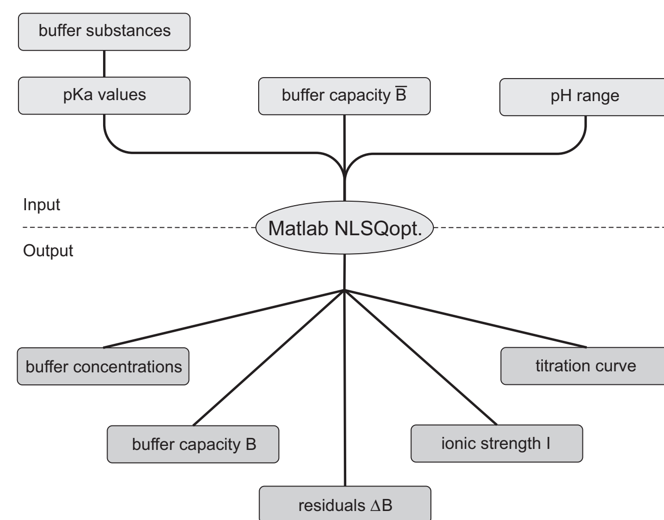


Fig. 1. The optimization method. Schematic overview of the in silico optimization process for buffer systems applicable for the formation of controllable, linear pH gradients in IEC.

Table 1
Buffer substances.

Basic buffer substances				Acidic buffer substances			
Substance	pKa_1	pKa_2	pKa_3	Substance	pKa_1	pKa_2	pKa_3
Piperidine	11.12[a]	–	–	CABS	10.70[c]	–	–
Methylamine	10.75[b]	–	–	CAPS	10.50[a]	–	–
1-Ethylpiperidine	10.45[a]	–	–	CAPSO	9.83[a]	–	–
1,2-Ethanediamine	9.93[b]	6.99[b]	–	CHES	9.39[a]	–	–
1,2-Propanediamine	9.82[a]	6.61[a]	–	AMPSO	9.14[a]	–	–
Piperazine	9.78[b]	5.52[b]	–	TABS	8.90[c]	–	–
2-Methylpiperazine	9.54[b]	5.24[b]	–	TAPS	8.44[a]	–	–
1-Methylpiperazine	9.16[b]	4.78[b]	–	EPPS	8.00[a]	–	–
Bis–tris propane	8.93[b]	6.59[b]	–	POPSO	7.80[a]	–	–
Morpholine	8.34[b]	–	–	TAPSO	7.64[a]	–	–
Tris	8.16[b]	–	–	HEPES	7.56[a]	–	–
1,4-Dimethylpiperazine	8.15[b]	4.04[b]	–	MOPS	7.18[a]	–	–
Triethanolamine	7.52[b]	–	–	MOPSO	6.90[a]	–	–
4-Methylmorpholine	7.34[b]	–	–	MES	6.10[a]	–	–
Bis–tris	6.22[b]	–	–	Acetate	4.76[a]	–	–
Hydroxylamine	5.67[b]	–	–	Succinate	4.21[a]	5.64[a]	–
Pyridine	5.23[a,a]	–	–	Formate	3.75[a]	–	–
–	–	–	–	Malate	3.40[a]	5.11[a]	–
–	–	–	–	Citrate	3.13[a]	4.76[a]	6.40[a]
–	–	–	–	Phosphate	2.15[a]	7.20[a]	12.35[a]

[a] Handbook of Chemistry and Physics, 89th ed., 2008, CRC Press.
[b] Measured pK_a value, at 25 °C.
[c] Thiel et al. [13].

2.3. Buffer substances

The buffer substances for the pH gradient IEC need to fulfill different criteria. The substances should not interact with the ion exchange resin and thus they have to be oppositely charged. Therefore, acidic substances are used for cation exchange chromatography (CEC), while basic substances are used for anion exchange chromatography (AEC). Another important criterion is that the substances do not interact with proteins, which is the case for ampholytes. We also decided to limit the substances to mono-, di-, and triprotic/-basic acids, because substances with a higher possible dissociation degree are rarely used in chromatography. Additionally, the substances have to be commercially available at an acceptable price and with analytical grade purity. In Table 1, a collection of useful buffer substances with their corresponding pKa values is shown.

3. Materials and methods

3.1. Chemicals and buffers

For the pH gradient CEC the chosen substances were MES, formic and acetic acid (Merck, Darmstadt, Germany), HEPPSO (Molekula, Dorset, UK), and MOPSO, TAPS, CHES, CAPS (Applichem, Darmstadt, Germany). The buffer for pH gradient AEC consisted of hydroxylamine, methylamine, 1,2-ethanediamine, 1,4-dimethylpiperazine from Merck (Darmstadt, Germany), 1-methylpiperazine from Sigma–Aldrich (St. Louis, MO, USA) and Bis–Tris from Molekula (Dorset, UK). Sodium chloride, hydrochloric acid and sodium hydroxide were obtained from Merck (Darmstadt, Germany). All substances were purchased in analytical grade. The used model proteins were purchased from Sigma–Aldrich (St. Louis, MO, USA), except glucose isomerase, which was purchased from Hampton Research (Hampton Research, Aliso Viejo, CA, USA). To set up the buffers for the pH gradient IEC, all substances were weighed in, dissolved in ultrapure water and split in two equal volumes. One part was titrated to the low pH extreme, the other to the high pH extreme, with the appropriate strong titrant. The pH adjustment was carefully performed with a freshly, five-point calibrated pH meter (HI-3220, Hanna Instruments, Woonsocket, RI, USA). The pH calibration buffers, pH 3, 5, 7, 9, and 11, were high precision standards from Hanna Instruments. After adjusting the pH, the buffers were brought to their final volume. All buffers have been filtered through 0.2 μm cellulose acetate filters (Sartorius, Goettingen, Germany). All buffer solutions were prepared with ultrapure water from a Arium water purification system (Sartorius, Goettingen, Germany). After preparation, the buffers were used for a maximum time period of two weeks.

3.2. Buffer optimization for pH gradient IEC

For the optimization of the buffer composition a mean buffer capacity of 10 mM was chosen. The pH range for the optimization was pH 10.5–3.5 for pH gradient AEC and 4.0–11.0 for pH gradient CEC. After optimizing the compositions, the buffers were prepared as described previously.

The applicability of the buffer systems was validated by examining the linearity of the resulting pH gradients. For the pH gradient CEC, a Mono S 4.6/100 column (GE Healthcare, Uppsala, Sweden) was used. The pH gradient AEC was run on a Mono Q 4.6/100 column (GE Healthcare, Uppsala, Sweden).

The experiments were done on an Aekta Purifier (GE Healthcare, Uppsala, Sweden) equipped with a pH electrode for online pH measurement. The pH electrode was calibrated with pH 3 and pH 12 high precision calibration standards. The column was equilibrated offline with ten column volumes (CV = 1.662 ml) of the application buffer. The gradient from 0 to 100% was performed in 15 column volumes. After running the gradient, a post-gradient, at 100% of the elution buffer was maintained for another five column volumes. The chromatographic run was executed with a flow rate of 1.5 ml/min.

To validate the reproducibility, three different experimental setups were run by three different persons. This means, for all three chromatographic runs the buffers were prepared fresh and the online-pH measurement was calibrated again. Each experiment was performed by a different person.

3.3. Elution-pH measurement of model proteins

The model proteins were prepared by dissolving 2 mg protein in 2 ml of the application buffer. Particulates in the protein solution were removed by centrifugation. The protein elution-pH values were determined by using the same chromatographic procedure as described in 3.2, but with an injection of 250 μl of the protein solution. The pH was monitored online, as well as the UV-absorption at 280 nm. To analyse the chromatograms of the pH gradient AEC the UV280 absorption of a blank run had to be subtracted first as the buffer showed a reproducible ghost peak at the beginning of each run probably resulting from minor impurities of the buffer substances. Afterwards, the proteins' elution-pH values could be determined from the peak maxima in the chromatograms. The Unicorn 5.2 software (GE Healthcare, Uppsala, Sweden) was used to analyse the chromatograms.

To validate the applicability of the method for the characterization of proteins in terms of repeatability as well as intermediate precision [14], the elution-pH values of four different model proteins were determined in three different experimental setups by a six time repeat measurement run by three different people.

After validation of the method's applicability, 22 model proteins were characterized by pH gradient IEC. Every model protein was analyzed by a repeat measurement on the cation and the anion exchange resin. The average protein's elution-pH value was then calculated for each chromatographic mode.

4. Results and discussions

4.1. Buffer optimization for pH gradient IEC

The aim was to generate buffer compositions for pH gradient AEC and CEC providing a broad pH range with low ionic strength. At first the buffer substances were chosen. With the known pK_a values and a mean buffer capacity of 10 mM, the MATLAB optimization procedure was applied for the minimization of the previously mentioned target function ΔB, varying the single buffer concentrations.

Table 2
Buffer systems for pH gradient IEC.

Substance	pK_{a1}	pK_{a2}	Conc.[mM]
AEC buffer pH 10.5–3.5			
Methylamine	10.75	–	9.8
1,2-Ethanediamine	9.93	6.99	9.1
1-Methylpiperazine	9.16	4.78	6.4
1,4-Dimethylpiperazine	8.15	4.04	13.7
Bis–tris	6.22	–	5.8
Hydroxylamine	5.67	–	7.7
Min ionic str. = 8.5 mM/max ionic str. = 104.3 mM			
CEC buffer pH 4.0–11.0			
CAPS	10.50	–	15.6
CHES	9.39	–	9.4
TAPS	8.44	–	4.6
HEPPSO	8.04	–	9.9
MOPSO	6.90	–	8.7
MES	6.10	–	11.0
Acetate	4.76	–	13.0
Formate	3.75	–	9.9
Min ionic str. = 8.3 mM/Max ionic str. = 89 mM			

The optimization resulted in the buffer compositions listed in Table 2. The course of the buffer capacity and the calculated titration curve of each buffer composition are visualized in Fig. 2. The high linearity of the calculated titration curve clearly proves that this approach was successful. The ionic strength of the buffer systems at the extreme pH values (Table 2) is acceptably low, leading to a minimal effect of the ionic strength on the chromatographic elution behavior of proteins.

4.2. Validation

To validate the applicability of the buffer systems, they have been applied on their correlating chromatographic resin. The resulting pH gradients were determined by measuring the pH online at the column outlet. The resulting pH gradients are shown in Fig. 3. Both gradients reached a linearity with $R^2 > 0.99$.

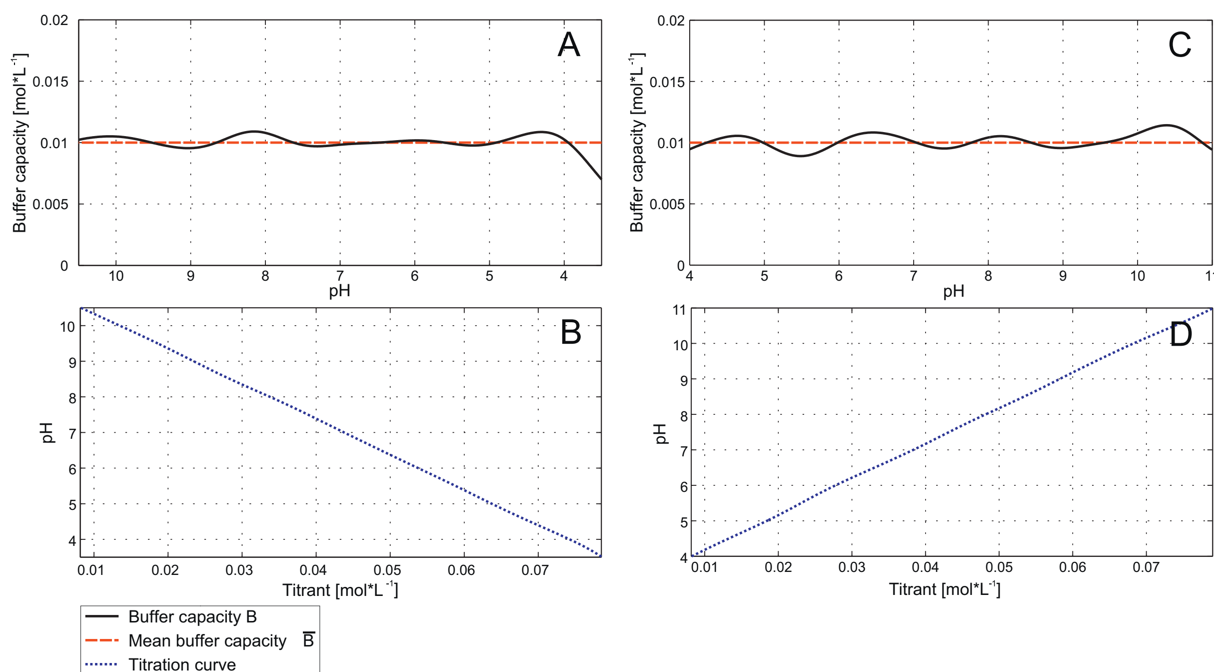


Fig. 2. Buffer calculations. Illustration of the course of the buffer capacity and the titration curve of the optimized buffer compositions described in Table 2. Optimization of the buffer systems and the calculation of the related plots were done in MATLAB. A and B: AEC buffer pH 10.5–3.5. (A) Calculated course of the buffer capacity. (B) Calculated titration curve. C and D: CEC buffer pH 4.0–11.0. (C) Calculated course of the buffer capacity. (D) Calculated titration curve.

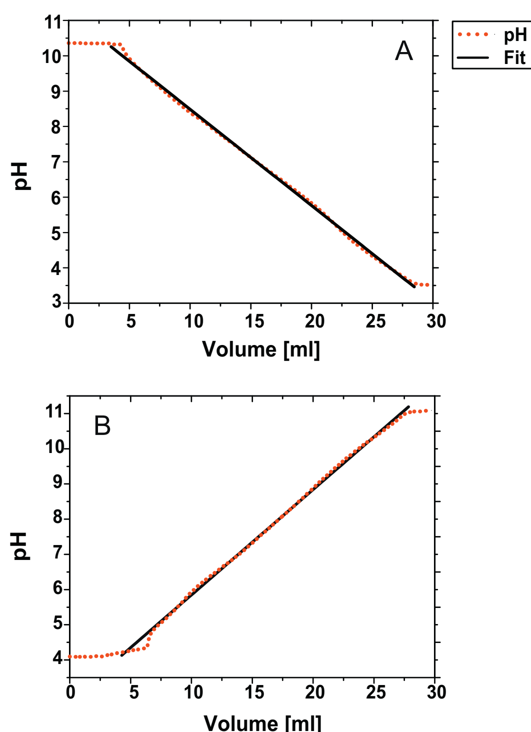


Fig. 3. pH gradients – linearity. Experimental determination of pH gradient linearity. pH gradients were formed by the application of each optimized buffer (Table 2) on the corresponding MonoQ/MonoS column. pH was monitored online at the column outlet with a delay volume from pump to pH probe of 3.5 ml. The gradient length was 24.9 ml with the starting point at 0 ml. The columns were equilibrated offline before the run. The linearity of the gradient was determined by linear regression. (A) AEC buffer applied on MonoQ column, linearity $R^2 > 0.99$. (B) CEC buffer applied on MonoS column, linearity $R^2 > 0.99$.

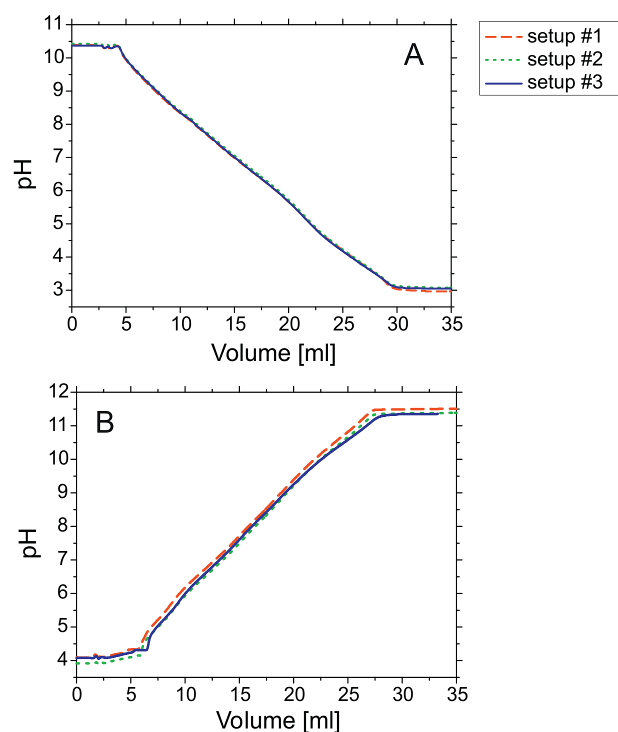


Fig. 4. pH gradients – reproducibility. Illustration of the overlay of three pH gradients (length = 24.9 ml, start at 0 ml) on a MonoS/MonoQ column formed by using the optimized buffer systems (Table 2). pH was monitored at the column outlet with a delay volume of 3.5 ml. Experiments were run as three independent setups by three different users. **A**: AEC buffer pH 10.5–3.0, 3× experimental setups. (B) CEC buffer pH 4.0–11.5, 3× experimental setups.

To reach high linearity at the extreme pH values 10 mM NaCl was added to suppress the exchange of the resins counterion: this means the exchange of Cl^- by OH^- at very basic pH values on the anion exchange resin or of Na^+ by H^+ at very acidic pH values on the cation exchange resin. Suppressing this ion exchange procedure by adding a small amount of salt, improved linearity greatly. The concentration of NaCl depends on the ion exchange rate of the resins counterion with H^+/OH^- and would therefore probably need adaption for other resins. Obviously, the ionic strength of 10 mM NaCl has to be added to the calculated buffers ionic strength.

However, there was still a small effect of the columns on the resulting pH gradients. It can be seen from the chromatograms (Fig. 3) that both gradients slightly deviate from linearity in the beginning and their starting points are delayed by 4–5 ml. This is little more than the gradient delay volume, which was 3.5 ml from the pump to the pH probe for the chosen setup. It is likely to be that this is still an effect caused by the described not completely suppressed ion exchange process. It seems that a small amount of H^+/OH^- ions still bound to the column and were then titrated off the adsorbent by the gradual increase of the elution buffer. This consequently would have leaded to delayed gradient starting points and an increased steepness in the beginning of the gradient, which correlates well with the presented results in Fig. 3. Therefore, a further increase of the amount of NaCl in the buffer would eliminate this effect. However, increasing the salt concentration further would effect the proteins' elution behaviour, which was not wanted. As a gradient linearity with $R^2 > 0.99$ was already reached, we decided that a further increase of the NaCl concentration would not be of any advantage.

The results of the validation, done by running the experiments in three different experimental setups by three persons, are shown in Fig. 4. The nearly identical course of the three pH gradients indicates the reproducibility of the method under common lab circumstances. Additionally we recognized that the gradients linearity could be maintained even when the range was extended by 0.5 pH units (AEC 10.5–3.0; CEC 4.0–11.5). This implies the minor disadvantage that the buffer capacity is lower, out of the optimized buffer systems pH range.

To validate the method for its actual purpose, being the separation and characterization of proteins with pH gradient IEC, the elution-pH values of four model proteins were determined in a six time repeat measurement in three different experimental setups by three different people. The experiments were done for both chromatographic modes. The model proteins for pH gradient AEC were cytochrome C, myoglobin, glucose oxidase and amyloglucosidase. For the pH gradient CEC, glucose oxidase, bovine serum albumine, alpha-chymotrypsinogen A and cytochrome C were chosen. The decision for the model proteins was made due to their clearly defined chromatographic peak in the applied chromatographic mode. The results are shown in Table 3. The results clearly

Table 3
Experimental validation of pH gradient IEC.

Protein	pH	Repeatability	Int. Prec.
1. pH gradient AEC			
RNAse A	9.38	±0.03	±0.04
Myoglobin	8.65	±0.01	±0.02
Glucose oxidase	4.68	±0.01	±0.06
Amyloglucosidase	3.93	±0.01	±0.04
2. pH gradient CEC			
Cytochrome C	10.25	±0.01	±0.09
α-Chymo-trypsinogen A	10.07	±0.01	±0.05
BSA	5.84	±0.00	±0.06
Glucose oxidase	4.34	±0.01	±0.09

indicate a high repeatability and intermediate precision with a standard deviation for both 0.1 pH units, in both chromatographic modes. Thus the experimental setup using the optimized buffer system clearly qualified for the specified purpose, meaning the separation and characterization of proteins with pH gradient IEC.

4.3. Elution-pH determination of model proteins

To characterize the elution behavior of proteins in pH gradient IEC, the validated method was used for an extensive characterization of 22 model proteins, with anion and cation exchange chromatography, comparing the proteins' elution behavior in the two chromatographic modes and literature values of experimentally determined pI values of the proteins.

In previous studies by Ahamed et al. [7], different model proteins were analyzed in terms of their elution behavior in pH gradient AEC, correlating the results with the pI values as well as the proteins titration curves. Their results showed that acidic (p*I* < 6) and basic proteins (p*I* > 8) elute roughly at their pI value while neutral proteins (p*I* ∼ 7) eluted at higher pH values in pH gradient AEC. Ahamed et al. [7] explained this phenomenon with the flat nature of the titration curve of neutral proteins. To our knowledge no study has been made, comparing the elution-pH value in pH gradient AEC and CEC to discover possible differences between the two chromatographic modes. Additionally the ionic strength of the buffer system, proposed by Ahamed et al. [7] was relatively high. The buffer's (pH 10.5–4.0 with 5 mM NaCl) calculated ionic strength was 10–198 mM charge equivalents. This means that the ionic stength of our optimized buffer systems is reduced roughly by one half, while providing a constant buffer capacity and also a linear, controllable pH gradient. This results in a reduced influence on the proteins retention behavior by ionic strength, meaning that these effects are minimized for the characterization of proteins.

The determined elution pH values as well as literature values of experimentally determined p*I*s are shown in Table 4. For better interpretation of the results, they were sorted in two groups, proteins with and without isoforms.

Table 5
Comparison of protein elution-pH values and their pI values.

Protein	ΔpH pI-AEC	ΔpH pI-CEC	ΔpH AEC-CEC
Cytochrome C (bovine)	+0.06	−0.24	−0.30
Cytochrome C (equine)	+0.01	−0.20	−0.30
Amyloglucosidase	−0.33	–	–
Glucose oxidase	−0.48	−0.14	+0.34
Lysozyme (human)	−0.50	−0.19	+0.31
α-Lactalbumin	−0.76	−0.81	−0.05
α-Chymotrypsinogen A	−0.09	−1.10	−1.19
Lysozyme (chicken)	–	+0.63	–
Glucose isomerase	+0.66	–	–
Hemoglobin A_0	−0.75	–	–

4.3.1. Isoform-free proteins

The identity of the detected chromatographic peak is clearly defined in both chromatographic modes as isoform free proteins are analyzed. This allows the direct comparison of the two elution-pH values and the isoelectric point. Interpretation of the results was done with regard to the differences between the three values, to gain insight into mechanistic differences between the three methods. The differences are shown in numbers for a better overview in Table 5. The determination of a protein's elution-pH value is exemplarily illustrated in Fig. 5. The illustration shows the analysis of the isoform-free protein cytochrome C by pH gradient CEC. To obtain the elution-pH value of the protein the pH at the peak maximum was determined.

The hemeproteins, **cytochrome C** from bovine or horse heart eluted merely at the same pH values, close to their p*I* values. Comparing the two IEC modes, the elution-pH values in pH gradient CEC are slightly shifted to higher pH values.

The enzyme **amyloglucosidase** is very acidic, therefore the protein was not retained in pH gradient CEC at pH 4.0 and eluted in the flow-through. The AEC elution-pH value is in the range of the proteins p*I*.

The acidic protein **glucose oxidase** eluted close to its pI values, which is lower than both elution-pH values. The AEC elution-pH value is a little higher than the corresponding CEC elution-pH value.

Table 4
Elution-pH of model proteins.

Protein	Organism	Ref.	p*I*	AEC elution-pH	CEC elution-pH
Cytochrome C	*Bos taurus*	[15]	10.01	9.95	10.25
Cytochrome C	*Equus caballus*	[15]	10.03	9.93	10.23
Amyloglucosidase	*Aspergillus niger*	S.I.	3.60	3.93	< 4.00
Glucose oxidase	*Aspergillus niger*	S.I.	4.2	4.68	4.34
Lysozyme	*Homo sapiens*	[16]	10.0	≈ 10.5	10.19
α-Lactalbumin	*Bos taurus*	S.I.	4.53	5.29	5.34
α-Chymotrypsinogen A	*Bos taurus*	[16]	8.97	8.88	10.07
Glucose isomerase	*Streptomyces rubignosus*	calc.	5.00	4.34	n.a.
Lysozyme	*Gallus gallus*	S.I.	11.35	> 10.5	10.72
Hemoglobin A_0	*Homo sapiens*	[16]	6.95	7.70	n.a.
RNAse A	*Bos taurus*	[17]	9.7	9.38	9.16
RNAse B	*Bos taurus*	[17]	9.7	9.38	8.83
beta-Lactoglobulin	*Bos taurus*	[16]	5.26, 5.34	4.25	5.97
Carbonic anhydrase	*Bos taurus*	[16][b]	5.89[a]	8.07, 8.23, 8.64[a]	6.48, 7.41, 10.66[a]
Myoglobin	*Equus caballus*	S.I.	6.8, 7.2	8.65, 8.96	n.a.
Thaumatin	*Thaumatococcus*	[18]	11.7 − 12	9.82[a], 10.02[a]	9.17[a], 9.49[a]
Serum albumin	*Homo sapiens*	[19]	4.8, 5.6	5.25, 5.35	6.06
Serum albumin	*Bos taurus*	[16]	4.98, 5.07, 5.18	5.18, 5.28, 5.32	5.79
Conalbumin	*Gallus*	[16]	5.62, 5.78[a], 6.05, 6.25[a], 6.50, 6.73[a]	5.89[a], 6.21[a], 6.72[a]	7.47
holo-Transferrin	*Bos taurus*	[20]	5.2, 5.4, 5.6, 5.7	5.64, 5.84, 5.98	6.54, 6.95
Ovalbumin	*Gallus gallus*	[21]	4.8, 4.9, 5.0	5.14[a], 5.23[a], 5.33[a], 5.49	4.89
Trypsin inhibitor	*Gallus gallus*	[22]	3.83, 4.01, 4.17, 4.28, 4.41	4.20, 5.17, 5.35[a], 5.70, 5.89	4.72
Catalase	*Bos taurus*	S.I.	5.40	5.96, 6.09[a]	5.44, 5.99[a], 6.19

S.I.: Supplier information, Sigma–Aldrich (St. Louis, MO, USA). Calc.: Calculated pI (Compute pI/Mw, http://web.expasy.org/compute_pi/). n.a.: not available, protein denatured in application buffer.

[a] Major isoform.

[b] Literature p*I* values incomplete.

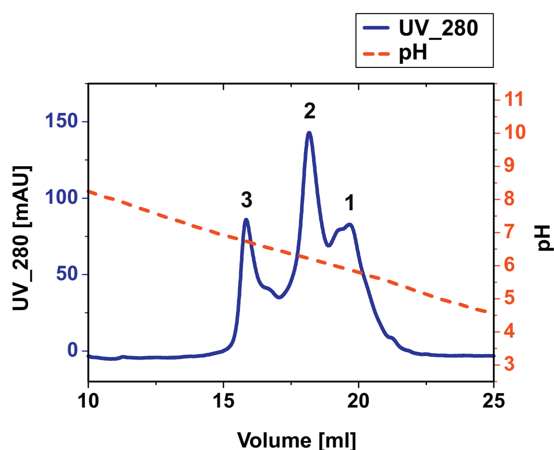


Fig. 5. pH gradient CEC of cytochrome C. CEC elution-pH determination of the isoform-free protein cytochrome. 250 μl of 1 mg/ml protein solution were injected on a MonoS column and analysed by a 24.9 ml pH gradient from pH 4.0 to 11.5, while monitoring the pH at the column outlet and the UV 280 nm signal. Elution-pH was determined by reading out the pH value at the determined peak maximum. Chromatograms were analysed with the Unicorn 5.2 software.

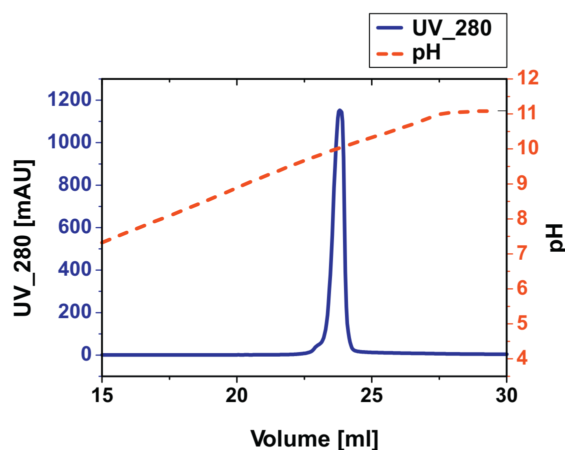


Fig. 6. pH gradient AEC of Conalbumin. AEC elution-pH determination of the isoforms of Conalbumin. 250 μl of 1 mg/ml protein solution were injected on a MonoQ column and analysed by a 24.9 ml pH gradient from pH 10.5 to 3.0, while monitoring the pH at the column outlet and the UV 280 nm signal. Elution-pH values were determined by reading out the pH values at the determined peak maxima. Chromatograms were analysed with the Unicorn 5.2 software.

Human milk **lysozyme** eluted at very basic pH values in both chromatographic modes. The protein's elution-pH in CEC is close to its p*I* but lower than in AEC.

While having two nearly identical elution-pH values, the p*I* of α-**lactalbumin** differs strongly from the proteins elution-pH values. Both elution-pH values are shifted about ~0.8 pH units to higher pH values.

The AEC elution-pH value of α-**chymotrypsinogen A**, is roughly in the range of its pI value. Surprisingly the elution-pH in CEC is more than one unit higher than in AEC.

Lysozyme from chicken egg is a very basic protein, which was therefore not retained in pH gradient AEC. The elution-pH value in pH gradient CEC is lower than the protein's p*I* and than the corresponding value in AEC.

The p*I* value of **glucose isomerase** is ~0.8 pH units higher than the corresponding AEC elution-pH. The CEC elution-pH could not be determined due to the known instability of the protein below pH 5.0 (Supplier information).

The hemeprotein **hemoglobin** A_0 is the major isoform of human hemoglobins. The protein's AEC elution-pH differs strongly from its p*I* value, both of them are in the neutral pH range. The proteins CEC elution-pH could not be determined due to denaturation of the protein in the application buffer at pH 4.0.

Summarizing the results leads to the following conclusions. The difference of the pI value and the elution-pH value varies from protein to protein, e.g. the elution-pH values of cytochrome C are very close to the proteins p*I*, while the elution-pH values of α-lactalbumin vary very strong from the proteins p*I*. A plausible explanation for this lies in the fundamental difference between isoelectric focusing and pH gradient IEC, the interaction with the stationary phase. Due to the threedimensionaltiy of the protein only a patch of the protein might be directly involved into the binding of the stationary phase. Depending on the strength of the attractive interaction between the resin and the interacting patch of the protein, this might result in a difference between the p*I* and the elution-pH value. The more anisotropic the proteins charge distribution is, the bigger the difference between the values would get. By means of this explanation it is very likely that the surface charge distribution of cytochrome C is more isotropic as the one of α-lactalbumin.

The same explanation is plausible for the elucidation of the differences between the elution-pH values of anion and cation exchange chromatography. Due to the oppositely charged adsorbers, different patches of the protein surface might interact with the different adsorber type. The resulting difference in the attractive interaction with the stationary phase concludes to a variation of the two elution-pH value.

One major drawback of using long range pH gradients is the denaturation of the protein at the extreme pH value of the application buffer. This problem might be easily fixed in most cases by using protein specific pH gradients with a narrower pH range and hence a less disruptive application buffer.

4.3.2. Proteins with isoforms

Many proteins have isoforms, resulting in charge variants of the single protein. Possible reasons are differences in the amino acid sequence, differences in the oxidative state, post-translational modifications, introducing a charge or masking charged amino acids and many more. Interpretation of the obtained results was done with regard to the separation behavior of the isoforms in the different methods.

The characterization of a protein with isoforms is exemplarily shown in Fig. 6. The illustration shows the analysis of the protein Conalbumin by pH gradient AEC. To obtain the elution-pH values of the single isoforms the pH values at the detected peak maxima were determined.

β-**Lactoglobuline** shows two known isoforms (A and B) in isoelectric focusing [16]. The proteins differ in their primary structure by two amino acids (A: Asp64; B: Gly64). The isoforms were not resolved by either of the two modes of long range pH gradient IEC. The resolution of the long range pH gradient seems to be too low, most probably using a pH gradient with a shorter pH range would resolve the two isoforms.

It is well known that **carbonic anhydrase** has many different isoforms. Up to 16 known isoforms exist in mammals [23] isoforms differing in their primary structure. The results of the pH gradient IEC of carbonic anhydrase from bovine erythrocytes show three well-separated major isoforms. Due to the inhomogeneity of the protein it is not a simple task to assign the peaks to isoforms. Also there is a big difference of the elution-pH between the two IEC modes, which makes it difficult to correlate the isoforms in between the chromatographic separations. Most probably the three major isoforms are related to carbonic anhydrase I–III, due to their higher abundancy in erythrocytes [24], whereby isoform II has the highest abundancy. Isoform II would therefore relate to the major isoform with the highest elution-pH value in both chromatographic modes.

Myoglobin showed one major and one minor isoform in pH gradient AEC. The two isoforms are natural variants, varying in their primary structure. Comparing the proteins AEC elution-pH with its pI shows the same result like reported previously. The elution-pH of the neutral protein is significantly higher than its p*I* [7]. Ahamed et al. explain the difference with the flat shape of the titration curve of neutral proteins, leading to a small change of charges as the pH is changed strongly. This means the attractive interaction of the protein with the resin at pH ~9 is already too low to retain the protein. The determination of the elution-pH on the cation exchange resin was not possible due to the denaturation of the hemeprotein at pH 4, which was also reported by others [25].

Thaumatins are extremely basic proteins with two known isoforms, **thaumatin I** and **thaumatin II** [26]. The two isoforms differ in their primary structure. By pH gradient IEC we were able to separate the two major isoforms. The measured elution-pH values differed greatly from the proteins p*I*, but literature values on the proteins p*I* are inconsistent. In another reference a pI range from 10.2 to 10.5 [27] is reported. This indicates that the well accepted p*I* value of 11.7–12.0 is likely to be wrong, which was already proposed by others [28]. Thus, the elution-pH values lie in the range of the proteins p*I*s.

Human serum albumin is known to form two isoforms due to different amounts of bound lipids, related to a conformational change [19]. The two isoforms are separated by isoelectric focusing and also by pH gradient AEC, but not by pH gradient CEC. A possible explanation is the bound fatty acids. The negatively charged molecules have a much stronger influence on the protein's interaction with the anion exchange resin, than with the cation exchange resin.

Bovine serum albumin has three known major isoforms due to different oxidative states of the intermolecular disulfide bonds resulting in the formation of multimeres [29]. Isoelectric focusing is able to separate three of them, which were also closely separated in pH gradient AEC, there was only one peak resolved in pH gradient CEC.

The chicken egg protein **conalbumin**, also known as ovotransferrin, has three major isoforms. Those are a result of the amount of bound iron ions, as the protein's p*I* drops with the amount of bound iron [30]. The elution-pH values decrease in the following order, iron-free, mono-ferric and the di-ferric version. pH gradient AEC of conalbumin shows the expected three major isoforms with almost exactly the same pH values than the p*I*s. pH gradient CEC was not able to separate the isoforms, also the determined elution-pH is significantly higher. The reason for this might be the fact, that at acidic pH values the iron ions are released from the protein [31]. Therefore the ferric-isoforms are removed in pH gradient CEC and only the iron-free protein is detected, which elutes at a higher pH value in pH gradient CEC. Comparing the AEC elution-pH values with the elution-pH values, determined by Ahamed et al. [7] (pH 6.7, 7.0, and 7.5) one will notice, that these values are slightly higher than the values presented here. The reason for this is most probably the lowered ionic strength of the used, optimized buffer, which leads to a reduced effect on the elution behavior of the proteins and consequently lower AEC elution-pH values.

Holo-transferrin, also known as siderophilin has four major isoforms, resulting from post-translational modification with sialic acid from mono- up to hexasialotransferrin. Di- to pentasialoferrine especially tetrasialotransferrin are the most abundant [32,20]. The references for the p*I* values [20] were determined from human holo-transferrin and are used here for comparison due to the unavailability of the same data from the bovine protein. Three major isoforms could be separated in pH gradient AEC, while only two were separated in pH gradient CEC. This difference in the selectivity of both chromatographic modes is likely to be a result of the post-translational modification with the negatively charged sialic acid. This modification has a stronger influence on the interaction with the anion exchange, than with the cation exchange resin. Another effect that might also be involved in the lower selectivity of pH gradient CEC is the partial denaturation of the protein at low pH values as it is known that the bound iron ions might be released at low pH values [33].

The hen-egg protein, **ovalbumin** has three known isoforms differing in their electrostatic properties a di-, a mono- and a non-phosphorylated version [21]. Three major isoforms are also detected in pH gradient AEC. A further, minor isoform is detected at a slightly higher elution-pH, probably this chromatographic peak is a result of the glycoforms of ovalbumin [34]. pH gradient CEC was not able to separate the different phosphoforms. This means the interaction with the cation exchange resin is barely affected by the negative charges introduced due to the phosphorylation.

Trypsin inhibitor, also known as ovomucoid, is a heavily glycosylated protein. The five different isoelectric isoforms are related to different glycoforms of the protein [21]. Only one elution-pH could be determined by cation exchange chromatography, while five were determined by pH gradient AEC. This indicates that the glycosylation does not have much influence on the proteins interaction with a negatively charged resin. Ovomucoid is glycosylated at Asn residues by various, negatively charged glycosylations with sialic acid or sulfated carbohydrates [35]. The different glycosylation degrees lead to a varying strength of attractive interaction of the different glycoforms with the anion, but not with the cation exchange resin. This concludes to the differing selectivity between the two chromatographic modes.

Ribonuclease A and **B** are two isoforms differing in the glycosylation pattern, whereby the B form is glycosylated at Asn34. Both isoforms have an identical pI and identical AEC elution-pH values, but interestingly they differ in the CEC elution-pH. The reason might be the positively charged amino acid, which is masked by the glycosylation and therefore not accessible by the cation exchange resin, thus the glycoform RNAse B elutes at a lower pH than the non-glycosylated RNAse A. This results are in accordance with previously reported results, stating that the two isoforms could be separated by CEC but not by AEC [36].

Catalase from bovine liver is uniform in isoelectric focusing, but in both pH gradient IEC modes, two to three isoforms were detected. The cause for this is likely to be the partial dissociation of the tetrameric protein at low [37] or high pH values [38] resulting in induced isoforms. To determine the detailed composition of the resulting protein fragments, further analysis would be needed.

Summarizing the results from the characterization of the different proteins via pH gradient ion exchange chromatography leads to the following conclusion. It became obvious that different modifications resulting in isoforms have a different impact on the separation efficiency of the single methods. This issue manifests in the differing selectivity of cation and anion exchange chromatography, e.g. the different phosphoforms of ovalbumin could be separated in pH gradient AEC but not CEC. This means that depending on the nature of the modification pH gradient AEC, CEC or isoelectric focusing might be the method of choice. Regarding the results it became clear that pH gradient IEC proved to be a potent tool to separate isoforms based on their different electrostatic properties.

5. Conclusions

In this work, we successfully demonstrated, that the in silico optimization of buffer compositions for pH gradient IEC is a fast and simple way to generate buffer compositions for well controllable pH gradients with low ionic strength. With this method we could successfully generate buffer compositions for long range pH gradients, spanning up to 7.5 pH units. The applicability of the buffer compositions was successfully validated by applying them on their

corresponding resins. The resulting pH gradients showed a high linearity and reproducibility. Additionally we collected a list of possible buffer substances, enabling us to generate optimized buffer compositions for nearly every pH range.

In the second part we successfully validated, the buffer compositions applicability for the characterization of proteins via pH gradient IEC. As a consequence, we applied pH gradient AEC and CEC for the characterization of 22 proteins, comparing the results with literature values of the proteins experimental p*I*s. The results clearly showed that there are major differences between the three methods, due to the proteins interaction with a charged adsorber surface. These differences often resulted in different selectivities of the three methods, motivating a selective use of the methods for specific separation problems. Additionally, pH gradient IEC clearly showed that it can be used as a real alternative for the characterization of protein charge variants.

Still there are many questions on the elution behaviour of proteins in pH gradient IEC left open for future studies. One interesting point would be to study the differences between salt and pH gradient IEC or a combination of both e.g. in terms of chromatographic resolution or robustness. The comparison of protein separation behaviour in pH gradient IEC using different columns, with different lengths and resins is also not treated by this article. The described methodology for the development of buffer systems for pH gradient IEC makes it possible to easily generate applicable buffer systems to work on those or further scientific questions.

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