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Review

Ion-exchange chromatography for the characterization of biopharmaceuticals

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ABSTRACT

Ion-exchange chromatography (IEX) is a historical technique widely used for the detailed characterization of therapeutic proteins and can be considered as a reference and powerful technique for the qualitative and quantitative evaluation of charge heterogeneity. The goal of this review is to provide an overview of theoretical and practical aspects of modern IEX applied for the characterization of therapeutic proteins including monoclonal antibodies (Mabs) and antibody drug conjugates (ADCs). The section on method development describes how to select a suitable stationary phase chemistry and dimensions, the mobile phase conditions (pH, nature and concentration of salt), as well as the temperature and flow rate, considering proteins isoelectric point (p*I*). In addition, both salt-gradient and pH-gradient approaches were critically reviewed and benefits as well as limitations of these two strategies were provided. Finally, several applications, mostly from pharmaceutical industries, illustrate the potential of IEX for the characterization of charge variants of various types of biopharmaceutical products.

Contents

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1. Introduction

Proteins and monoclonal antibodies (mAbs) are an emerging class of therapeutic agents currently being developed by many pharmaceutical companies [1]. Due to the increasing number of approved therapeutic proteins in the pharmaceutical area and the number of biosimilars (or follow-on-biologics) potentially entering the market, the need for analytical techniques for their detailed characterization has increased. Several characteristics of protein-based therapy contribute to its success by improving the risk–benefit ratio. These characteristics include improved tolerance, good efficacy, high specificity, and limited side effects. However, the intrinsic micro-heterogeneity is of major concern with biomolecules and should be critically evaluated because differences in impurities and/or degradation products could lead to health implications [2]. Furthermore, producing biosimilars is more challenging than manufacturing generic small molecule based pharmaceuticals [3].

In general, the identity, heterogeneity, impurity content, and activity of each new batch of therapeutic proteins has to be thoroughly investigated before release. This examination is achieved using a wide range of analytical methods, including ion-exchange chromatography (IEX), reversed-phase liquid chromatography (RPLC), hydrophobic interaction chromatography (HIC), size exclusion chromatography (SEC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), capillary isoelectric focusing (cIEF), capillary zone electrophoresis (CZE), circular dichroism (CD), Fourier transform infrared spectroscopy (FT-IR), fluorescence spectrophotometry (FL), and mass spectrometry (MS). The goal of this multi-method strategy is to demonstrate the similarity between production batches by precisely characterizing the primary, secondary, and tertiary structure of the proteins [4,5].

IEX is a historical and non-denaturing technique widely used for the characterization of charge variants of therapeutic proteins and is considered as a reference technique for the qualitative and quantitative evaluation of charge heterogeneity of therapeutic proteins [1]. The history and continuous evolution of IEX was reviewed by Lucy [6]. Among the different IEX modes, cation-exchange chromatography (CEX) is the most widely used for protein purification and characterization [7]. CEX is considered as the gold standard for charge sensitive analysis, but method parameters, such as column type, mobile phase pH, and salt concentration gradient, often need to be optimized for each individual protein [8]. IEX separates charge variants by differential interactions on a charged support. The number of possible charge variants increases with the molecular weight of the analyzed sample. In addition, changes in charge may be additive or subtractive, depending on any modifications. Thus, IEX profiles become more complex, and the overall resolution of individual variants may be lost [1]. This property is particularly apparent for large biomolecules. Therefore, not only the intact but also the reduced or digested forms (limited proteolysis or peptide mapping) of therapeutic proteins are commonly characterized by IEX.

In this review, we focus on the possibilities of IEX chromatography for the characterization of therapeutic proteins. Moreover, the aim of this review is to detail the theoretical and practical aspects of modern IEX. Last, method development approaches and applications are also reviewed and explained.

2. Theoretical aspects of IEX

2.1. Salt-gradient based separations

IEX separates proteins based on differences in the surface charge of the molecules, with separation being dictated by the protein interactions with the stationary phase [9]. As a classical mode of IEX, a linear salt-gradient is regularly applied for the elution. Several models for chromatographic retention of ion-exchange adsorbents have been proposed in the past years [10]. The retention models can be divided into stoichiometric and non-stoichiometric models. Stoichiometric models describe the multi-faceted binding of the protein molecules to the stationary phase as a stoichiometric exchange of mobile phase protein and bound counter-ions [11]. This stoichiometric displacement model (SDM) predicts that the retention of a protein under isocratic, linear conditions is related to counter-ion concentration. This model was extended to describe protein retention under linear gradient elution conditions (LGE model) [12], as well as under non-linear protein adsorption conditions (steric mass action (SMA) model) [13,14] for isocratic and gradient elution mode. Another extension of the stoichiometric model for the ion-exchange adsorption which accounts for charge regulation was developed recently [15,16].

Even if stoichiometric models are capable of describing the behavior of ion-exchange chromatographic systems, they assume that the individual charges on the protein molecules interact with discrete charges on the ion-exchange surface. In reality, retention through ion-exchange is more complex and primarily due to the interaction of the electrical fields of the protein molecules and the chromatographic surface [11]. Therefore, several non-stoichiometric models for describing protein retention as a function of the salt concentration in the mobile phase have also been proposed [17–20]. Quantitative structure–property relationship (QSPR) models have been derived for protein retention modeling in IEX by means of different numerical approaches that attempt to correlate retention to functions of descriptors derived from the three-dimensional structure of the proteins [21–23]. More recently, theories used in colloid and surface chemistry to describe electrostatic and other interactions have also been applied to describe retention properties of proteins in IEX [24–28].

The work of Snyder and co-workers showed that IEX systems follow non-linear solvent strength (LSS) type retention mechanism [29,30]. Consequently, solute-specific correction factors are required to use LSS model for retention predictions, thereby limiting the applicability of the LSS model. The non-linearity of LSS model was assessed by comparing the elution data to the stoichiometric displacement model (SDM) commonly used in IEX. The retention factor (k) can be written in the following way according to the SDM model:

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

where K is the distribution constant, z is associated with the protein net charge or number of binding sites (effective charge) and C is the salt concentration (that determines the ionic strength). This model is probably the most accepted one and is useful from a practical point of view. The non-linearity of Eq. (1) is most pronounced for small values of z [30]. If $z > 6$ (which is very often the case of therapeutic proteins), an LSS type model may provide reliable data for retention factor (retention time) [31]. Fig. 1A shows experimentally observed $\log k$ versus C plot for $z = 1$, while Fig. 1B shows some calculated $\log k$–C plots for various z values.

Proteins are eluted in order of increasing binding charge (correlates more or less with the isoelectric point (pI)) and equilibrium constant. The retention of large proteins in salt-gradient mode is strongly dependent on the salt concentration (gradient steepness or gradient time) – due to the relatively high z value – and a small change could lead to significant shift in retention. Therefore, isocratic conditions are impractical, and gradient elution is preferred in real-life proteins separations. For linear salt-gradient in IEX, the

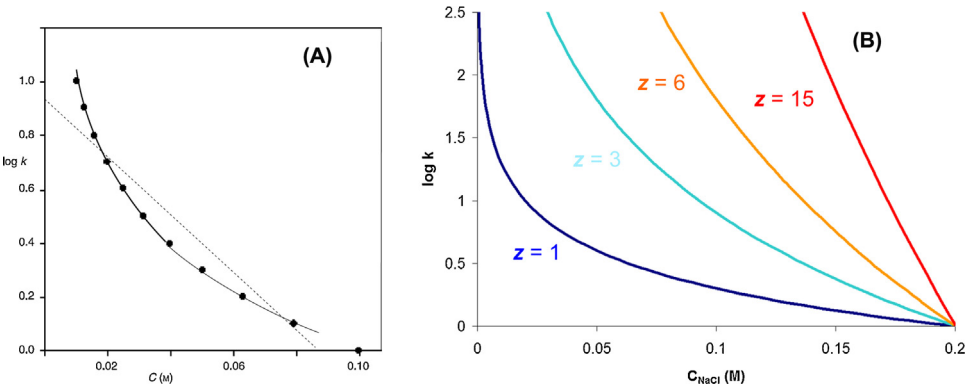

Fig. 1. log k–C plots: isocratic non-LSS ion-exchange retention compared with LSS model with $z = 1$ and $1 \leq k \leq 10$ (A) and some theoretical log k–C plots assuming several z values (B).
Fig. 1A was adapted from Ref [30], with permission.

salt concentration varies with time during the gradient, therefore Eq. (1) can be rewritten as:

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

where C_0 is the salt concentration at the beginning of the gradient (initial mobile phase composition) and ΔC is the change in the salt concentration during the gradient.

In analogy with RPLC, the following general equation can be written for salt-gradient based IEX separations in the gradient elution mode [31]:

$$k* = \frac{t_g}{1.15[t_0|z|\log(C_f/C_0)]} \quad (3)$$

where k^* is the median value of k during gradient elution when the band has reached the column mid-point, t_0 is the column dead time and C_f is the concentration of the counter-ion at the end of the gradient program. Please note that both RPLC and IEX separations vary with gradient conditions in a similar fashion. However, because of the differences in the dependence of k on the mobile phase composition C in IEX (log–log relationship) versus RPLC (log–linear relationship), the LSS model is theoretically not applicable for IEX. Nevertheless, as shown in Fig. 1, the higher the z, the lower the deviation from non-linearity is. It was currently shown, that LSS approach can be applied for large proteins (mAbs) possessing an important number of charges [32].

2.2. pH-gradient based separations

Ion-exchange chromatofocusing represents a useful alternative to linear salt-gradient elution IEX, in particular for separating protein isoforms with minor differences in the isoelectric point (p*I*). Chromatofocusing is performed on an ion-exchange column employing a pH gradient that can be generated internally within the column [33,34] or by external mixing of a high-pH and a low-pH buffer using a gradient pump system [35–37]. Highly linear, controllable, and wide-range pH gradients can be generated [37–40].

The number of applications reported at the analytical scale is large, but the number of publications dealing with the mathematical modeling of linear pH gradient elution in IEX is rather limited [9]. To describe the elution behavior of proteins in linear pH gradient IEX, a pH dependence parameter has to be incorporated into the ion-exchange model.

In pH-gradient mode, the proteins net charge is modified during the pH gradient, due to protonation–deprotonation of the functional groups. In CEX, the protein is expected to elute at, or close to its p*I*. According to theory, when applying pH-gradient elution mode and low ionic strength mobile phase, the chromatographic bands should be focused in narrower peaks enabling higher resolution compared to a pH-gradient performed at high ionic strength. The width of a protein peak along a linear pH-gradient expressed in pH units can be written as follows [33,34,40]:

$$(\Delta pH)^2 \approx \frac{D(dpH/dV)}{\varphi(dZ/dpH)} \quad (4)$$

where D is the diffusion coefficient of the analyte, dpH/dV is the gradient slope, φ is the Donnan potential and dZ/dpH is the change in protein net charge along the pH gradient. Since the Donnan potential depends on the ionic strength, a peak focusing effect is expected at lower ionic strength. In agreement with this expectation, pH gradients at low ionic strength showed better resolution for mAb variants compared to pH gradients performed at high ionic strength [40].

The applied pH range clearly determines the proteins that can possibly be eluted. Retention times and peak widths depend on the gradient steepness, as both are function of dZ/dpH.

The effect of gradient steepness (gradient time) on the retention of large proteins (intact mAbs and their variants) was recently studied and showed an LSS-like linear behavior [41]. The dependence of apparent retention factor (k_{app}) on gradient steepness (time) in pH-gradient based IEX separation is shown in Fig. 2.

In pH-gradient IEX mode, the use of a mixture of amine buffering species in the high-pH range and a mixture of weak acids in the low-pH range is quite common [38,39,42]. In such a system,

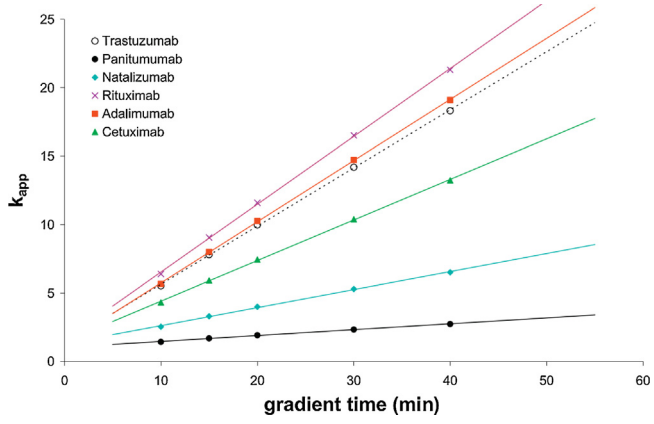

Fig. 2. The dependence of apparent retention factor (k_{app}) on gradient steepness (time) in pH gradient based IEX separation. The applied pH range was from pH = 5.6 to pH = 10.2 at 0.6 mL/min flow-rate on a 100 mm × 4.6 mm CEX column.
Adapted from Ref [40], with permission.

maintaining linearity of the pH gradient slope may be somewhat difficult. It was shown that an appropriate mixture of Tris base, piperazine and imidazole provides a linear pH gradient from pH 6–9.5 [8]. Triethylamine and diethylamine based buffer systems also offered linear pH gradient in the pH range of 7.5–10.0 [40]. For mass spectrometric (MS) detection, 5 mM ammonium hydroxide in 20% methanol yielded a reasonable pH gradient in a limited pH range (between 9.5 and 10.5) [40]. Zhang et al. [43] applied a salt-mediated improved pH gradient that was used in a wide pH range (between 5 and 10.5). In their study, a 0.25 mM/min sodium-chloride gradient was performed together with the pH gradient.

One of the benefits of pH-gradient based IEX is that the salt concentration can be kept low, yielding less buffer interferences (e.g. on-line or off-line two-dimensional LC). In addition, pH-gradient based separation using a CEX column was found to be a multi-product charge sensitive separation method for large therapeutic proteins (mAbs) [43,44].

3. Method development in IEX chromatography

Positively charged molecules can be separated using CEX columns, typically packed with 3–10 μm particles and containing negatively charged acidic functional groups. These columns bind cationic species such as protonated bases through ionic interaction. In anion-exchange (AEX) mode, the stationary phase carries positively charged basic functional groups that are capable of binding anions (e.g. ionized carboxylic acids). The mobile phase usually contains a buffer to maintain stable pH and varying the salt concentration (counter-ion) to control the retention of sample ions. The charge of the counter-ion has the same sign as the sample ions, therefore it can be used to control the retention of protonated bases in CEX or ionized acids in AEX.

The strength of the interaction is determined by the number and location of the charges on the analyzed molecules and on the functional groups. By increasing the salt concentration, the samples with the weakest ionic interactions start to elute from the column first. Molecules having a stronger ionic interaction require a higher salt concentration and elute later in the gradient.

In the pH-gradient mode, the ionic strength of the mobile phase is kept low and constant, while the pH is varied thanks to a linear gradient.

3.1. The impact of stationary phase

Regarding the stationary phase, there are two main aspects: (1) the strength of interaction and associated retention (strong or weak ion-exchanger) and the (2) achievable peak widths (efficiency).

Both cation and anion exchangers can be classified as either weak or strong exchangers. Weak cation exchangers are comprised of a weak acid that gradually loses its charge as the pH decreases (e.g. carboxymethyl groups), while strong cation exchangers are comprised of a strong acid that is able to sustain its charge over a wide pH range (e.g. sulfopropyl groups). On the other hand, strong anion exchangers contain quaternary amine functional groups, while weak anion exchanger possesses diethylaminoethane (DEAE) groups. Strong anion exchangers remain under ionized form in the pH range below 12, while strong cation exchangers are ionized at pH > 2.

As a rule of thumb, it is preferred to begin the method development with a strong exchanger to enable working over a broad pH range. Strong exchangers are also useful if the maximum resolution occurs at an extreme pH. (However, silica based ion-exchangers can be operated only in a restricted pH range. In contrast, polymeric ion-exchangers can be used in a wide pH range.)

In the case of proteins, the cation exchange mode is well suited, but a strong anion exchanger can be applied to bind the proteins if their p*I* is below pH 7. Weak exchangers can only be useful in a second instance, if the selectivity of strong ion exchangers is unsatisfactory. However, it is important to keep in mind that the ion exchange capacity of weak ion exchangers varies with pH.

Commercially available IEX columns are based on silica or polymer particles. Both porous and non-porous particles are available but for large molecules which possess low diffusivity, non-porous materials are clearly preferred to avoid the unwanted band broadening effects of the transparticle mass transfer resistance (C-term of the van Deemter equation). Highly cross-linked non-porous poly(styrene–divinylbenzene) (PS/DVB) particles are most frequently used in protein separations due to their pH stability ($2 \leq pH \leq 12$). Those materials can now withstand pressure drop of up to a 500–600 bar and can be routinely used beyond 400 bar. Columns packed with 10, 5 or 3 μm non-porous particles are often used, but sub-2 μm materials are also available since recently. On those columns high peak capacity can be attained even with large biomolecules. However some limitations can be expected in terms of loading capacity and retention when applying these non-porous materials. Table 1 summarizes the most popular state-of-the-art IEX columns applied for the separation of protein charge variants.

3.2. The impact of mobile phase composition

In the salt-gradient mode, the mobile phase buffer pH must be between the p*I* of the charged molecule (e.g. therapeutic protein) and the pK_a of the charged functional group at the surface of the stationary phase. In CEX, using a strong cation exchanger with a pK_a of 1.2, a molecule with a p$I \sim 8$ (e.g. mAbs) may be eluted with a mobile phase pH buffer of ~ 6. In AEX, a molecule with a p$I \sim 6$ may be run with a mobile phase buffer at pH 8 when the pK_a of the solid support is beyond 10.

In the CEX mode, increasing the mobile phase buffer pH will cause the molecule to become less protonated (less positively charged). Therefore, the protein forms weaker ionic interaction with the negatively charged stationary phase groups, which results in a retention decrease. On the contrary, decreasing the pH manifests in higher retention. In AEX mode, – oppositely – decreasing the mobile phase pH causes the molecule to become more protonated (more positively and less negatively charged), therefore a decrease in retention is expected.

The most often applied pH range for proteins IEX separations is between 5.5 and 7.0, however in some cases low pH around 3.5 is required to reach appropriate selectivity and retention. The most frequently used buffers for protein separations are 2-(N-morpholino)ethanesulfonic acid (MES), phosphate and citrate. MES is useful between pH 5.5 and 6.8 (p$K_a \sim$6.15), phosphate is applied for pH between 6.7 and 7.6 (p$K_a \sim$7.2), while citrate provides high buffer capacity for pH between 2.6 and 3.7 (p$K_a \sim$3.1). Other additives such as malonic acid, acetic acid or formic acid have also been reported for a limited number of applications. The buffer concentration is typically comprised between 10 and 50 mM and allows a sufficient buffer capacity.

After selecting the mobile phase pH and buffer, the salt-gradient has to be optimized. Typically sodium- or potassium-chloride are used for proteins characterization, using a salt gradient from 0 to 0.2–0.5 M. The protein samples are injected onto the column under conditions where it is sufficiently retained. Then, a gradient of linearly increasing salt concentration is applied to elute the sample components from the column. It is finally important to notice that the gradient steepness has a strong impact on retention and selectivity and should therefore be systematically optimized.

In the pH-gradient mode, the main difficulty is to perform linear and robust pH gradients. The use of a mixture of amine buffering

Table 1
Properties of the most popular state-of-the-art IEX columns available for protein separations.

	Column name		Chemistry	Particle size/macropore size	Max temperature (°C)	pH range	Max pressure (bar)
Monoliths	Proswift (Thermo)	SAX-1S	Strong anion exchange (quaternary amine)	Information not available	70	2–12	70
		WAX-1S	Weak anion exchange (tertiary amine)		60		
		WCX-1S	Weak cation exchange (carboxylic acid)		60		
		SCX-1S	Strong cation exchange (polymethacrylate)		60		
Packed	TSKgel (Tosoh)	SCX	Strong cation exchange (sulfonic acid)	5	45	2–14	50
		SuperQ-5PW	Strong cation exchange (trimethylamino)	10		2–12	
		SP-STAT	Strong cation exchange (sulfopropyl)	7, 10		3–10	
		Q-STAT	Strong anion exchange (quaternary ammonium)	7, 10		3–10	
	Bio Mab (Agilent)		Weak cation exchange (carboxylate)	1.7	80	2–12	270
				3			410
				5			550
				10			680
	Antibodix (Supelco, Sepax)		Weak cation exchange (carboxylate)	1.7	80	2–12	270
				3			410
				5			550
				10			680
	Protein-Pak Hi Res IEX (Waters)	SP	Strong cation exchange (sulfopropyl)	7	60	3–10	100
		CM	Weak cation exchange (carboxymethyl)	7			100
		Q	Strong anion exchange (quaternary ammonium)	5			150
	MAbPac SCX-10 (Thermo)		Strong cation exchange (sulfonic acid)	3	60	2–12	480
				5			480
				10			200
	Bio-Pro (YMC)	QA	Strong anion exchange (quaternary ammonium)	5	60	2–12	30
		QA-F					120
		SP	Strong cation exchange (sulfopropyl)				30
		SP-F					120
	Poly (PolyLC)	CAT A	Weak cation exchange (polyaspartic acid)	5	Ambient	Information not available	Information not available
		WAX LP	Weak anion exchange (polyethyleneimine)				

species in the high-pH range and a mixture of weak acids in the low-pH range is quite common [38,39,42]. As previously discussed, the most often used buffer components are Tris base, piperazine, imidazole, triethylamine, diethylamine and ammonium hydroxide [8,40,43]. Finally, a 0.25 mM/min sodium-chloride gradient was successfully performed concomitantly with a pH-gradient for the characterization of mAbs possessing isoelectric points (p*I*) between 6.2 and 9.4, to highlight the interest of pH gradient separation over salt gradients [43].

3.3. *The impact of sample* pI

The charge of proteins depends on the number and type of ionizable amino acid groups. Lysine, arginine and histidine residues have a positively charged side chain group when ionized, whereas glutamic acid and aspartic acid residues are negatively charged when ionized. Each ionizable side chain groups has its own pK_a. Therefore, the overall number of charges on a particular protein at a given pH depends on the number and type of ionizable amino acid groups. Proteins tend to have different charges at a given pH and so can be fractionated on the basis of their net and accessible charges. Each protein has a p*I* value, which corresponds to the pH value where it has no net charge. Then, when pH is equal to p*I*, the protein will not bind to the ion-exchange resin. Below this pH value, the protein has a net positive charge and binds to a cation exchanger, while above this pH, it has a net negative charge and binds to an anion exchanger. In practice, proteins are stable and functionally active within a fairly narrow pH range, so the choice of ion exchanger is often dictated by the pH stability of the desired protein. If the protein is stable at pH values below its p*I*, a cation exchanger should be used if it is stable at pH values above its p*I*, then an anion exchanger phase has to be chosen.

The p*I* of the protein also determines the mobile phase pH. The p*I* of therapeutic proteins distribute between 3.6 and 11.0, and among them, mAbs possess p*I* values between 6 and 10. For salt-gradient based CEX mode, the mobile phase pH should preferably be at least 1–2 units below the p*I* of the sample, to maintain appropriate retention. In AEX mode – oppositely – the pH has to be set at least 1–2 units above the p*I* of the protein.

In the pH gradient mode – performed on CEX columns – the starting pH should be below the p*I* of the less retained protein, while the final pH has to be somewhat higher than the p*I* of the most retained protein.

3.4. The impact of temperature

The effect of temperature on retention factor (k) is generally expressed in liquid chromatography with the Gibbs free energy or van't Hoff equation:

$$\log\ k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \log\ \beta \quad (5)$$

where ΔH represents the enthalpy change associated with the transfer of the solute between phases, ΔS the corresponding entropy change, R the molar gas constant, T the absolute temperature and β the phase ratio of the column. When log (k) is plotted against $1/T$, the enthalpy is given by the slope of the curve. With regular compounds, these plots generally follow a linear relationship. However, non-linear dependence of log (k) versus $1/T$ over a wide range of temperature was noticed by different authors using silica-based as well as non silica-based stationary phases [45]. The effect of temperature on the retention of partially ionized compounds which may exist in two forms (i.e. molecular and ionized forms) can also be described with Eq. (5). However, both enthalpy and entropy are expected to be different for the two forms and as a result, both H and S can vary with temperature when both forms are present to a significant extent [45]. With large biomolecules, the effect of temperature on retention becomes more complex. Depending on the stability of the secondary structure, the molecules unfold to various extents and hence interact with the stationary phase with various strengths [46]. Due to the different conformation-dependent responses of proteins at elevated temperatures, the change in retention can be difficult to assess [47,48]. In RPLC separation of proteins, temperature is a useful parameter for adjusting selectivity. In IEX separations of proteins, the impact of temperature was found to be especially important for peak capacity (and therefore for resolution), but has a limited impact on selectivity [32,41]. It seemed that in both salt- and pH-gradient based separations, the temperature does not modify severely selectivity, but impact the achievable peak capacity. Therefore, in some cases, temperature optimization could also be of importance during the IEX method development procedure.

3.5. Optimization procedure

In contrast with RPLC, the method development in IEX was mostly based on trial and error or "one factor at time" (OFAT) approaches. However, there are some guidelines available from column providers, which explain the basic rules for method screening (e.g. column selection, buffer selection. . .).

Bai et al. showed the dependence of retention and selectivity of IgG antibodies on mobile phase pH, stationary phase type and salt-gradient steepness in CEX mode [49]. They studied the effect of the three variables independently, and found that mobile phase pH was the most important parameter in CEX separations of proteins. It had the biggest impact on the separation and therefore should be determined first [49]. It was also found that (i) peak width of IgG-s mostly depends on the type of the stationary phase and (ii) resolution can be tuned by changing the gradient steepness. Fig. 3 shows the impact of salt-gradient steepness on the separation of IgG proteins.

The mobile phase linear velocity also has a strong influence on the separation quality of large proteins [50,51]. Indeed, the longitudinal diffusion is negligible with large molecules, while band broadening is mostly determined by the mass transfer resistance. Therefore, low flow rate is always preferred for high resolution separations, but a compromise has to be found between resolution and analysis time.

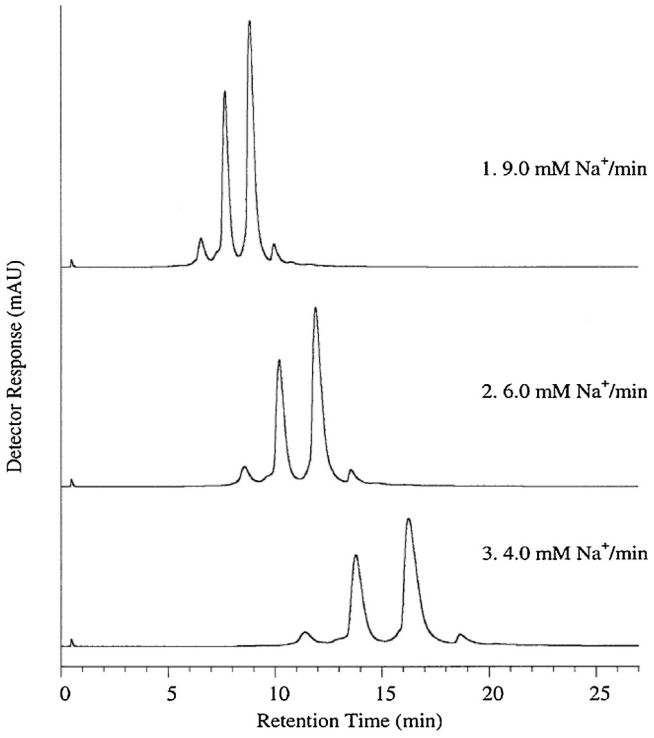

Fig. 3. The effect of salt-gradient slope on the retention, selectivity and peak width in CEX separation. Sample: IgG1, mobile phase: 40 mM phosphate pH 6.5, applying a 0–0.4 M NaCl gradient.
Adapted from Ref [49], with permission.

The influence of salt type can also be important. Its effect on the retention of bovine serum albumin was reported by Al-Jibbouri [52].

Computer assisted method development and optimization in RPLC protein separations is quite common [53,54] and was also recently applied in ion-exchange mode. Because of the system non-linearity, finding the optimum for process optimization is challenging [55]. Thiemo et al. developed a software called ChromX for the estimation of parameters, chromatogram simulation, and process optimization [55]. ChromX provides numerical tools for solving various types of chromatography models, including the model combination of Transport Dispersive Model (TDM) and SMA. Similarly to RPLC method development, a non-LSS and LSS type computer assisted method development procedure was recently reported for both salt- and pH-gradient modes in agreement with Quality by Design (QbD) concept [32,41].

For the salt-gradient based protein separation, it seemed that temperature was not a relevant parameter for tuning selectivity and should be kept at 30 °C, to achieve high resolving power (elevated peak capacity) [32]. Because the relationship between apparent retention factors and gradient time (slope) can be described with a linear function, only two initial gradient runs of different slopes are required for optimizing the salt gradient program. For pH dependence, a second order polynomial model (i.e. based on three initial runs) is preferred to describe k versus pH dependence. When combining the experiments in a design of experiments (DoE), it appeared that method optimization can be performed rapidly, in an automated way thanks to a HPLC modeling software, using two gradient times and three mobile phase pH (e.g. 10 and 30 min gradient on a 100 mm long standard bore column at pH = 5.6, 6.0 and 6.4). Such a procedure can be applied routinely and the time spent for method development would be only around 9 h. The relative error in retention time prediction was lower than 1%, making this approach highly accurate [32]. Fig. 4A shows a generic DoE for

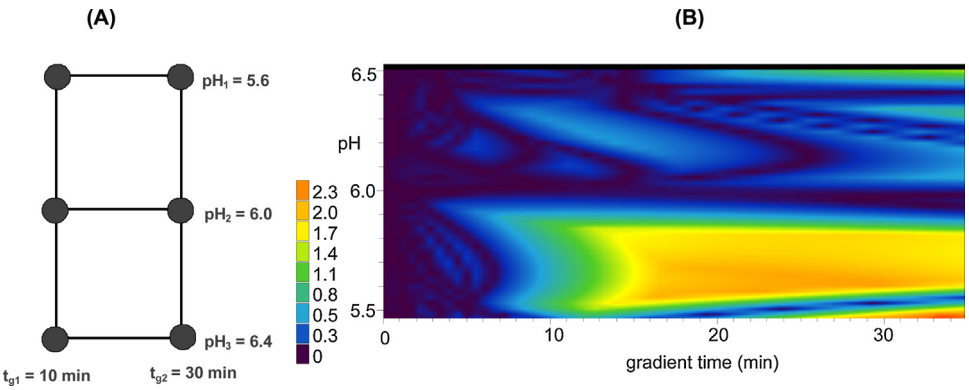

Fig. 4. Design of experiments (A) and resolution map (B) for the optimization of salt-gradient based CEX separation of mAbs (t_g–pH model). Column: YMC BioPro SP-F (100 mm × 4.6 mm). Mobile phase "A" 10 mM MES, "B" 10 mM MES + 1 M NaCl. Flow rate: 0.6 mL/min, gradient: 0–20%B, temperature: 30 °C. Gradient times: t_{g1} = 10 min, t_{g2} = 30 min, pH_1 = 5.6, pH_2 = 6.0, pH_3 = 6.4. On the resolution map, red-orange colors show the highest resolution while the dark-blue areas indicate the co-elution of peaks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Adapted from Ref [31], with permission.

the method development of salt-gradient based CEX separation of mAbs (possessing a wide range of p*I* between 6.7 and 9.1), while Fig. 4B represents the obtained resolution map showing the critical resolution of the peaks to be separated as a function of method parameters.

In the pH-gradient mode, it was found that the retention of large proteins can be accurately modeled as a function of gradient steepness and mobile phase temperature [41]. Because the retention models were always linear, only four initial experiments (2 gradients times at 2 temperatures) were required to model the behavior in CEX pH-gradient. Then, only ~6 h were required to find out the optimal conditions on a 100 × 4.6 mm column [41].

4. Application of IEX for protein separations

Today, IEX is mainly applied to separate protein charge variants and isoforms. IEX may be useful at different levels of protein analysis, including (i) the analysis of intact proteins (top down approach), (ii) the analysis of partly digested large protein fragments (middle down approach) and (iii) the characterization of tryptic digests (peptide mapping or bottom up approach).

In the case of therapeutic proteins characterization, IEX is mostly used to separate C-terminal lysine variants/truncation, N-terminal glutamine-pyroglutamate variants, deamidated forms (asparagine forms a succinimide intermediate that results in two products of its hydrolysis, either aspartate or isoaspartate), glycoforms especially sialic acid variants (usually attached at terminal positions of glycan molecules). In addition, IEX also separates the products of the PEGylation reaction according to the extent of conjugation, and provides the separation for the isomeric forms of PEGylated proteins (which differ from one another by the location of the conjugation site within the polypeptide chain) [55,56].

4.1. Characterization of charge variants of therapeutic proteins

Peptide mapping is commonly used to demonstrate protein identity. In the last phases of pharmaceutical development and in quality assurance/control (QA/QC), peptide mapping of the protein drugs serves as a primary protein QC method. Although reversed-phase separation is the typical choice for separating peptides, high-resolution ion-exchange chromatography is an alternative method that provides additional information and a different selectivity [57]. As example, a strong CEX column was used for the peptide mapping of cytochrome C tryptic digest [58] and Imamura et al. applied CEX for the peptide mapping of hemoglobin to identify its charge variants [59]. IEX was widely used in the past for peptide mapping, but today it is less popular and is mostly replaced by RPLC, due to the inherent incompatibility of IEX with MS detection.

IEX is definitely one of the most powerful techniques to separate deamidated forms of native proteins. Deamidation is a source of nonenzymatic protein degradation, and should be strictly monitored during the course of formulation development [60]. As example, CEX was successfully applied to separate the deamidated analogs of recombinant granulocyte colony-stimulating factor (G-CSF, filgrastim), as illustrated in Fig. 5. Weitzhandler et al. [61] presented the baseline separation of cytochrome C variants (bovine, horse, rabbit) and two deamidation products of ribonuclease A, such as the Asp^{67} and $isoAsp^{67}$ forms. Gotte et al. [62] applied CEX for the separation of ribonuclease B deamidated forms. Abzalimov et al. [55] applied the combination of IEX and top-down tandem MS for the structural characterization of protein–polymer conjugates and to assess heterogeneity of a small PEGylated protein and mapping conjugation sites. Finally, Ganzler et al. showed the separation of oxidized and deamidated PEGylated-G-CSF using CEX [63].

4.2. Separation of mAb variants

MAbs are a specific class of therapeutic proteins and gained significant interest over the past few years. Then, a dedicated section was devoted to their IEX based separation and characterization.

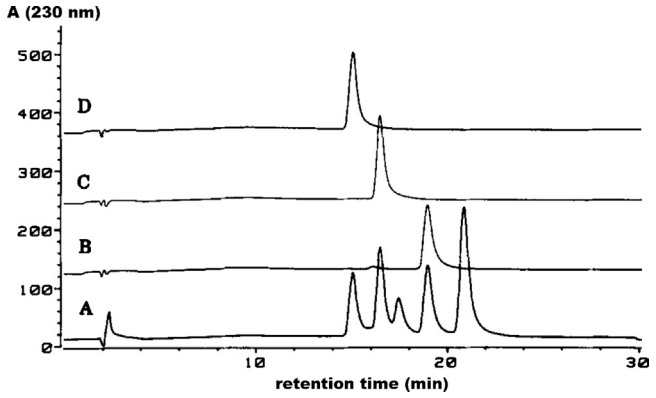

Fig. 5. Ion-exchange HPLC of rhG-CSF and the deamidated analogs. (A) $Gln^{11,20,67}$ → Glu analog, Gln^{67} → Glu analog, f-met rhG-CSF, $Gln^{11,20}$ → Glu analog, and met rhG-CSF (from left); (B) $Gln^{12,21}$ → Glu analog; (C) Gln^{67} → Glu analog; (D) $Gln^{11,20,67}$ → Glu analog.
Adapted from Ref [60], with permission.

The mAbs are glycoproteins that belong to the immunoglobulin (Ig) superfamily, which can be divided into five isotypes: IgA, IgD, IgE, IgG, and IgM. Because only IgGs are produced for therapeutic purposes through genetic engineering, the terms recombinant mAb and IgG are often used interchangeably. IgGs are large tetrameric glycoproteins measuring approximately 150 kDa that are structurally composed of four polypeptide chains: two identical heavy chains (HC, ~50 kDa) and two identical light chains (LC, ~25 kDa) connected through several inter- and intra-chain disulfide bonds at their hinge region [64]. Each chain is composed of structural domains according to their size and function, giving the constant, variable, and hypervariable regions. Differences between the HC constant domains are responsible for the IgG sub-classes (i.e., IgG1, IgG2, IgG3, and IgG4).

Functionally, mAbs consist of two regions, the crystallizable fraction (Fc) and the antigen-binding fraction (Fab) [65]. Fc (~50 kDa) is composed of two truncated HCs and is responsible for the effector functions, such as complement fixation and receptor binding. The Fc sequence also has a conserved N-glycosylation site, which is generally occupied by a biantennary oligosaccharide accounting for significant effects on the activity and efficacy of the IgGs [66]. The Fab domain (~50 kDa) is composed of the LC and the remaining portion of the HC. This domain is primarily involved in antigen binding [65].

There are several common modifications leading to antibody charge variants (or isoforms) on the peptide chains (e.g., deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine oxidation, or glycosylation variants) and size variants on the peptide chains (e.g., aggregation or incomplete formation of disulfide bridges). The combination of these micro-heterogeneity sources in the peptide chains significantly increases the overall micro-heterogeneity of an entire IgG. Therefore, the complete characterization of an intact mAb is difficult to achieve. In this context, various enzymes, such as pepsin, papain, Lys-C or IdeS are often used to obtain mAb fragments and facilitate the investigation of its micro-heterogeneity. Papain is primarily used to cleave IgGs into three fragments at the HC hinge region to create one Fc and two identical Fab fragments of ~50 kDa each, while pepsin and IdeS generates $F(ab')_2$ fragments of ~100 kDa. These types of digestion are called limited proteolysis (LP).

Moorhouse et al. [56] was among the first ones to describe the potential of IEX for mAb characterization. Papain-digested mAb samples were successfully separated and the corresponding fragments were identified thanks to MS detection. The C-terminal lysine variability of the Fc and the N-terminal glutamine-pyroglutamate variability of the Fab were observed. A recent study demonstrated the suitability of IEX for studying complex degradation processes involving various IgG1 molecules [67]. Assignment of covalent degradations to specific regions of the mAbs was facilitated using Lys-C and papain to generate Fab and Fc fragments. This method was particularly useful for characterizing protein variants formed in the presence of salts under accelerated storage conditions. The usefulness of this assay was further illustrated by characterization of light-induced degradations of mAb formulations. Another study presented the importance of IEX in the analysis of oxidized mAb samples [68]. Both CEX and AEX approaches were used and found suitable for the separation of the more basic oxidized variants of the intact mAbs. Fig. 6 displays multiple acidic and basic isoforms obtained in AEX mode, typical of recombinant mAb drug products. Vlasak et al. [69] combined CEX, papain digestion and a panel of MS techniques to identify asparagine deamidation in the light chain region of a humanized IgG1 mAb. Another study also presented and proved the importance of CEX (salt-gradient based) in mAb characterization and showed the separation of different isoforms [70].

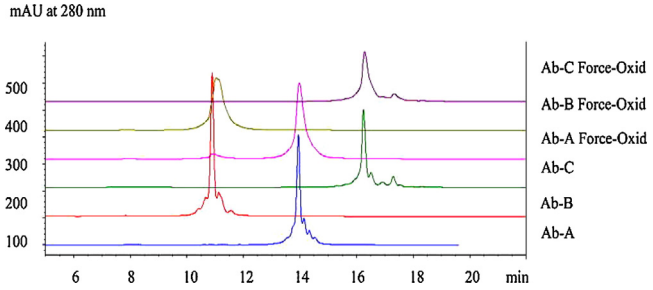

Fig. 6. CEX of XOMA 3AB antibodies. The three XOMA 3AB reference antibodies (Ab-A, Ab-B, and Ab-C) display multiple acidic and basic isoforms typical of recombinant monoclonal antibody drug products. The forced oxidized samples of each of these antibodies exhibit broad peak profiles indicating underlying structural heterogeneity.

Adapted from Ref [68], with permission.

A pH-gradient based separation using CEX chromatography was evaluated in a recent study [8]. The method was shown to be robust for mAbs and suitable for its intended purpose of charge heterogeneity analysis. Simple mixtures of defined buffer components were used to generate the pH gradients that separated closely related antibody species. Validation characteristics, such as precision, linearity and robustness were demonstrated. The stability-indicating capability of the method was determined using thermally stressed antibody samples [8]. Another recent study showed the applicability of a shallow pH gradient through CEX monolithic column and demonstrated relatively high resolution separation of mAb charge variants in three different biopharmaceuticals [40]. Zhang et al. [43] presented a multiproduct charge sensitive separation method for 16 mAbs possessing p*I*-s between 6.2 and 9.4. This salt-mediated pH-gradient IEX method was demonstrated to be robust under various chromatographic conditions and capable of assessing manufacturing consistency and monitoring degradation of mAbs. As an illustration, Fig. 7 shows the charge heterogeneity profile of 16 mAbs.

High-performance cation-exchange chromatofocusing methods were developed for the variants of neutral to acidic antibodies, and base-line separation of a variety of antibody variants was achieved [71]. The high resolution achieved indicated that the methods developed were useful alternatives to isoelectric focusing for characterizing the charge heterogeneity of mAbs variants.

The fast and efficient separation of cetuximab Fab and Fc variants were recently reported in both salt and pH-gradient CEX mode [32,41]. Fourteen charge variants were separated in only 17 min.

A systematic study compared the possibilities of IEX modes and showed that these approaches are cost- and time saving alternative to classical protein analysis methods (e.g. gel electrophoresis). The authors predicted that in the next step, further biologicals, e.g. antibodies, will be analyzed and quantified mostly with IEX and RPLC in the native as well as in its denatured form, respectively [72]. Another study also found benefits of IEX compared to electrophoretic methods such as the possibility of being automated and better quantitative results [73].

Besides the separation of mAbs' charge variants, pH-gradient based IEX chromatography can also be applied to evaluate the p*I* of intact mAbs [74].

Besides recombinant mAbs, IEX seems to be a promising technique for the characterization of other antibody-related products such as bispecific antibodies, recombinant polyclonal antibodies (pAbs) and Fc-fusion proteins [75–80].

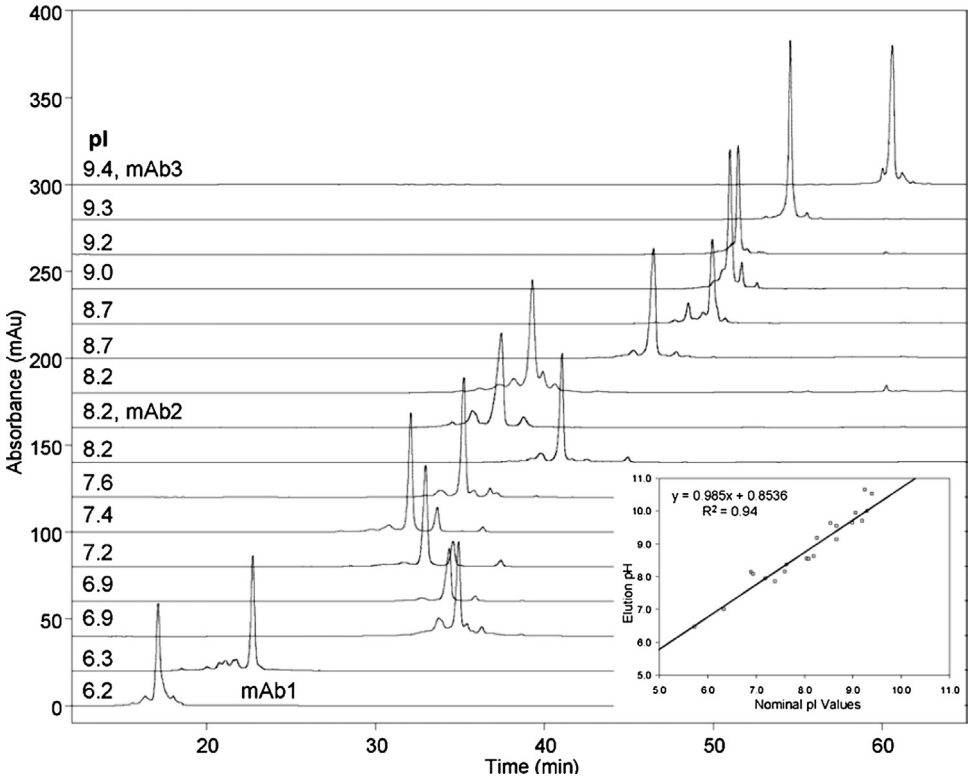

Fig. 7. The charge heterogeneity profiles of 16 mAbs with p*I* ranging from 6.2 to 9.4 obtained with the salt-mediated pH-gradient based IEX method. Adapted from Ref [43], with permission.

4.3. Analysis of antibody–drug conjugates

Antibody–drug conjugates (ADCs) or immunoconjugates, are becoming another increasingly important class of therapeutic agents undergoing clinical investigations for treatment of various cancer [81]. ADCs are produced through the chemical linkage of a potent small molecule cytotoxin (drug) to a mAb and have more complex and heterogeneous structures than the corresponding antibodies [82]. ADCs are constructed from three components: a mAb that is specific to a tumor antigen, a highly potent cytotoxic agent and a linker species that enables covalent attachment of the cytotoxin to the mAb through either the protein or the glycan. The primary sites used for protein-directed conjugation are the amino groups of lysine residues or the sulfhydryl groups of the inter-chain cysteine residues [82]. Depending on the characteristics of the drug, the linker and the conjugation site (i.e., lysine, inter-chain sulfhydryl, carbohydrate), the methods commonly used to characterize the parent mAb may not be applicable to the ADC or may give significantly different information.

Attachment of an uncharged linker and drug through lysine residues decreases the net positive charge by one for each bound drug-linker. In this case, separation based on charge, such as using IEX or iso-electric focusing (IEF), results in profiles that characterize the drug load, rather than proving information about the underlying mAb [83]. Despite the utility of these methods, there are only a few published reports of charge-based assays applied to ADCs.

For gemtuzumab ozogamicin, the IEX profile showed that most of the calicheamicin was on approximately half of the antibody while 45–65% of the product was a low conjugated fraction, essentially unconjugated antibody [83,84].

Thiomabs are antibodies with an engineered unpaired cysteine residue on each heavy chain that can be used as intermediates to generate ADCs [85]. Multiple charge variant peaks were observed during CEX analysis of several different thiomabs. This charge heterogeneity was due to cysteinylation and/or glutathionylation at the engineered and unpaired cysteines through disulfide bonds formed during the cell culture process [85].

In a recent study, the effects of chemical conjugation on the electrostatic properties of Fc-conjugates were estimated [86]. To minimize the effects of post-translational modifications (e.g., deamidation), a single Fc charge variant was isolated prior to conjugation of a fluorescent probe, to the side chains of lysine residues. The resulting Fc-conjugates were assessed by a variety of analytical techniques, including IEX, to determine their charge properties [86].

5. Perspectives in IEX

5.1. Decreasing the particle size

The Ultra-High Pressure Liquid Chromatography (UHPLC) technology was originally developed for RPLC applications, but it is now also available for SEC [87] and IEX operations due to the availability of 1.7 and 3 μm non-porous particles (PS/DVB) [88,89].

Applying 1.7 and 3 μm particles may open a new level of performance in IEX, but it has to be kept in mind that on very fine particles, the separation quality is improved at the cost of pressure (and temperature gradients attributed to frictional heating effects). Therefore, there is a risk of creating on-column degradation when analyzing temperature or pressure sensitive proteins under high pressure (i.e., >300 bar) conditions, as reported for RPLC and SEC [90,91]. The other disadvantage of sub-2 μm IEX separations is that currently there are only a very limited number of commercially available stationary phases.

Columns packed with these very fine particles are stable up to 600 bar that could be beneficial for both fast and high resolution separations. On short columns, high throughput separations can be achieved by applying high flow rates. On the other hand, the separation power can be improved by increasing the column length (e.g. coupling columns in series). With relatively long columns (e.g.

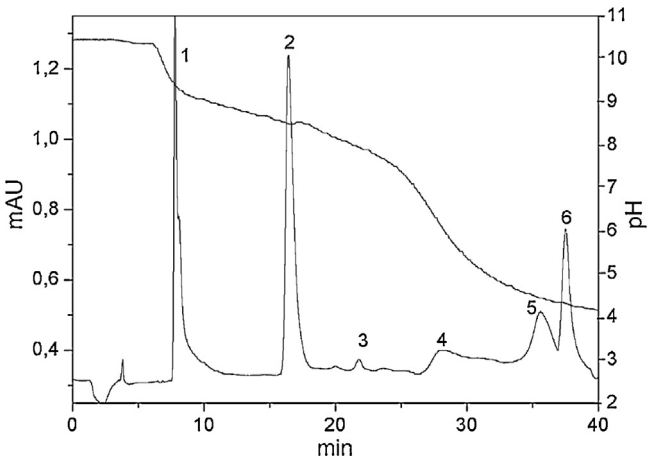

Fig. 8. Separation of a standard protein mixture on a 0.32 mm ID × 25 cm strong anion exchanger capillary column using pH-gradient mode. The elution order of the proteins: cytochrome C (1), myoglobin basic band (2), myoglobin acidic band (3), conalbumin (4), and β-lactoglobulin B (5) and A (6).
Adapted from Ref [43], with permission.

20–40 cm) employed at low flow rates, the peak capacity can be improved at the cost of analysis time.

5.2. Capillary IEX

To improve the sensitivity of protein variants determination or handle very small amounts of samples, the use of capillary columns in IEX appears to be a promising approach. However, several key modifications to a commercially available liquid chromatography system are required to reduce the system volume and associated extra-column band broadening, which could be critical for capillary IEX operation. Until now, the number of applications in this field is rather limited, but a 0.32 mm I.D. IEX capillary column was successfully applied in pH-gradient mode as the first dimension in a 2D separation of standard protein mixtures [92]. Fig. 8 shows the chromatogram observed with a 0.32 mm ID × 25 cm strong anion exchanger capillary column using pH-gradient mode.

5.3. Monolithic columns

Monolithic stationary phases are promising materials to improve chromatographic performance [93,94].

A monolithic column can be defined as a continuous solid matrix, porous in nature and containing interconnected flow paths. Various types of inorganic (e.g. silica, zirconia, carbon, titania) and organic (e.g. polymethacrylate, polyacrylamide, poly(styrene-divinylbenzene...) monoliths can be prepared but only polymethacrylate, poly(styrene–divinylbenzene), and silica-based monoliths are commercially available (mostly for RPLC separations).

The large flow-through channels and essentially nonporous surfaces support fast mass transfer, especially for large molecules (possessing slow diffusion), resulting in high resolution or fast separations. These channels also provide high permeability, allowing the use of high linear velocities.

Monoliths are generally classified as organic and silica-based monoliths. The organic monoliths are usually applied for the separations of biomolecules, including oligonucleotides, peptides, and intact proteins such as protein isoforms [95,96]. They are better accepted for protein separations than inorganic monoliths because of their biocompatibility. However, low surface area and binding capacity, swelling and shrinkage in some solvents, as well as deficiency in mechanical stability are their major drawbacks. Contrarily, silica-based monoliths are well adapted to the analysis of small molecules. They consist of a single rod of silica with two types of pores: macropores, which enable low flow resistance, and mesopores, which ensure enough surface area to reach high separation efficiency and loadability.

Currently, only 5 cm long organic monoliths containing strong and weak exchangers are available in either 4.6 or 1.0 mm ID format [97].

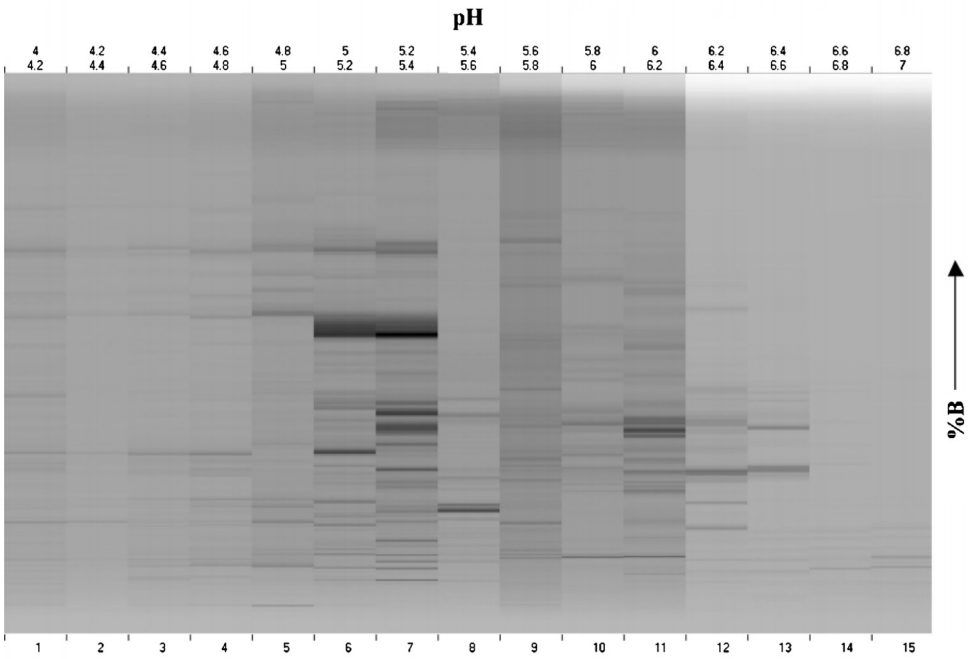

Fig. 9. The 2D protein expression map of *E. coli* bacterial lysate. The *x* axis is in p*I* unit from pH 4.0 to 7.0 (measured by pH-gradient IEX) and the *y* axis displays increasing hydrophobicity (%B) (measured by RPLC).
Adapted from Ref [98], with permission.

5.4. Two dimensional separations

IEX is widely used for the separation of large biomolecules and can be sometimes combine with other HPLC modes for two-dimensional (2D) or multistep separations.

The most promising combination of LC modes for protein analysis is probably IEX as the first and RPLC as the second dimension in 2D setups. In this configuration, it becomes possible to acquire MS information from an IEX separation that is hardly feasible in uni-dimensional IEX, except when using MS compatible mobile phases in IEX [40]. This 2D setup enables the use of ESI-TOF-MS analysis of intact proteins or MS/MS sequencing or other methods.

A multidimensional method has been applied by Zheng et al. for the separation and comparison of *E. coli* bacteria lysates using pH gradient based IEX coupled with RPLC [98]. The method provided a 2D map of protein expression with proteins categorized by both p*I* and hydrophobicity. This 2D method offered significantly higher loadability than conventional techniques while maintaining a high degree of resolution. Fig. 9 shows the 2D protein expression map of *E. coli* bacterial lysate.

Pepaj et al. [92] showed another application of IEX for multidimensional separation. A capillary IEX column was applied in pH-gradient mode as the first dimension in a 2D separation of standard protein mixtures [92].

Comprehensive 2D LC was recently proposed as a novel tool for peptide mapping of therapeutic mAbs in both R&D and routine (QA/QC) environments [99]. It was illustrated by the analysis of the tryptic digest of trastuzumab applying a commercially available 2D-LC system. Among three different LC × LC combinations, CEX × RP, was reported as an important tool for antibody characterization.

6. Conclusion

Because IEX is a non denaturating strategy, it will certainly remain one of the gold standards for the characterization of charge variants of therapeutic proteins. As illustrated in this review, IEX is a valuable strategy for the analysis of biopharmaceuticals and allows appropriate resolving power even for closely related substances. Currently, there are two approaches used in IEX, namely salt-gradient and pH-gradient, and both have their advantages and drawbacks. Today, one of the main limitations of pH-gradient IEX strategy is the high cost of commercial buffers, which prevent a more widespread use of this approach.

When dealing with therapeutic proteins, a good starting point for method development may be to use polymeric non porous stationary phase bonded with strong cation exchanger (sulfonic acid group). To select the best mobile phase conditions (i.e. pH, salt nature and concentration), it may be of importance to consider the p*I* of the proteins.

To date, IEX has been successfully employed for the characterization of charge variants on the peptide chains (e.g., deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine oxidation, or glycosylation variants) of numerous therapeutic proteins including mAbs and ADCs.

At the end, there are two main limits in IEX: (i) the limited resolving power of this technique which provides high selectivity but relatively poor kinetic performance (low peak capacity can be attained compared to RPLC). To improve this aspect, IEX columns packed with smaller particle sizes in the range sub-2 μm–3 μm have been recently commercialized by a few providers and offers significantly higher peak capacity than 5–10 μm particles, and (ii) the lack of compatibility with mass spectrometry, due to the presence of high concentration of non volatile salts, which prevent the precise identification of charge variants. To resolve this issue, the on-line combination of IEX and RPLC in a comprehensive 2D-LC setup appears as a promising solution and will certainly be more and more applied in a close future.

References

[1] S. Fekete, A.L. Gassner, S. Rudaz, J. Schappler, D. Guillarme, Analytical strategies for the characterization of therapeutic monoclonal antibodies, Trends Anal. Chem. 42 (2013) 74–83.
[2] C.K. Schneider, Monoclonal antibodies – regulatory challenges, Curr. Pharm. Biotechnol. 9 (2008) 431–438.
[3] P.A. Scolnik, mAbs: a business perspective, mAbs 1 (2009) 179–184.
[4] Q. Zhao, T.A. Stadheim, L. Chen, M.W. Washabaugh, in: Z. An (Ed.), Therapeutic Monoclonal Antibodies, John Wiley & Sons, Inc, Hoboken, NJ, 2009, p. 525.
[5] A. Beck, H. Diemer, D. Ayoub, F. Debaene, E. Wagner-Rousset, C. Carapito, A. Van Dorsselaer, S. Sanglier-Cianferani, Analytical characterization of biosimilar antibodies and Fc-fusion proteins, Trends Anal. Chem. 48 (2013) 81–95.
[6] C.A. Lucy, Evolution of ion-exchange: from Moses to the Manhattan project to modern times, J. Chromatogr. A 1000 (2003) 711–724.
[7] J. Svasti, C. Milstein, The disulphide bridges of a mouse immunoglobulin G1 protein, J. Biochem. 126 (1972) 837–850.
[8] J.C. Rea, G.T. Moreno, Y. Lou, D. Farnan, Validation of a pH gradient based ion-exchange chromatography method for high-resolution monoclonal antibody charge variant separations, J. Pharm. Biomed. Anal. 54 (2011) 317–323.
[9] M. Schmidt, M. Hafner, C. Frech, Modeling of salt and pH gradient elution in ion-exchange chromatography, J. Sep. Sci. 37 (2014) 5–13.
[10] J. Ståhlberg, Retention models for ions in chromatography, J. Chromatogr. A 855 (1999) 3–55.
[11] T. Bruch, H. Graalfs, L. Jacob, C. Frech, Influence of surface modification on protein retention in ion-exchange chromatography – evaluation using different retention models, J. Chromatogr. A 1216 (2009) 919–926.
[12] S. Yamamoto, K. Nakanishi, R. Matsuno, Ion-Exchange Chromatography of Proteins, Marcel Dekker, New York, 1988.
[13] S.R. Gallant, S. Vunnum, S.M. Cramer, Optimization of preparative ion-exchange chromatography of proteins: linear gradient separations, J. Chromatogr. A 725 (1996) 295–314.
[14] C.A. Brooks, S.M. Cramer, Steric mass-action ion exchange: displacement profiles and induced salt gradients, AIChE J. 38 (1992) 1969–1978.
[15] H. Shen, D.D. Frey, Effect of charge regulation on steric mass-action equilibrium for the ion-exchange adsorption of proteins, J. Chromatogr. A 1079 (2005) 92–104.
[16] H. Shen, D.D. Frey, Charge regulation in protein ion-exchange chromatography: development and experimental evaluation of a theory based on hydrogen ion Donnan equilibrium, J. Chromatogr. A 1034 (2004) 55–68.
[17] G.S. Manning, Limiting laws and counterion condensation in polyelectrolyte solutions I. Colligative properties, J. Chem. Phys. 51 (1969) 924–933.
[18] G.S. Manning, Limiting laws and counterion condensation in polyelectrolyte solutions III. An analysis based on the Mayer ionic solution theory, J. Chem. Phys. 51 (1969) 3249–3252.
[19] W.R. Melander, Z. El Rassie, Cs. Horvath, Interplay of hydrophobic and electrostatic interactions in biopolymer chromatography: effect of salts on the retention of proteins, J. Chromatogr. 469 (1989) 3–27.
[20] I. Mazsaroff, L. Varady, G.A. Mouchawar, F.E. Regnier, Thermodynamic model for electrostatic-interaction chromatography of proteins, J. Chromatogr. 499 (1990) 63–77.
[21] C.B. Mazza, N. Sukumar, C.M. Breneman, S.M. Cramer, Prediction of protein retention in ion-exchange systems using molecular descriptors obtained from crystal structure, Anal. Chem. 73 (2001) 5457–5461.
[22] G. Malmquist, U.H. Nilsson, M. Norrman, U. Skarp, M. Strömgren, E. Carredano, Electrostatic calculations and quantitative protein retention models for ion exchange chromatography, J. Chromatogr. A 1115 (2006) 164–186.
[23] W.K. Chung, Y. Hou, A. Freed, M. Holstein, G.I. Makhatadze, S.M. Cramer, Investigation of protein binding affinity and preferred orientations in ion exchange systems using a homologous protein library, Biotechnol. Bioeng. 102 (2009) 869–881.
[24] B. Jönsson, J. Ståhlberg, The electrostatic interaction between a charged sphere and an oppositely charged planar surface and its application to protein adsorption, Colloids Surf. B: Biointerfaces 14 (1999) 67–75.
[25] J. Ståhlberg, B. Jönsson, Cs. Horvath, Theory for electrostatic interaction chromatography of proteins, Anal. Chem. 63 (1991) 1867–1874.
[26] J. Ståhlberg, B. Jönsson, C.S. Horvath, Combined effect of coulombic and vander-waals interactions in the chromatography of proteins, Anal. Chem. 64 (1992) 3118–3124.
[27] J. Ståhlberg, B. Jönsson, Influence of charge regulation in electrostatic interaction chromatography of proteins, Anal. Chem. 68 (1996) 1536–1544.
[28] C.M. Roth, K.K. Unger, A.M. Lenhoff, Mechanistic model of retention in protein ion-exchange chromatography, J. Chromatogr. A 726 (1996) 45–56.
[29] R.W. Stout, S.I. Sivakoff, R.D. Ricker, L.R. Snyder, Separation of proteins by gradient elution from ion-exchange columns: optimizing experimental conditions, J. Chromatogr. 353 (1986) 439–463.
[30] M.A. Quarry, R.L. Grob, L.R. Snyder, Prediction of precise isocratic retention data from two or more gradient elution runs. Analysis of some associated errors, Anal. Chem. 58 (1986) 907–917.

[31] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, second ed., John Wiley & Sons Inc, 1997.
[32] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part I: Salt gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 33–44.
[33] L.A.Æ. Sluyterman, O. Elgersma, Chromatofocusing: isoelectric focusing on ion exchange columns. I. General principles, J. Chromatogr. 150 (1978) 17–30.
[34] L.A.Æ. Sluyterman, J. Wijdenes, Chromatofocusing: isoelectric focusing on ion exchange columns. II. Experimental verification, J. Chromatogr. 150 (1978) 31–44.
[35] L.A.Æ. Sluyterman, J. Wijdenes, Chromatofocusing: IV. Properties of an agarose polyethyleneimine ion exchanger and its suitability for protein separation, J. Chromatogr. 206 (1981) 441–447.
[36] A. Rozhkova, Quantitative analysis of monoclonal antibodies by cation-exchange chromatofocusing, J. Chromatogr. A 1216 (2009) 5989–5994.
[37] X. Kang, D. Frey, High-performance cation-exchange chromatofocusing of proteins, J. Chromatogr. A 991 (2003) 117–128.
[38] L. Shan, D.J. Anderson, Effect of buffer concentration on gradient chromatofocusing performance separating proteins on a high-performance DEAE column, J. Chromatogr. A 909 (2001) 191–205.
[39] L. Shan, D.J. Anderson, Gradient chromatofocusing versatile pH gradient separation of proteins in ion-exchange HPLC: characterization studies, Anal. Chem. 74 (2002) 5641–5649.
[40] M. Talebi, A. Nordbog, A. Gaspar, N.A. Lacher, Q. Wang, X.Z. He, P.R. Haddad, E.F. Hilder, Charge heterogeneity profiling of monoclonal antibodies using low ionic strength ion-exchange chromatography and well-controlled pH gradients on monolithic columns, J. Chromatogr. A 1317 (2013) 148–154.
[41] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part II: pH gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 282–289.
[42] Y. Liu, D.J. Anderson, Gradient chromatofocusing high-performance liquid chromatography: I. Practical aspects, J. Chromatogr. A 762 (1997) 207–217.
[43] L. Zhang, T. Patapoff, D. Farnan, B. Zhang, Improving pH gradient cation-exchange chromatography of monoclonal antibodies by controlling ionic strength, J. Chromatogr. A 1272 (2013) 56–64.
[44] D. Farnan, G.T. Moreno, Multiproduct high-resolution monoclonal antibody charge variant separations by pH gradient ion-exchange chromatography, Anal. Chem. 81 (2009) 8846–8857.
[45] S. Heinisch, J.-L. Rocca, Sense and non-sense of high-temperature liquid chromatography, J. Chromatogr. A 1216 (2009) 642–658.
[46] Y. Chen, C.T. Mant, R.S. Hodges, Temperature selectivity effects in reversed-phase liquid chromatography due to conformation differences between helical and non-helical peptides, J. Chromatogr. A 1010 (2003) 45–61.
[47] C.T. Mant, Y. Chen, R.S. Hodges, Temperature profiling of polypeptides in reversed-phase liquid chromatography: I. Monitoring of dimerization and unfolding of amphipathic α-helical peptides, J. Chromatogr. A 1009 (2003) 29–43.
[48] C.T. Mant, B. Tripet, R.S. Hodges, Temperature profiling of polypeptides in reversed-phase liquid chromatography: II. Monitoring of folding and stability of two-stranded α-helical coiled-coils, J. Chromatogr. A 1009 (2003) 45–59.
[49] L. Bai, S. Burman, L. Gledhill, Development of ion exchange chromatography methods for monoclonal antibodies, J. Chromatogr. A 22 (2000) 605–611.
[50] T. Ishihara, S. Yamamoto, Optimization of monoclonal antibody purification by ion-exchange chromatography, Application of simple methods with linear gradient elution experimental data, J. Chromatogr. A 1069 (2005) 99–106.
[51] S. Yamamoto, E. Miyagawa, Retention behaviour of very large biomolecules in ion-exchange chromatography, J. Chromatogr. A 852 (1999) 25–30.
[52] S. Al-Jibbouri, The influence of salt type on the retention of bovine serum albumin in ion-exchange chromatography, J. Chromatogr. A 1139 (2007) 57–62.
[53] S. Fekete, S. Rudaz, J. Fekete, D. Guillarme, Analysis of recombinant monoclonal antibodies by RPLC: toward a generic method development approach, J. Pharm. Biomed. Anal. 70 (2012) 158–168.
[54] S. Fekete, J.L. Veuthey, D. Guillarme, New trends in reversed-phase liquid chromatographic separations of therapeutic peptides and proteins: theory and applications, J. Pharm. Biomed. Anal. 69 (2012) 9–27.
[55] R.R. Abzalimov, A. Frimpong, I.A. Kaltashov, Structural characterization of protein–polymer conjugates. I. Assessing heterogeneity of a small PEGylated protein and mapping conjugation sites using ion exchange chromatography and top-down tandem mass spectrometry, Int. J. Mass Spectrom. 312 (2012) 135–143.
[56] K.G. Moorhouse, W. Nashabeh, J. Deveney, N.S. Bjork, M.G. Mulkerrin, T. Ryskamp, Validation of an HPLC method for the analysis of the charge heterogeneity of the recombinant monoclonal antibody IDEC-C2B8 after papain digestion, J. Pharm. Biomed. Anal. 16 (1997) 593.
[57] D. Zhenyu, X. Qun, J. Rohrer, High-resolution cation-exchange alternative to peptide mapping for protein ID and QA/QC, Dionex-Thermo, application update 188 (2113708-AU188), 2012, pp. 1–4.
[58] S. Waite, M. McGinley, Peptide separations by cation exchange chromatography using Luna SCX, Application note TN-1024, Phenomenex Inc., Torrance, CA, USA, 2005, pp. 1–2.
[59] T. Imamura, J. Sugihara, E. Yokota, M. Kagimoto, Y. Naito, T. Yanase, Analytical peptide mapping by ion-exchange high-performance liquid chromatography: application to haemoglobin variants, J. Chromatogr. 305 (1984) 456–460.
[60] C.L. Clogston, Y.R. Hsu, T.C. Boone, H.S. Lu, Detection and quantitation of recombinant granulocyte colony-stimulating factor charge isoforms: comparative analysis by cationic-exchange chromatography, isoelectric focusing gel electrophoresis, and peptide mapping, Anal. Biochem. 202 (1992) 375–383.
[61] M. Weitzhandler, D. Farnan, J.S. Rohrer, N. Avdalovic, Protein variant separations using cation exchange chromatography on grafted, polymeric stationary phases, Proteomics 1 (2001) 179–185.
[62] G. Gotte, M. Libonati, D.V. Laurents, Glycosylation and specific deamidation of ribonuclease B affect the formation of three-dimensional domain-swapped oligomers, J. Biol. Chem. 278 (2003) 46241–46251.
[63] Z.S.K. Szekeres, M. Olajos, K. Ganzler, Analytical aspects of biosimilarity issues of protein drugs, J. Pharm. Biomed. Anal. 69 (2012) 185–195.
[64] D.R. Mould, K.R.D. Sweeney, The pharmacokinetics and pharmacodynamics of monoclonal antibodies – mechanistic modeling applied to drug development, Curr. Opin. Drug Discov. Dev. 10 (2007) 84–96.
[65] G.M. Edelman, B.A. Cunningham, W.E. Gall, P.D. Gottlieb, U. Rutishauser, M.J. Waxdal, The covalent structure of an entire gamma G immunoglobulin molecule, J. Immunol. 173 (2004) 5335–5342.
[66] R. Jefferis, Glycosylation of recombinant antibody therapeutics, Biotechnol. Prog. 21 (2005) 11–16.
[67] H. Lau, D. Pace, B. Yan, T. McGrath, S. Smallwood, K. Patel, J. Park, S.S. Park, R.F. Latypov, Investigation of degradation processes in IgG1 monoclonal antibodies by limited proteolysis coupled with weak cation-exchange HPLC, J. Chromatogr. B 878 (2010) 868–876.
[68] G. Teshima, M.X. Li, R. Danishmand, C. Obi, R. To, C. Huang, J. Kung, V. Lahidji, J. Freeberg, L. Thorner, M. Tomic, Separation of oxidized variants of a monoclonal antibody by anion-exchange, J. Chromatogr. A 1218 (2011) 2091–2097.
[69] J. Vlasak, M.C. Bussat, S. Wang, E.W. Rousset, M. Schaefer, C.K. Hamour, M. Kirchmeier, N. Corvaïa, R. Ionescu, A. Beck, Identification and characterization of asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody, Anal. Biochem. 392 (2009) 145–154.
[70] A. Beck, M.C. Bussat, N. Zorn, V. Robillard, C.K. Hamour, S. Chenu, L. Goetsch, N. Corvaıa, A.V. Dorsselaer, J.F. Haeuw, Characterization by liquid chromatography combined with mass spectrometry of monoclonal anti-IGF-1 receptor antibodies produced in CHO and NS0 cells, J. Chromatogr. B 819 (2005) 203–218.
[71] X. Kang, J.P. Kutzko, M.L. Hayes, D.D. Frey, Monoclonal antibody heterogeneity analysis and deamidation monitoring with high-performance cation-exchange chromatofocusing using simple, two component buffer systems, J. Chromatogr. A 1283 (2013) 89–97.
[72] S. Grotefend, L. Kaminski, S. Wroblewitz, S.E. Deeb, N. Kuhn, S. Reichl, M. Limberger, S. Watt, H. Watzig, Protein quantitation using various modes of high performance liquid chromatography, J. Pharm. Biomed. Anal. 71 (2012) 127–138.
[73] N. Lingg, M. Berndtsson, B. Hintersteiner, M. Schuster, M. Bardor, A. Jungbauer, Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range: Validation of the method parameters, J. Chromatogr. A 1373 (2014) 124–130.
[74] S. Fekete, A. Beck, E. Wagner, K. Vuignier, D. Guillarme, Adsorption and recovery issues of recombinant monoclonal antibodies in reversed phase liquid chromatography, J. Sep. Sci. (2014), http://dx.doi.org/10.1002/jssc.201400996 (in press).
[75] A. Beck, T. Wurch, C. Bailly, N. Corvaia, Antibody-related products (definition): strategies and challenges for the next generation of therapeutic antibodies, Nat. Rev. Immunol. 10 (2010) 345–352.
[76] F. Debaene, E. Wagner-Rousset, O. Colas, D. Ayoub, N. Corvaïa, A. Van Dorsselaer, A. Beck, S. Cianférani, Time resolved native ion-mobility mass spectrometry to monitor dynamics of IgG4 Fab arm exchange and bispecific monoclonal antibody formation, Anal. Chem. 85 (2013) 9785–9792.
[77] A.F. Labrijn, J.I. Meesters, P. Priem, R.N. de Jong, E.T. van den Bremer, M.D. van Kampen, A.F. Gerritsen, J. Schuurman, P.W. Parren, Controlled Fab-arm exchange for the generation of stable bispecific IgG1, Nat. Protoc. 10 (2014) 2450–2463.
[78] L.S. Nielsen, A. Baer, C. Müller, K. Gregersen, N.T. Mønster, S.K. Rasmussen, D. Weilguny, A.B. Tolstrup, Single-batch production of recombinant human polyclonal antibodies, Mol. Biotechnol. 45 (2010) 257–266.
[79] A. Beck, J.M. Reichert, Fc-fusion proteins. Therapeutic Fc-fusion proteins and peptides as successful alternatives to antibodies, MAbs 3 (2011) 415–416.
[80] D. Ayoub, W. Jabs, A. Resemann, W. Evers, C. Evans, L. Main, C. Baessmann, E. Wagner-Rousset, D. Suckau, A. Beck, Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques, MAbs 5 (2013) 699–710.
[81] P.D. Senter, Potent antibody drug conjugates for cancer therapy, Curr. Opin. Chem. Biol. 13 (2009) 235–244.
[82] A. Wakankar, Y. Chen, Y. Gokarn, F.S. Jacobson, Analytical methods for physicochemical characterization of antibody drug conjugates, mAbs 3 (2011) 161–172.
[83] J. Moran, Presentation at 224th American Chemical Society National Meeting, Boston, MA, August 18–22, 2002.
[84] A. Kunz, 2004. Calicheamycin derivative carrier conjugates, United States Patent Application Publication, No. US 2004/01929200 A1.
[85] X. Chen, M. Nguyen, F. Jacobson, J. Ouyang, Charge-based analysis of antibodies with engineered cysteines, from multiple peaks to a single main peak, Mabs 6 (2009) 563–571.

[86] N.J. Boylan, W. Zhou, R.J. Proos, T.J. Tolbert, J.L. Wolfe, J.S. Laurence, Conjugation site heterogeneity causes variable electrostatic properties in fc conjugates, Bioconj. Chem. 24 (2013) 1008–1016.
[87] S. Fekete, A. Beck, J.L. Veuthey, D. Guillarme, Theory and practice of size exclusion chromatography for the analysis of protein aggregates, J. Pharm. Biomed. Anal. 101 (2014) 161–173.
[88] Agilent Technologies Inc., Agilent Bio MAb columns, 2010, Data sheet: 5973-1744.
[89] Sepax Technologies, Cation exchange chromatography, 2012, Application note: 1-877-SEPAX-US.
[90] S. Fekete, S. Rudaz, J.L. Veuthey, D. Guillarme, Impact of mobile phase temperature on recovery and stability of monoclonal antibodies using recent reversed-phase stationary phases, J. Sep. Sci. 35 (2012) 3113–3123.
[91] S. Fekete, K. Ganzler, D. Guillarme, Critical evaluation of fast size exclusion chromatographic separations of protein aggregates, applying sub-2 μm particles, J. Pharm. Biomed. Anal. 78–79 (2013) 141–149.
[92] M. Pepaj, S.R. Wilson, K. Novotna, E. Lundanes, T. Greibrokk, Two-dimensional capillary liquid chromatography: pH gradient ion exchange and reversed phase chromatography for rapid separation of proteins, J. Chromatogr. A 1120 (2006) 132–141.
[93] G. Guiochon, Monolithic columns in high-performance liquid chromatography, J. Chromatogr. A 1168 (2007) 101–168.
[94] K. Cabrera, Applications of silica-based monolithic HPLC columns, J. Sep. Sci. 27 (2004) 843–852.
[95] S. Eeltink, B. Wouters, G. Desmet, M. Ursem, D. Blinco, G.D. Kemp, A. Treumann, High-resolution separations of protein isoforms with liquid chromatography time-of-flight mass spectrometry using polymer monolithic capillary columns, J. Chromatogr. A 1218 (2011) 5504–5511.
[96] F. Detobel, K. Broeckhoven, J. Wellens, B. Wouters, R. Swart, M. Ursem, G. Desmet, S. Eeltink, Parameters affecting the separation of intact proteins in gradienst-elution reversed-phase chromatography using poly(stryrene-co-divinylbenzene) monolithic capillary columns, J. Chromatogr. A 1217 (2010) 3085–3090.
[97] Dionex, ProSwift ion-exchange monolith columns for biomolecule analysis, 2008, Application note: LPN 2020 PDF 02/08.
[98] S. Zheng, K.A. Schneider, T.J. Barder, D.M. Lubman, Two-dimensional liquid chromatography protein expression mapping for differential proteomic analysis of normal and O157:H7 *Escherichia coli*, Bio Tech. 35 (2003) 1202–1212.
[99] G. Vanhoenacker, I. Vandenheede, F. David, P. Sandra, K. Sandra, Comprehensive two-dimensional liquid chromatography of therapeutic monoclonal antibody digests, Anal. Bioanal. Chem. 407 (2015) 355–366.