



HAL
open science

Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range: Validation of the method parameters

Nico Lingg, Martina Berndtsson, Beate Hintersteiner, Manfred E. Schuster,
Muriel Bardor, Alois Jungbauer

► **To cite this version:**

Nico Lingg, Martina Berndtsson, Beate Hintersteiner, Manfred E. Schuster, Muriel Bardor, et al.. Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range: Validation of the method parameters. *Journal of Chromatography A*, 2014, 1373, pp.124-130. 10.1016/j.chroma.2014.11.021 . hal-01842178

HAL Id: hal-01842178

<https://normandie-univ.hal.science/hal-01842178>

Submitted on 10 May 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range: Validation of the method parameters

Nico Lingg^{a,b}, Martina Berndtsson^{a,c}, Beate Hintersteiner^a, Manfred Schuster^d,
Muriel Bardor^{b,e,1}, Alois Jungbauer^{a,*,1}

^a Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Muthgasse 18, A-1190 Vienna, Austria

^b Bioprocessing Technology Institute, Agency for Science, Technology and Research (A*STAR), 20 Biopolis Way, No. 06-01 Centros, 138668 Singapore, Singapore

^c Department of Pure and Applied Biochemistry, Lund University, Faculty of Engineering, Lund, Sweden

^d Apeiron Biologics AG, Campus-Vienna-Biocenter 5, A-1030 Vienna, Austria

^e Normandie Université, Laboratoire Glyco-MEV, EA 4358, Institut de Recherche et d'Innovation Biomédicale (IRIB), 76821 Mont-Saint-Aignan Cedex, France

Cation exchange chromatography has been routinely used for the quantification of monoclonal antibody (mAb) charge heterogeneity. A previously developed method utilizing pH gradients for the elution instead of salt gradients was validated according to current guidelines proposed by the ICH. The linearity, stability, accuracy, precision and the lower limit of quantification have been determined, using pure charge variant standards. The method is valid for the quantification of mAb samples with a charge heterogeneity between 1% and 50%. Three different approaches to obtaining pure standard material for the validation of bio-analytical methods for the quantification of charge heterogeneity of IgG are presented. These methods are based on salt gradient elution, pH gradient elution and displacement in cation exchange chromatography.

1. Introduction

Monoclonal antibodies (mAb) are a highly researched class of proteins which represent a very profitable part of the biopharmaceutical industry [1]. Indeed, their ability to target a wide range of antigens, e.g. other proteins, carbohydrates, and the strong effector functions they can elicit, e.g. antibody dependent cellular cytotoxicity, complement dependent cytotoxicity, make them an ideal molecule to be used as a therapeutic [2]. Immunoglobulin G (IgG) is the most commonly used isotype. It is a rather large protein with ~150 kDa and consists of four subunits. In order for the protein to function properly it requires proper folding, correct disulfide bonds and correct *N*-glycosylation for effector functions [3,4]. Other post-translational modifications, e.g. methionine oxidation, C-terminal lysine processing, deamidation of asparagine and isomerization of aspartic acid influence the behavior of mAb, as such they can be considered a critical quality attribute (CQA) [5,6]. The emergence

of novel purification strategies demands sophisticated analytical methods to ensure comparability of the product [7–11], and such analytical methods should be validated for their intended purpose [12,13].

In order to facilitate this goal, we decided to validate our previously presented pH gradient method for the analysis of antibody charge heterogeneity based on cation exchange chromatography (CEX) [14]. This method is intended to be used for the determination of acidic and basic charge variants, relative to the main charge variant found in a monoclonal antibody batch. In order to validate the method parameters, we purified mAb charge variants, to be used as a standard. The preparative separation of charge variants requires methods with a high resolving power. In this work, we describe different approaches to obtain pure standard material using CEX. The charge variants will be referred to as the main charge variant (MCV) and the acidic charge variants (ACV). The MCV is the main peak eluting in the chromatogram, and is usually not unmodified antibody, it is rather the antibody with the most common modifications [15]. The ACV are simply all charge variants eluting before the MCV in CEX. Basic charge variants could not be purified in sufficient amounts to allow usage as standard material, but the method should be valid for all charge variants. Isoelectric focusing

* Corresponding author. Tel.: +43 1 47654 6226; fax: +43 1 47654 6675.

E-mail address: alois.jungbauer@boku.ac.at (A. Jungbauer).

¹ These authors contributed equally to this work.

Table 1

Summary of the salt gradient elution experiments performed on a semi-preparative ProPac WCX-10 column. [M] is the modifier (NaCl) concentration, with the indices *i* and *f* meaning initial and final respectively.

Buffer	[NaCl] buffer A (mM)	[NaCl] buffer B (mM)	pH	[M] _i (mM)	[M] _f (mM)	Gradient slope (CV)
30 mM MES	0	1000	6.0	50	350	20
10 mM phosphate	50	100	7.0	50	100	5
10 mM phosphate	50	100	7.0	70	77	5
10 mM phosphate	50	100	7.0	63	70	5
10 mM phosphate	50	100	7.0	63	65.5	5
10 mM phosphate	50	100	7.0	60	65.5	5

Table 2

Summary of the displacement chromatography experiments performed on a Mono S column.

Buffer	pH	Displacer (mM)	Load (mg/ml)	Load (% DBC ₁₀)
40 mM MES, 15 mM NaCl	5.5	5	35	50
30 mM MES, 100 mM NaCl	6.1	5	14.9	60
30 mM MOPSO, 15 mM NaCl	7.0	5	33.6	60

(IEF) was used as an orthogonal method to check the purity of the standards.

Our first approach for the preparative scale separation of charge variants was standard CEX elution chromatography, using NaCl as the mobile phase modifier. This method has been used successfully for decades [16] and requires only a simple setup. The next approach was displacement CEX [17], which has been shown to be a method capable of high resolution separations of mAb charge variants [18]. With the advent of high performance displacers, this approach was very promising. Our last approach focused on the scale up of our own pH gradient elution CEX method into preparative scale, which we have previously shown to have a higher resolution than standard NaCl gradient elution CEX [14].

In order for this method to be used as a standard for the quantification of mAb charge heterogeneity, we validated the key performance parameters of the method, by following the recommendations for the validation of bioanalytical chromatographic methods presented by Hartmann et al. [12], while using the terminology from Peters et al. in this work [19]. In detail the following parameters have been determined: selectivity, calibration model (linearity), stability, accuracy (bias), precision (repeatability, intermediate precision) and the lower limit of quantification (LLOQ). This was done in a similar fashion to the work published by Tscheliesnig and Jungbauer [20] and Rozhkova [21]. The upper limit of quantification (ULOQ) was not determined, instead a charge heterogeneity of 50% was taken as the upper limit.

Our final goal was to provide evidence that our previously presented method is indeed valid for the quantification of mAb charge variants.

Other methods, such as imaging capillary isoelectric focusing (iCIEF), can also be used for quantification of mAb charge variant content and are well established in the biopharmaceutical industry [22,23]. Such methods have shown great inter-laboratory robustness and are reliable methods [24].

2. Material and methods

2.1. Materials and chemicals

All chemicals were of analytical grade, unless stated otherwise. 3-Morpholino-2-hydroxypropanesulfonic acid (MOPSO), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), *N,N*-bis(2-hydroxyethyl)glycine (bicine), 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-(*N*-Morpholino)ethanesulfonic acid (MES), NaH₂PO₄, Tergitol (70% in water) and isopropanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). NaCl,

Table 3

The buffer system used throughout this work for the creation of linear pH gradients.

	HEPES	Bicine	CAPSO	CAPS	NaCl	pH
Buffer A [mM]	5.5	4.2	9.5	0.8	6.3	8.0
Buffer B [mM]	0.0	10.5	2.5	7.0	0.0	10.5

Na₂HPO₄, NaOH, Urea and glycerol was purchased from Merck (Darmstadt, Germany). Pharmalyte 3–10 was purchased from (GE Healthcare, Uppsala, Sweden). Histidine, research grade (>98.5% purity) was from Serva (Heidelberg, Germany). Tween 20 was obtained from Bio-Rad (Hercules, CA, USA). ExpellTM SP1 (Sachem Inc., Austin, TX, USA) was used as the displacer in displacement chromatography experiments.

2.2. Preparative chromatography

The mAb used for the validation was a chimeric IgG 1, provided by Apeiron Biologics (Vienna, Austria). An ÄKTA Explorer 100 (GE Healthcare) was used for all preparative chromatography steps. The outlet was monitored at 280 nm.

2.2.1. Salt gradient elution

A Dionex ProPac WCX-10 column, 9 × 250 mm (Thermo Fisher, Sunnyvale, CA, USA) was used. The flow rate was 2.5 ml/min (236 cm/h). The buffers used for the salt gradient experiments are described in Table 1. The method used consisted of a 1 CV equilibration step at 0% B, 10 ml injections via sample loop, a 1 CV wash step at 0% B, a 1 CV gradient to the initial elution conductivity and the elution gradient with varying gradient length, found in Table 1.

2.2.2. Displacement chromatography

For displacement chromatography, a Mono S column, 4 × 200 mm (GE Healthcare, Uppsala, Sweden) was used. The flow rate was 0.25 ml/min (76 cm/h). Before each experiment, the dynamic binding capacity (DBC) at 10% breakthrough was determined for each mobile phase. The buffers and conditions used for displacement chromatography can be found in Table 2. The displacement experiments were performed according to Zhang et al. [18] and McAtee and Hornbuckle [17].

2.2.3. pH gradient elution

For salt gradient and pH gradient preparative experiments, a Dionex ProPac WCX-10 column, 9 × 250 mm (Thermo Fisher, Sunnyvale, CA, USA) was used. The flow rate for the ProPac column was 2.5 ml/min (236 cm/h). The buffers used for pH gradient experiments are found in Table 3. The various gradients used are detailed in Table 4. The collected acidic and main fractions from each

Table 4
Outline of the pH gradient elution method used for the purification of the standard material.

Experiment	Wash (CV)	Initial pH	Gradient (CV)	Final pH	Gradient (CV)	Final pH
1st round	1	8.0	14	9.8		
2nd round, acidic	1	8.0	2	8.9	10	9.4
2nd round, main	1	8.9	10	9.4		

run were concentrated and buffer exchanged with Amicon Ultra-15 Centrifugal Filter Units, 50 kDa (Merck Millipore, Darmstadt, Germany). The diafiltration buffer was 20 mM histidine, 0.1% Tween 20, pH 6.0. The gradient for the second purification round of the ACV standard consisted of two segments, a steeper initial gradient and a shallower gradient to separate the ACV from the main charge variant. The concentrated and buffer exchanged mAb was adjusted to a concentration of 2 mg/ml and used as the MCV and ACV standards.

2.3. Isoelectric focusing

Immobilized pH gradient (IPG)-polyacrylamide-gels (size: 125 × 260 × 1 mm) were cast on a GELbond-PAG-film backing (GE Healthcare), polymerized at 50 °C for 2 h, washed and dried following a procedure previously described by Westermaier [25]. The desired pH gradient from 7.0 to 11.0 was obtained by graphic interpolation from a recipe previously published by Gorg et al. [26].

Directly before use, the gel was cut in 3 mm strips, which were rehydrated for 2 h in a solution containing 6 M urea, 2% tergitol, 2% pharmlayte 3–10, 10% glycerol and 16% isopropanol.

The rehydrated strips were put onto the IPGphor (GE Healthcare) using the manifold. The electrodes were positioned on the acidic and basic ends of the strips with wetted paper wicks between gel and electrode to ensure good contact. The samples, 15 µg of mAb, were applied by cup loading under a covering layer of paraffin and electrophoresis was performed overnight (at 150 V for 1 h, followed by 300 V for 3 h, 3500 V for 18 h). After electrophoresis, the strips were stained with Coomassie blue G250.

2.4. Analytical chromatography

An Agilent 1220 Infinity LC System (Santa Clara, CA, USA) equipped with a SIM sample-cooler (Scientific Instruments Manufacturer GmbH, Oberhausen, Germany) was used for all validation experiments. The column used was a Dionex ProPac WCX-10, 4 × 250 (Thermo Fisher). The column oven and the auto-sampler temperatures were set to 30 °C and 10 °C, respectively. These temperatures were chosen due to instrument limitations.

The samples for the calibration model, the accuracy, precision and the stability experiments were prepared by mixing appropriate volumes of ACV and MCV standard. The outlet was monitored at 280 nm. The flow rate was 1 ml/min (477.5 cm/h). The buffers were the same as those used for the preparative experiments in Table 3. A gradient from 35% to 55% buffer B in 8 CV was used. The chromatograms were then integrated with the Chemstation software suite (Agilent).

3. Results and discussion

3.1. Creation of charge variant standard

In order to validate the method parameters, pure IgG charge variant standards were necessary. For such preparations, different CEX approaches were conceived and tested. The first approach was cation exchange chromatography combined with salt gradient elution.

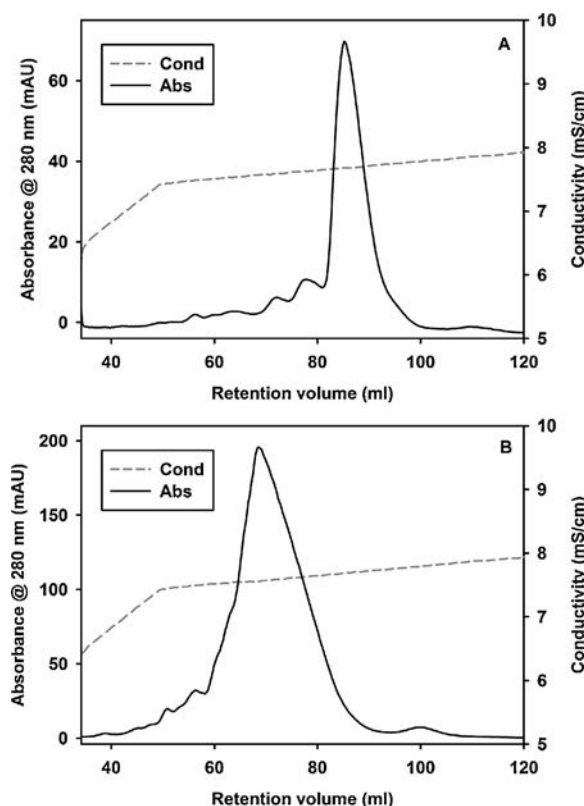


Fig. 1. Chromatogram of the semi-preparative separation of charge variants using salt gradient elution. Panel (A) shows a load of 3 mg (~190 µg/ml column) and panel (B) shows a load of 15 mg (~950 µg/ml column). A drastic decrease in resolution is apparent at higher loading. Stationary phase: Dionex ProPac WCX-10 column, 9 × 250 mm. Flow rate: 2.5 ml/min (236 cm/h). Mobile phase: 10 mM phosphate, pH 7.0, buffer A with 50 mM NaCl, buffer B with 100 mM NaCl. Gradient from 60 to 65.5 mM NaCl in 5 CV.

3.1.1. Salt gradient elution

Both the Mono S and the ProPac WCX-10 columns were tested, but the latter column was used for further optimization due to the higher resolution, owing to the faster mass transfer.

The method was optimized by testing elution behavior at different pH values and adjusting the salt gradient, the conditions tested for the optimization can be found in Table 1. The elution was optimized by running increasingly narrow gradients around the elution peaks of interest, thereby increasing the resolution. Optimization experiments were performed with 190 µg/ml injections, with a total volume of 10 ml, but subsequent scale up to 1.9 mg/ml was unsuccessful. Fig. 1 shows the optimized salt gradient with 190 µg/ml (Fig. 1A) and 950 µg/ml (Fig. 1B) injections. A drastic decrease in resolution was observed even under relatively low loading amounts (<1 mg/ml stationary phase). Due to the low binding capacity observed at pH 7, we did not attempt separation at a higher pH. Since scale up of salt gradient elution failed, we pursued displacement chromatography as a method for creating the standard.

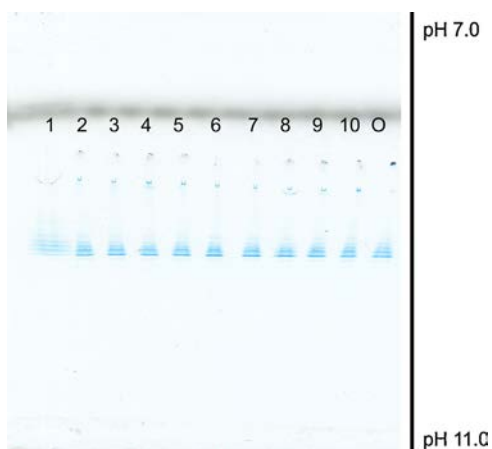


Fig. 2. IPG IEF of the displacement fractions from a displacement experiment on a Mono S column, 5×200 mm. Flow rate: 0.25 ml/min (76 cm/h). Mobile phase: 30 mM MOPSO, 15 mM NaCl, pH 7.0. Displacer: 5 mM Expell SP1. Lanes 1–10 contain displacement fractions with higher numbers denoting later eluting fractions. O is the initial sample. No difference in any fraction could be observed. No marker was used, since only the isoelectric point (pI) relative to the initial sample was relevant.

3.1.2. Displacement chromatography

Displacement chromatography was tested at three different conditions, as described in Table 2. Expell SP1 was chosen as the displacer, as it has proven to be useful in IgG separations [17,18,27]. No separation of charge variants was detectable after analysis with

IEF. A representative result from the pH 7.0 displacement run is shown in Fig. 2. Due to the disappointing results in all three displacement experiments, we decided to scale up our pH gradient elution method to a semi-preparative scale.

3.1.3. pH gradient elution

Finally preparative separation of the charge variants using pH gradient elution was used, lacking an orthogonal method for creating the standard. The initial mAb sample was purified as shown in Table 4 and acidic and main peak fractions were collected, as shown in Fig. 3(A). After concentration and buffer exchange of the eluate with ultra-/diafiltration, the standards were used in a second round of purification using a shallower gradient to remove the remaining unwanted charge variants. All three chromatograms can be seen in Fig. 3, including the fractionation that was used to obtain the pure standard material. It should be noted that the linearity of the pH gradients stays almost unaffected, even with higher loads of antibody. The gradients used can be found in Table 4.

The standards obtained this way will be referred to as main charge variant (MCV) and acidic charge variants (ACV). Because of non-orthogonality of the method used for creating the standard, IEF was used to confirm that the standard material indeed consisted of purified and separated charge variants. Fig. 4 shows the original mAb sample and both of the standards used in this work. No main band can be detected in the ACV sample, and only a faint band with a lower isoelectric point (pI) is visible in the MCV sample. It was concluded that these two samples will be of sufficient purity to validate the analytical method.

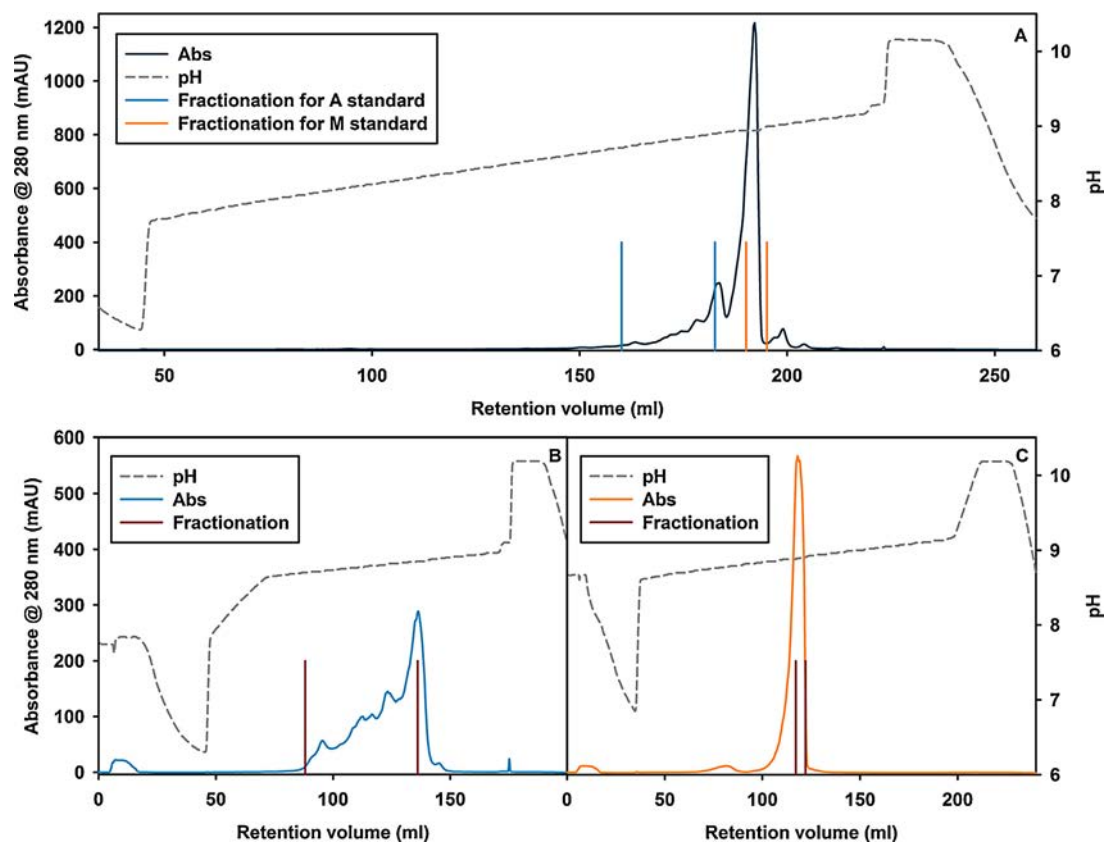


Fig. 3. Chromatograms of the preparative pH gradient elution separation of charge variants on a ProPac WCX-10, 9×250 mm column. Flow rate: 2.5 ml/min (236 cm/h). Mobile phase A: 5.5 mM HEPES, 4.2 mM Bicine, 9.5 mM CAPSO, 0.8 mM CAPS, 6.3 mM NaCl, pH 8.0, mobile phase B: 10.5 mM Bicine, 2.5 mM CAPSO, 7.0 mM CAPS, pH 10.5. Panel (A) shows the initial purification and fractionation for the acidic (blue) and the main (orange) standard. Gradient from pH 8.0 to 9.8 in 14 CV. After concentration and buffer exchange those fractions were used in a second round of purification to further increase the purity. Panels (B) and (C) show the purification and fractionation of the second round for acidic and main standard, respectively. For the acidic standard a 2 CV gradient from pH 8.0 to 8.9 was followed by a 10 CV gradient to pH 9.4. For the main standard a 10 CV gradient from pH 8.9 to 9.4 was used. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



Fig. 4. IEF gel of the initial mAb (O), the acidic charge variants (ACV) and the main charge variant (MCV). A faint band is visible with a pI lower than the main band in the MCV standard. No main band can be detected in the ACV standard.

3.2. Testing of the standard material

The purified MCV and ACV standards were analyzed using the analytical pH gradient elution method to check if the standards are of sufficient purity to be used for the validation and compared with the original mAb sample. A gradient from 35% to 55% buffer B (pH ~8.9–9.4, see Table 3 for composition) in 8 CV was used. This specific gradient was able to separate the charge variants, as shown in Fig. 5, while allowing to reduce the run time. Therefore, it was used for the whole validation. Both standards are of over 99% purity and elute at different times, as shown in Fig. 5C. It was concluded from these experiments that MCV and ACV will be suitable standard material for the subsequent validation experiments.

3.3. Calibration model

In order to confirm the linearity of the calibration model and to find the linear range of the method, spiking experiments were performed. MCV was spiked with ACV, to obtain relative abundances from 1% to 50% acidic species. The calibration model was tested by fitting a linear model to the data obtained with eight concentration levels evenly spaced across the calibration range with three replicates [19]. The results can be found in Table 5 and Fig. 6. The results of the linear regression gave a slope of 157.9 and an intercept of 143.0 with a correlation of regression of 0.9996. The residuals were equally (Fig. 7) distributed and the RSD was lower than 10%

Table 5
Calibration results.

ACV content (%)	Response (AU*s)	RSD (%)
0	97.2	7.7
1	280.2	6.7
3	632.2	1.4
5	945.3	0.3
10	1719.7	0.6
20	3406.9	0.6
40	6355.5	4.4
50	8079.8	1.0

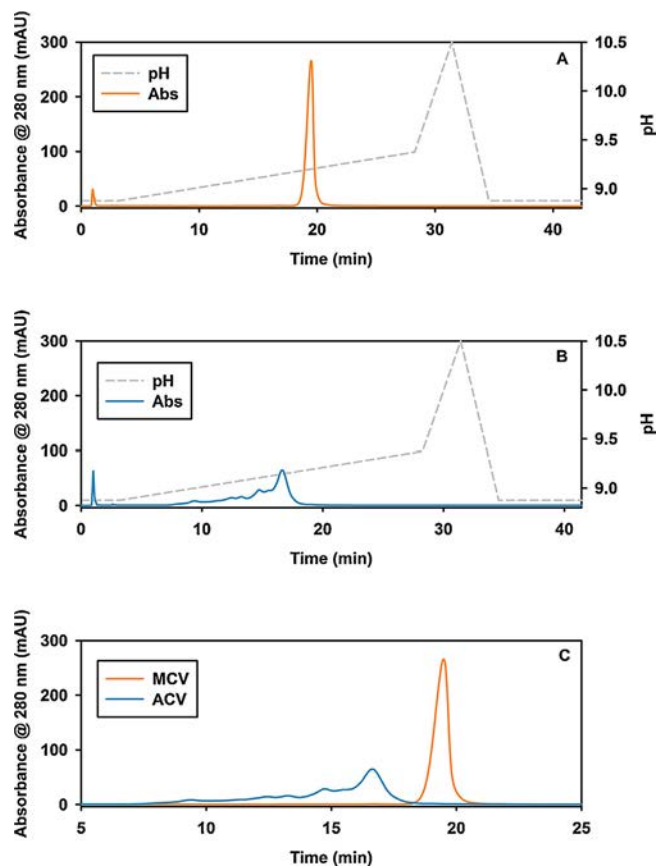


Fig. 5. Evaluation experiments for the suitability of the standard. The MCV (orange) and ACV (blue) standards were tested using the pH gradient elution method. Stationary phase: Dionex ProPac WCX-10, 4 × 250 mm. Flow rate: 1 ml/min (477.5 cm/h). Mobile phase A: 5.5 mM HEPES, 4.2 mM Bicine, 9.5 mM CAPSO, 0.8 mM CAPS, 6.3 mM NaCl, pH 8.0, mobile phase B: 10.5 mM Bicine, 2.5 mM CAPSO, 7.0 mM CAPS, pH 10.5. A gradient from 35% to 55% B in 8 CV was used. Panel (A) and (B) show the chromatograms of the MCV and ACV standard, respectively. The gray dashed line represents the pH at the column inlet, not a measured signal. Panel (C) shows the overlay of both chromatograms from 5 to 25 min. The samples are of high purity and are suited for the use as a charge variant standard. The small initial peak is a solvent peak, consisting of the histidine and Tween 20 of the mAb buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

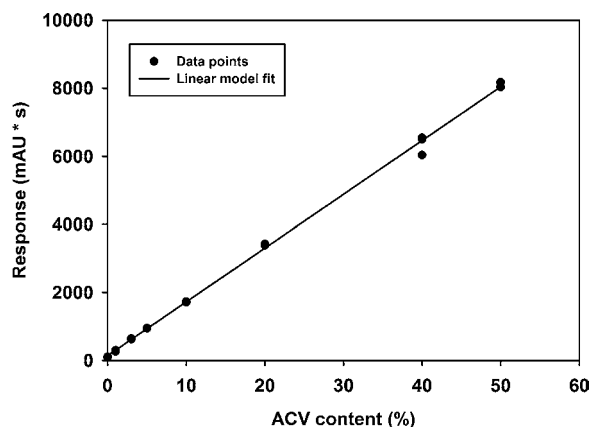


Fig. 6. The calibration curve for the acidic charge variant (ACV) content range of this study.

Table 6
Results of the accuracy and precision measurement.

Relative amount of spiked ACV (%)	Average relative charge variant content measured (%)	Bias (%)	Standard deviation	Relative standard deviation (%)
1	0.82	-17.6	0.07	8.9
25	25.37	1.5	0.38	1.5
50	49.96	-0.1	0.32	0.6

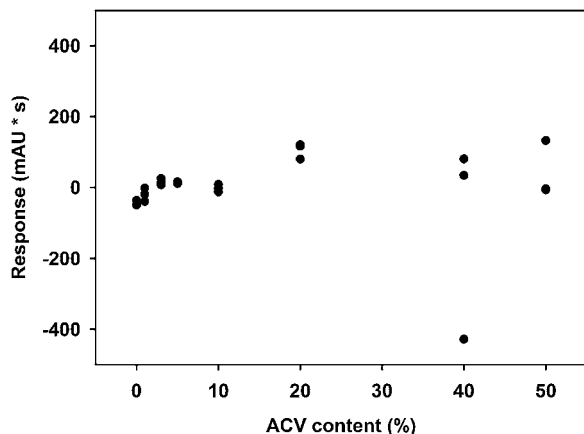


Fig. 7. Residuals of the calibration curve.

over the whole range. One value with an unusually large residual was observed, but Grubb's outlier test ($\alpha = 5\%$) determined that this was not an outlier [12]. ANOVA was used to confirm the validity of a linear calibration model, with the following results: linearity F test ($\alpha = 1\%$), $F^* = 16640.41 > F = 7.95$, which leads to the acceptance of linear model; lack of fit F test ($\alpha = 1\%$), $F^* = 1.23 < F = 4.20$, which leads to the acceptance of the null hypothesis of good model fit.

3.4. Accuracy and precision

The accuracy and bias were determined by the statistical evaluation of duplicate experiments performed on eight days at three different concentrations, as recommended by Peters et al. and Hartmann et al. [12,19]. The concentration levels selected were the LLOQ, the ULOQ and the median, i.e. 1%, 50% and 25% ACV, respectively. The precision was determined as the overall RSD, with acceptance criteria being 20% RSD for the lower limit of quantification and 15% for the other two spiking levels. The accuracy was determined as the deviation of the grand means from the respective reference values, with acceptance criteria being $\pm 20\%$ bias for the lower limit of quantification and $\pm 15\%$ for the other two spiking levels [19,28,29]. The results from measuring at the LLOQ, the median and the ULOQ can be seen in Table 6. Both the bias and the RSD are within the acceptance criteria. The bias at the LLOQ is rather high, with -17.6% , suggesting that quantification of charge variants below 1% in relative abundance will not be practical. This also served as confirmation of our estimation of the LLOQ for this method.

3.5. Selectivity

We assume that this method will be used for the determination of charge heterogeneity of purified monoclonal antibodies only, and as such the components that might be expected to be present in the samples analyzed are small molecules, such as buffering compounds, amino acids, sugars and detergents. As such, selectivity, "the ability to measure the analyte in the presence of components which might be expected" [19], has been shown previously [14].

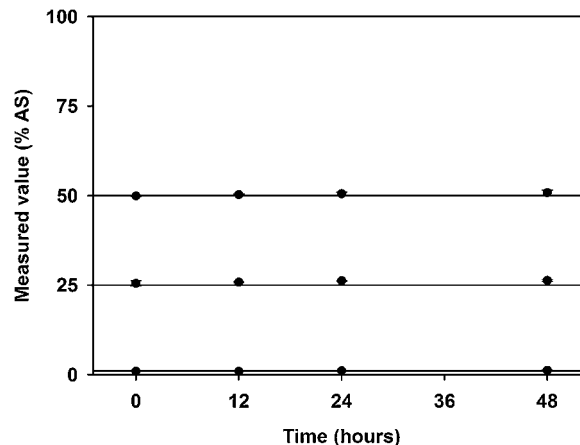


Fig. 8. Results from the stability experiments over 48 h. Samples with 1%, 25% and 50% ACV were measured in triplicates at 0, 12, 24 and 48 h.

3.6. Stability

The stability of mAb samples inside the autosampler, at 10°C , and the stability of the pH gradient buffers was tested over a time frame of 48 h. Samples were measured in triplicates at 0, 12, 24 and 48 h. The distribution of the measured values over time can be seen in Fig. 8. Those results demonstrated that the samples and the method are stable for 48 h and the method can therefore be used over night or over the weekend.

4. Conclusions

We presented the development of a highly linear pH gradient elution method in our previous work [14]. In the present complementary work, we have validated key method parameters, such as the accuracy and precision. The method uses commercially available stationary and mobile phase components, which allows others to implement this method easily. Other methods such as IEF provide a higher resolution than our method, but can only be considered as semi-quantitative method, cannot be automated as easily and cannot be scaled up to semi-preparative scale. The method for the quantification of mAb charge variants is valid for the quantification of purified mAb samples with a charge variant content as low as 1%. The linearity of the calibration range was confirmed through statistical testing and also showed an excellent coefficient of correlation of 0.9996. 1% charge variants was confirmed as the LLOQ, with an accuracy (bias) of -17.6% and a precision of 8.9%. For samples measured at the median or the ULOQ, the method had excellent accuracy and precision of below 2%. We have previously presented methods for the creation of gradients from pH 7.0 to pH 10.5. The big advantage of our method is that the linearity of the gradient is still high even when a narrower gradient is being used to increase the resolution. This means that our method can be easily customized for the analysis of different mAb, just by varying the initial and the final buffer concentration. In this study, we have used an antibody with a chromatographic pI of 9.2 and have adjusted our method gradient from 35% to 55% buffer B accordingly, which corresponded to a pH gradient from 8.9 to 9.4.

Compared to iCIEF, our method shows comparable precision [23], but no thorough validation could be found in which accuracy of iCIEF was determined. We conclude that our method is comparable to other standard methods. The biggest difficulty in performing this study was the production of the standard material. Our attempts at using standard salt gradient elution cation exchange chromatography proved unfruitful. We were unable to find conditions under which we had a sufficient resolution with an adequate protein load. The approach presented by Rozhkova [21], i.e. cation exchange chromatography combined with salt gradient elution, only worked for very small loading amounts for our mAb and additionally did not achieve the resolution we observed in pH gradient elution experiments. Unfortunately Rozhkova does not present analytical data of her standard material as well as more detailed information about loading amounts. Accordingly it is difficult to compare the two methods.

Our next approach, displacement chromatography [17], did not yield a separation of charge variants, under any conditions. This is in stark contrast to the results that Zhang et al. [18] have presented previously. Eventually, we used a preparative pH gradient elution method to prepare our standards, and confirmed their identity as charge variants by IEF. This approach proved to be highly effective in separating large amounts. It has previously been shown that induced pH gradients can be used for the preparative separation of mAb charge variants [30–32], and we are happy to report that our external pH gradients are able to achieve the same feat. Solubility problems due to elution close to the pI were not observed, even at the preparative scale used in this work, making this a high resolution method for the preparative separation of protein charge variants.

Acknowledgments

We would like to thank Dr. Anne Tscheliessnig for her help with the statistical analysis and Nikolaus Hammerschmidt and Peter Satzer for their critical review of the manuscript. This work was supported by the Austrian Research Promotion Agency (FFG). Nico Lingg was a fellow from the A*STAR Research Attachment Programme (ARAP). The collaboration between the Glyco-MEV, University of Rouen, France and BTI, A*STAR, Singapore was supported by the Merlion 2011 initiative program called Glyco-TOOLS. We would like to thank GE Healthcare for providing the Mono S columns used in this study.

References

- [1] J.G. Elvin, R.G. Couston, C.F. van der Walle, Therapeutic antibodies: market considerations, disease targets and bioprocessing, *Int. J. Pharm.* 440 (2013) 83–98.
- [2] M. Schuster, A. Nechansky, R. Kircheis, Cancer immunotherapy, *Biotechnol. J.* 1 (2006) 138–147.
- [3] N. Lingg, P. Zhang, Z. Song, M. Bardor, The sweet tooth of biopharmaceuticals: importance of recombinant protein glycosylation analysis, *Biotechnol. J.* 7 (2012) 1462–1472.
- [4] A.M. Goetze, M.R. Schenauer, G.C. Flynn, Assessing monoclonal antibody product quality attribute criticality through clinical studies, *mAbs* 2 (2010) 500–507.
- [5] H. Liu, G. Gaza-Bulseco, D. Faldu, C. Chumsae, J. Sun, Heterogeneity of monoclonal antibodies, *J. Pharm. Sci.* 97 (2008) 2426–2447.
- [6] M.M. van Beers, M. Bardor, Minimizing immunogenicity of biopharmaceuticals by controlling critical quality attributes of proteins, *Biotechnol. J.* 7 (2012) 1473–1484.
- [7] N. Hammerschmidt, A. Tscheliessnig, R. Sommer, B. Helk, A. Jungbauer, Economics of recombinant antibody production processes at various scales: industry-standard compared to continuous precipitation, *Biotechnol. J.* 9 (2014) 766–775.

- [8] T. Barroso, A. Hussain, A.C. Roque, A. Aguiar-Ricardo, Functional monolithic platforms: chromatographic tools for antibody purification, *Biotechnol. J.* 8 (2013) 671–681.
- [9] S.M. Cramer, M.A. Holstein, Downstream bioprocessing: recent advances and future promise, *Curr. Opin. Chem. Eng.* 1 (2011) 27–37.
- [10] R. Sommer, P. Satzer, A. Tscheliessnig, H. Schulz, B. Helk, A. Jungbauer, Combined polyethylene glycol and CaCl₂ precipitation for the capture and purification of recombinant antibodies, *Process Biochem.* 49 (2014) 2001–2009.
- [11] A. Tscheliessnig, P. Satzer, N. Hammerschmidt, H. Schulz, B. Helk, A. Jungbauer, Ethanol precipitation for purification of recombinant antibodies, *J. Biotechnol.* 188C (2014) 17–28.
- [12] C. Hartmann, J. Smeyers-Verbeke, D.L. Massart, R.D. McDowall, Validation of bioanalytical chromatographic methods, *J. Pharm. Biomed. Anal.* 17 (1998) 193–218.
- [13] ICH, ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology, Q2(R1), ICH, Geneva, Switzerland, 2005 http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf
- [14] N. Lingg, E. Tan, B. Hintersteiner, M. Bardor, A. Jungbauer, Highly linear pH gradients for analyzing monoclonal antibody charge heterogeneity in the alkaline range, *J. Chromatogr. A* 1319 (2013) 65–71.
- [15] Y. Du, A. Walsh, R. Ehrick, W. Xu, K. May, H. Liu, Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies, *mAbs* 4 (2012) 578–585.
- [16] G. Carta, A. Jungbauer, *Protein Chromatography; Process Development and Scale-Up*, John Wiley & Sons, Inc., Weinheim, Germany, 2010.
- [17] C.P. McAtee, J. Hornbuckle, Isolation of monoclonal antibody charge variants by displacement chromatography, *Curr. Protoc. Protein Sci.* (2012), 69:8.10:8.10.1–8.10.13.
- [18] T. Zhang, J. Bourret, T. Cano, Isolation and characterization of therapeutic antibody charge variants using cation exchange displacement chromatography, *J. Chromatogr. A* 1218 (2011) 5079–5086.
- [19] F.T. Peters, O.H. Drummer, F. Musshoff, Validation of new methods, *Forensic Sci. Int.* 165 (2007) 216–224.
- [20] A. Tscheliessnig, A. Jungbauer, High-performance monolith affinity chromatography for fast quantitation of immunoglobulin G, *J. Chromatogr. A* 1216 (2009) 2676–2682.
- [21] A. Rozhkova, Quantitative analysis of monoclonal antibodies by cation-exchange chromatofocusing, *J. Chromatogr. A* 1216 (2009) 5989–5994.
- [22] C.L. Anderson, Y. Wang, R.R. Rustandi, Applications of imaged capillary isoelectric focussing technique in development of biopharmaceutical glycoprotein-based products, *Electrophoresis* 29 (2012) 1538–1544.
- [23] Z. Susic, D. Houde, A. Blum, T. Carlage, Y. Lyubarskaya, Application of imaging capillary IEF for characterization and quantitative analysis of recombinant protein charge heterogeneity, *Electrophoresis* 29 (2008) 4368–4376.
- [24] O. Salas-Solano, B. Kennel, S.S. Park, K. Roby, Z. Susic, B. Boumajny, S. Free, A. Reed-Bogan, D. Michels, W. McElroy, P. Bonasia, M. Hong, X. He, M. Ruesch, F. Moffatt, S. Kiessig, B. Nunnally, Robustness of iCIEF methodology for the analysis of monoclonal antibodies: an interlaboratory study, *J. Sep. Sci.* 35 (2012) 3124–3129.
- [25] R. Westermeier, *Electrophoresis in Practice: A Guide to Methods and Applications of DNA and Protein Separations*, John Wiley & Sons, Inc., Weinheim, Germany, 2004.
- [26] A. Gorg, C. Obermaier, G. Boguth, A. Csordas, J.J. Diaz, J.J. Madjar, Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins, *Electrophoresis* 18 (1997) 328–337.
- [27] L.A. Khawli, S. Goswami, R. Hutchinson, Z.W. Kwong, J. Yang, X. Wang, Z. Yao, A. Sreedhara, T. Cano, D. Tesar, I. Nijem, D.E. Allison, P.Y. Wong, Y.H. Kao, C. Quan, A. Joshi, R.J. Harris, P. Motchnik, Charge variants in IgG1: isolation, characterization, in vitro binding properties and pharmacokinetics in rats, *mAbs* 2 (2010) 613–624.
- [28] V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Bioanalytical method validation—a revisit with a decade of progress, *Pharm. Res.* 17 (2000) 1551–1557.
- [29] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies, *Pharm. Res.* 09 (1992) 588–592.
- [30] T.A. Vetter, G. Ferreira, D. Robbins, G. Carta, Mixed-beds of strong and weak anion exchange resins for protein separations with step-induced pH gradients, *Sep. Sci. Technol.* 49 (2014) 477–489.
- [31] T.A. Vetter, G. Ferreira, D. Robbins, G. Carta, Predicting retention and resolution of protein charge variants in mixed-beds of strong and weak anion exchange resins with step-induced pH gradients, *Sep. Sci. Technol.* 49 (2014) 1775–1786.
- [32] T.M. Pabst, G. Carta, N. Ramasubramanian, A.K. Hunter, P. Mensah, M.E. Gustafson, Separation of protein charge variants with induced pH gradients using anion exchange chromatographic columns, *Biotechnol. Prog.* 24 (2008) 1096–1106.