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Received March 31, 2017
Revised August 5, 2017
Accepted August 5, 2017

Research Article

Optimization of capillary zone electrophoresis for charge heterogeneity testing of biopharmaceuticals using enhanced method development principles

CZE is a well-established technique for charge heterogeneity testing of biopharmaceuticals. It is based on the differences between the ratios of net charge and hydrodynamic radius. In an extensive intercompany study, it was recently shown that CZE is very robust and can be easily implemented in labs that did not perform it before. However, individual characteristics of some examined proteins resulted in suboptimal resolution. Therefore, enhanced method development principles were applied here to investigate possibilities for further method optimization. For this purpose, a high number of different method parameters was evaluated with the aim to improve CZE separation. For the relevant parameters, design of experiments (DoE) models were generated and optimized in several ways for different sets of responses like resolution, peak width and number of peaks. In spite of product specific DoE optimization it was found that the resulting combination of optimized parameters did result in significant improvement of separation for 13 out of 16 different antibodies and other molecule formats. These results clearly demonstrate generic applicability of the optimized CZE method. Adaptation to individual molecular properties may sometimes still be required in order to achieve optimal separation but the set screws discussed in this study [mainly pH, identity of the polymer additive (HPC versus HPMC) and the concentrations of additives like acetonitrile, butanolamine and TETA] are expected to significantly reduce the effort for specific optimization.

Keywords:
Capillary zone electrophoresis / Charge heterogeneity testing / Design of experiments / Monoclonal antibody
DOI 10.1002/elps.201700145

1 Introduction

During recent years protein based biopharmaceuticals like antibodies, new bispecific formats or fusion proteins became very important remedies for cancer therapies [1], for treating malfunctions of the immune system [2], or for treatment of genetic defects [3]. For quality control of these molecules, robust analytical techniques are needed to obtain information about stability trends and consistency of the production process.

Quality attributes of protein pharmaceuticals can be classified into several different groups. One important group comprises modifications that alter the charge profile of the molecule. By deamidation [4] a carboxylic group is generated which increases acidity. In contrast, aspartate-glycine motives are prone to succinimide formation [5] that increases the p*I* of the affected species. Also many other modifications like glutamine/pyroglutamate conversion [6], sialylation of glycan structures [7], C-terminal lysine heterogeneity [8] or oxidation of disulfide bonds [9] can influence the charge profile.

For ensuring comprehensive quality assessment, understanding of these critical quality attributes (CQAs) is important. They have to be identified and kept within acceptance criteria that ensure therapeutic efficacy and patient safety.

CE is a separation technique which has become an important and well accepted tool for fragment analysis (CE-SDS, [10]) and charge heterogeneity testing (CZE [11], [12] and IEF [13]) of biopharmaceuticals. Today, these techniques are well established in the Quality Control (QC) environment of most biopharmaceutical companies. They are used for release and stability testing under good manufacturing procedure (GMP), and also for process characterization / process validation studies (PC/PV) and for formulation development. CE is well accepted by regulatory agencies (European

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Abbreviations: CQA, critical quality attribute; **DoE**, design of experiments; **EACA**, ε-amino-caproic acid; **HPC**, hydroxypropyl cellulose; **HPMC**, hydroxypropyl methyl cellulose; **IEF**, isoelectric focusing; **MAb**, monoclonal antibody; **QC**, quality control; **TETA**, triethylenetetramine; **USP**, united states pharmacopoeia

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Colour Online: See the article online to view Figs. 1, 4–6 in colour.

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Pharmacopoeia (EP) or US Pharmacopoeia (USP)). The present study is intended to deepen the understanding of CZE.

CZE can be used for the separation of ions [14], small molecules [15], peptides [16], proteins [11] and carbohydrates [17]. The capillary is filled with a specific separation matrix (also known as background electrolyte or BGE); afterwards the sample is injected and separated within an electric field [18]. The separation is based on different charge to hydrodynamic radius ratios of the analytes that result in different migration velocities and splitting of the analytes in discrete zones. In case of EOF-driven separation CZE separates both anionic and cationic solutes into the same direction towards the detection window. Even neutral solutes flow to the detection window but are not separated. For proteins polarity is usually positive at the capillary inlet and negative at the capillary outlet with the detection window.

A well suited CZE method for charge heterogeneity testing of biopharmaceuticals was described in literature [11]. This method uses 400 mM EACA (ε-amino-caproic acid), 2 mM of triethylenetetramine (TETA) and 0.05% of hydroxypropyl methyl cellulose (HPMC) with a pH of 5.7 as BGE. UV detection is performed at 214 nm. Charges of protein analytes are mainly carried by carboxyl and primary amino groups. At pH 5.7 the carboxyl groups (pKa around 4-4.5) are deprotonated and negatively charged, whereas the primary amino groups are still positively charged (pKa around 10). That means that at pH 5.7 protein analytes are zwitter-ionic. pH changes of the BGE may have an influence on the overall net charge of investigated compounds and should be considered for method optimization.

EACA is a zwitter-ionic acid. Except for a small portion (pKa: 4.43 (-COOH), 10.75 (-NH2)) it is neutral at pH 5.7 (according to Henderson–Hasselbalch [19]). Therefore, reduction of analyte interaction with the capillary wall by a high concentration of EACA can be enabled without detrimental Joule heating. In addition, positively charged TETA interacts with the negatively charged silanolate of the capillary wall (dynamic coating) and thereby reduces EOF and also interaction of the analytes with the capillary wall (reduced peak tailing). In summary, TETA and EACA improve the quality of CZE separations. Low UV absorption of the background electrolyte does enable high sensitivity detection at 214 nm (due to high ampholyte background absorption low UV detection is not possible for IEF).

In spite of very successful separations with this method there were still resolution problems with some protein pharmaceuticals. This observation motivated a selection of relevant method parameters followed by DoE studies. DoE is a computer aided procedure to approximate the relationship between parameters that influence CZE separation and the separation results. This approach originates from the work of R.A. Fisher in the early 20th century [20]. Resulting cause-and-effect relationships can be used to optimize CZE separation by adjusting relevant parameters for response optimization [21].

The 'one parameter a time' approach alters only one parameter per time while holding all the other parameters constant. However, this approach does not show the interaction between different parameters. In order to gain a more comprehensive view on the interaction between parameters, a simultaneous variation is recommended. For an efficient setup most relevant parameters should be selected in a first step.

The steps to perform a successful DoE are typically described by two distinct phases: the screening phase is used to select the most relevant parameters. Afterwards, for the second DoE phase, the experimental setup, i.e. the range and spatial arrangement of relevant parameters should be defined. The number of dimensions of this arrangement depends on the number of parameters. In case of three parameters the parameter combinations can be depicted as points within (center point) and on the surface of a three dimensional cube. The number of surface points that is required for enabling a meaningful cause-and-effect matrix depends on the interaction between the parameters. If there is no interaction, fractional factorial designs are sufficient (only half of the cube corners are included). Linear interaction between the parameters requires a full factorial design (all cube corners are included), whereas a quadratic interaction between the parameters requires composite designs (all cube corners and all center points of the cube areas are included) [21].

CZE DoEs were performed with selected parameter combinations. Responses like resolution or number of peaks were obtained from these experiments. Parameters and responses were then used as input variables for mathematical regression analyses and allowed the approximation of a multidimensional equation for parameter response interaction. In this study we used 16 antibodies, new bispecific formats or fusion proteins for the verification of the obtained models.

2 Materials and methods

CZE separations were performed with a SCIEX PA800plus System (Brea, CA, USA) that was equipped with a UV detector, a 214 nm filter (cat.no. 144437, SCIEX), a temperature controlled auto sampler (±2°C), and a 30 kV power supply.

Optimization of CZE separations was started according to the published standard method [11]: before each run capillaries were rinsed with 0.1 M HCl (acidic wash solution; cat. no. 109973, Merck KGaA, Darmstadt, Germany) for 1 min (60 psi) and subsequently for 1 min (50 psi) with the separation buffer (400 mM EACA (cat. no. A-7824, Sigma-Aldrich), 2 mM of TETA (cat. no. H-7509, Sigma-Aldrich) and 0.05% of HPMC (cat. no. 90460, Sigma-Aldrich) adjusted to pH 5.7 by acetic acid (cat. no. 45730, Merck)). The monoclonal antibody (MAb) samples (F. Hoffmann La Roche, Basel, Switzerland) were pre-diluted to 1 mg/mL or 3.5 mg/mL with water (treated with Milli-Q). 100 μL of each sample were transferred into PCR autosampler vials. Samples were injected into a bare fused silica capillary (Beckman Coulter cat. no. TSP040375) using a pressure of 0.5 psi for 10 s. Polarity was positive

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(capillary inlet) to negative (capillary outlet). Instrument control, data acquisition and data evaluation were performed with 32 Karat 10.1 software (SCIEX, Brea, CA, USA).

Selection of relevant parameters for subsequent DoE designs was performed by modification of the originally published method [11] (often by structurally similar compounds with in case of buffer compounds slightly different pKa values): ε-amino-capronic acid was replaced by 5-amino-valeric acid (cat.no. 123188, Sigma-Aldrich), β-alanine (cat.no. 05160, Sigma-Aldrich), 4-methylamino benzoic acid (cat.no. 119695, Sigma-Aldrich) or phosphate buffer (cat.no. 72883, Sigma-Aldrich). TETA was substituted by tetraethylene-pentamine (cat.no. T11509, Sigma-Aldrich), poly(ethyleneimine)solution (cat.no. 482595, Sigma-Aldrich) or hexadimethrine bromide (cat.no. H9268, Sigma-Aldrich). HPMC was replaced by HPC (cat. no. 191892, Sigma-Aldrich). In order to account for different stacking effects sample pre-dilution was performed in water or tris aminomethane (cat.no. 252859, Sigma-Aldrich). Original pH of 5.7 was varied in the range of 4.7 to 7.3. Alternatively an amine coated capillary was tested (cat.no. 477431, SCIEX). The capillary length from inlet to detector window/outlet was 20/30 cm, 30/40 cm or 40/50 cm. The inner diameter was 40 or 50 μm. The capillary temperature was adjusted to 15, 20, 30, or 40°C. The separation voltage was adjusted to 5, 10, 15, 20, or 30 kV. Different additives were tested: 1-aminohexane (cat.no. 53130, Sigma-Aldrich), 6-aminohexanoic acid (cat. no. A-7824, Sigma-Aldrich), acetonitrile (cat. no. 100030, Merck), ammonium chloride (cat.no.: 1.01143.0050, VWR International AG), butanolamine (cat. no. 07191, Sigma-Aldrich St. Louis, MO, USA), diaminohexane (cat.no. 8.04323, Merck), ethanolamine (cat.no. 141-43-5, BASF), hexanolamine (cat.no. 208673, Fluorchem), HPC (cat. no. 191892, Sigma-Aldrich), L-2,4-diaminobutyric acid (cat.no. 32830, Sigma-Aldrich), phosphate buffer (cat.no. 72883, Sigma-Aldrich), propanolamine (cat.no. A76400, Sigma-Aldrich), sodium chloride, tetrahydrofuran (cat.no. 1.09731, Merck), trifluoroacetic acid (cat.no. 302031, Sigma-Aldrich), Tween 20 (cat.no.93773. Sigma-Aldrich), and Urea (cat.no. 1.08488, Merck).

DoE optimization was performed with MODDE (version 11.0.1; MKS Umetrics; Umeå, Sweden). Different method parameters (parameters) (e.g. pH of BGE) were correlated with their responses (e.g. resolution). This allowed the prediction of responses and optimization of parameters for better CZE separation.

3 Results and discussion

3.1 Parameter selection

CZE optimization started with the screening phase for selection of relevant parameters. For this purpose one parameter per time was modified and was compared with the original method according to He et al., 2011 [11]. Altogether 40 different parameters were tested during pre-screening (most important parameters are shown in Table 1). Due to the high number of investigated factors the one factor per time approach within the ranges in Table 1 was applied. The ranges were limited and did not allow for a further statistical evaluation but clearly demonstrated the relevance of each single parameter for CZE separation. This allowed a more efficient setup of subsequent elaborate DoE studies (Table 2) with crucial parameters only. In the scope of this article, only the results for the most crucial parameters can be shown (results are presented in Fig. 1). The effects by these parameters may be explained as follows:

(1) The addition of charged additives, such as ammonium chloride, sodium chloride and butanolamine resulted in an improved resolution and in slower movement of the analytes (Fig. 1I; Fig. 1V). This means that the current increase by these additives (lower resistance) is higher than the overall current increase. Improved resolution may be attributed to reduced EOF, altered hydrodynamic radius and to reduced interaction of the analytes with the capillary wall.
(2) Acetonitrile can act as solvent for hydrophobic parts of the analyte. Reduction of unwanted hydrophobic interactions between different charge species may explain the better resolution in the presence of moderate amounts of acetonitrile (Fig. 1II). However, altered conductivity or altered solubility may explain a decrease of resolution in the presence of higher amounts of acetonitrile.
(3) The pH value influences the charge to hydrodynamic radius ratio that directly determines the analyte's mobility and migration velocity. Therefore, it has a strong effect on the separation profile (Fig. 1III, [11]); by increasing the pH, the number of positive charges decreases while the higher relative portion of negative charges reduces the analyte's velocity towards the negative cathode (Fig. 1VI).
(4) In case of more basic MAb1 (pI∽9.3), substantial improvement could be achieved by replacement of HPMC by HPC (Fig. 1IV). In comparison to HPC HPMC has additional multiple methyl groups in its polymeric core structure. This explains its higher viscosity (2500–5000 cP) in comparison to that of HPC (150–400 cP) (online catalogue information from Sigma Aldrich). On the one hand, HPMC causes a prolonged migration time. On the other hand, lower resolution may be caused by a deterioration of the charge based separation by increased polymeric sieving. In this study, it was observed that basic antibodies are more affected by this effect. That may result from their faster migration in combination with an intensified deterioration effect.
(5) The variation of the TETA concentration has a big influence on the separation quality as well (not shown in Fig. 1; see also [11]). As discussed before, positively charged TETA interacts with the negatively charged silanolates of the capillary wall (dynamic coating) and thereby reduces EOF and also interaction of the analytes

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Table 1. Parameters that were tested for parameter pre-screening (for several variants also the pH value was varied in addition). Not all of these parameters did result in promising pre-screening results. Only most promising parameters, i.e. replacement of HPMC by HPC, variation of TETA and EACA concentrations, variation of pH and addition of butanolamine were chosen for final DoE evaluation

EACA [mM]	TETA [mM]	HPMC [%]	Modification
400	2	0.05	Addition of 20, 40, and 60 mM 6-aminohexanol
400	2	0.05	Addition of 5, 10, 20% acetonitrile
400	2	0.05	Addition of 5, 10, 20% tetrahydrofuran
400	2	0.05	Addition of 0.05% of TWEEN 20
400	2	0.05	Addition of 10 mM, 20 mM, 30 mM L-2,4-diaminobutyric acid dihydrochloride
400	2	0.05	Addition of 0.05% of hydroxypropyl cellulose
400	2	0.05	Addition of 5, 10, 20% of trifluoroacetic Acid
400	2	0.05	Addition of 10, 100 mM UREA
400	2	0.05	Addition of 10, 20, 30 mM ammonium Chloride
400	2	0.05	Addition of 10, 20, 30 mM ethanloamine
400	2	0.05	Addition of 10, 20, 30 mM propanolamine
400	2	0.05	Addition of 10, 20, 30 mM butanolamine
400	2	0.05	Addition of 10, 20, 30 mM diaminohexane
400	2	0.05	Addition of 10, 20, 30 mM 1-aminohexane
400	2	0.05	Addition of 10, 20, 30 mM sodium chloride
400	2	0.05	Addition of 10, 20, 30 mM hexanolamine + 5, 10, 20% acetonitrile
400	2	0.05	Addition of 5, 10, 20, 30 mM TRIS(hydroxyl-methyl) aminomethane
400	2	0.05	Addition of 4, 40% of phosphate buffer
400	-	0.05	2 mM spermidine instead of TETA
400	-	0.05	2 mM spermine instead of TETA
-	2	0.05	400 mM 5-amino -valeric acid instead of EACA
400	-	0.05	2 mM of tetraethylene-pentamine instead of TETA
400	-	0.05	-
-	2	0.05	400 mM β-alanine instead of EACA
400	2	-	0.05% of hydroxypropyl cellulose instead of HPMC
400	-	0.05	0.0002, 0.002, 0.01, 0.02, 0.07, 0.1, 0.2% poly(ethyleneimine)solution instead of TETA
400	-	0.05	2 mM, 5 mM TRIS(hydroxyl-methyl)aminomethane instead of TETA
400	-	0.05	0.0002, 0.002, 0.005, 0.01, 0.02% hexadimethrine bromide instead of TETA
-	2	0.05	4, 40% phosphate buffer instead of EACA

with the capillary wall. This reduces peak tailing and improves the resolution of CZE separations.

3.2 Design of experiments

On the basis of the first parameter screen and the originally published method according to He et al., 2011 [11] the study proceeded with DoE studies. Thereby, interaction of critical parameters was investigated. In total, 13 DoE studies with 136 combinations of 40 parameters were performed. Only the most relevant of the obtained results can be discussed in the context of this manuscript.

For DoE modelling 14 responses were correlated with the parameters discussed in Section 3.1. Highest values were preferred for the '*Total Number of Peaks'*, the '*Average of USP Resolutions'* (ratio of the sum of the resolution and the total number of peaks) and the '*Sum of the USP Resolutions*', whereas smallest values were preferred for the '*Width of main peak'* (was calculated at the base of the component peak by the tangent method), the '*USP width of main Peak*' according to United States Pharmacopeia and the '*Corrected Area Percent of the main peak*'. For QC testing a low value for '*Corrected Area Percent of the main peak*' is considered to be the optimum, because under these conditions there is an optimal separation of impurities or related substances. Some qualitative responses were included that represent the analyst's view of the separation quality and should result in highest scoring values between 0 and 5: '*Qualitative Response for the Number of Peaks*', '*Qualitative Response for the Shape of Peaks*', '*Qualitative Response for the Resolution of Peaks*' and '*Qualitative Response*' (overall evaluation). These additional parameters were added in order to compensate for observations like triangular peaks by electrodispersion, unacceptable separation in some parts of the electropherogram, baseline fluctuations/drifts and/or method artifacts that cannot be covered by the other quantitative responses. Values of 0, 3 and 5 mean very low, middle-rate and excellent quality. Values 2 and 4 allow for further grading in between. Qualitative grading is performed for visual corroboration of DOE modelling. Since some variations showed an improvement of either acidic or basic forms the '*number of peaks'* and the '*sum of USP resolutions'* were also calculated separately for the acidic and the basic region.

For MAb1 (IgG4, pI ~ 9.3) the effect of the pH value (5.4, 5.7 and 6.0), of the acetonitrile concentration (0, 3, and 6% v/v), of the TETA concentration (1, 3, and 5 mM) and the

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Table 2. Setup of DoE studies that resulted in the finally proposed '*compromise*' and '*highest number of peaks*' methods for MAb1 and the '*compromise*' method for MAb2

Fixed Parameters:	Values:	Factors for DoE testing:	Ranges:
MAb1			
Concentration of sample	3.5 mg/mL	pH Value	5.4 – 5.7 – 6.0
Concentration of EACA	400 mM	Concentration of acetonitrile	0–3 –6%
Concentration of HPC	0.05%	Concentration of TETA	1 mM–3 mM– 5 mM
Length of the Capillary	30/20 cm	Concentration of butanolamine	0 mM– 30 mM– 60 mM
Temperature	20°C		
Voltage	30 kV		
MAb2			
Concentration of sample	3.5 mg/mL	pH Value	5.5 – 6.2 – 6.7
Concentration of EACA	400 mM	Concentration of acetonitrile	0– 2.5– 5%
Concentration of HPMC	0.05%	Concentration of TETA	1 mM– 2 mM– 3 mM
Length of the Capillary	30/20 cm	Concentration of butanolamine	0 mM– 30 mM– 60 mM
Temperature	20°C		
Voltage	30 kV		

butanolamine concentration (0, 30 and 60 mM) was investigated in a full factorial design using Resolution V+® software (according to MODDE, MKS Umetrics; Umeå, Sweden) (Table 2). The MAb concentration (3.5 mg/mL), the EACA concentration (400 mM), the HPC concentration (0.05% w/v), the capillary length (30/20 cm), the capillary temperature (20°C) and the separation voltage (30 kV) were fixed. Due to pre-screening results HPMC according to He et al. [11] was replaced by HPC. After complementation with some extra separations (as proposed by the software; central composite design: $2^n+nx2+3 \rightarrow$ with 4 factors: $16+8+3 = 27$ (+ 1 Center Point = 28), 28 combinations of the four varied parameters were available. The resulting fitting parameters for most relevant parameters like number of peaks and resolution showed that the obtained model for correlation of parameters and responses was good and reliable. For these parameters the goodness of fit was around 0.95, goodness of prediction was around 0.9 and reproducibility was around 1 (according to MODDE (version 11.0.1; MKS Umetrics; Umeå, Sweden).

The coefficient plots for different responses (data not shown) demonstrated that the TETA concentration, the pH value, the acetonitrile and butanolamine concentrations and/or their combinations have an influence on the separation profile.

Subsequently, two different mathematical optimizations of the CZE method parameters were performed by the definition of acceptable ranges for relevant responses and by different weighting of these responses in the range of 0 to 1 (Table 3): one with the goal of improving all the 14 responses listed above by higher weighting of most relevant responses (highest weighting factor of 1 for the '*number of peaks*', the '*number of acidic peaks*', the '*number of basic peaks*', the '*sum of USP resolutions*' and the '*overall qualitative response*') and one to get only the highest number of peaks without focusing on the other responses (weighting of 1 only for the '*number of peaks*', the '*number of acidic peaks*' and the '*number of basic peaks*'). Both strategies were followed since on the one hand a higher number of peaks may resolve further relevant charge species and on the other hand inclusion of more responses is expected to result in a more robust method.

The obtained model predicted different sets of parameter for the two optimization strategies. The strategy that considered all responses resulted in the so called '*compromise method*': 400 mM EACA, 4 mM of TETA, 0.05% HPC, 24 mM of butanolamine and 0.4% of acetonitrile at a final pH of 5.7. The strategy that considered the number of peaks only resulted in the so called '*highest number of peaks method*': 400 mM EACA, 4.4 mM TETA, 0.05% HPC, 47 mM of butanolamine and 6% acetonitrile at a final pH of 6.0.

The DoE contour plots presented, reflect the most important responses (Fig. 2). The TETA concentration is plotted on the x-axis of the contour plots, whereas the butanolamine concentration is always plotted on the y-axis. The colors represent the scale of each response (third dimension) whereby red is the highest number and blue is the lowest (response ranges are also shown in Table 3). The response contour plots for the '*number of peaks*', the '*sum of USP resolutions*', the '*qualitative response (resolution)*', the '*width of main peak*', the '*number of acidic peaks*', and the '*number of basic peaks*' are shown in Fig. 2I–VI, respectively. The gray shaded range of the plots including TETA are actually out of the investigated range and extrapolated. The black stars indicate the position of the '*compromise method*', the white stars the position of the '*highest number of peaks method*'. In order to obtain the profiles of three different acetonitrile concentrations (0, 3, and 6%) and three different pH values (5.4, 5.7 and 6), a total of 3×3, i.e. 9, contour plots are required for each response. This enables a parallel presentation of response values in dependence of four parameters.

The graphs in vertical direction show that, at 3% acetonitrile, the number of acidic peaks is lower. Changes of response values in horizontal direction arise from the pH of the separation buffer. An increasing pH value results in a higher number of peaks (graph becomes more red) and in a lower resolution (graph becomes more orange). The pH has almost no influence on the qualitative response and the

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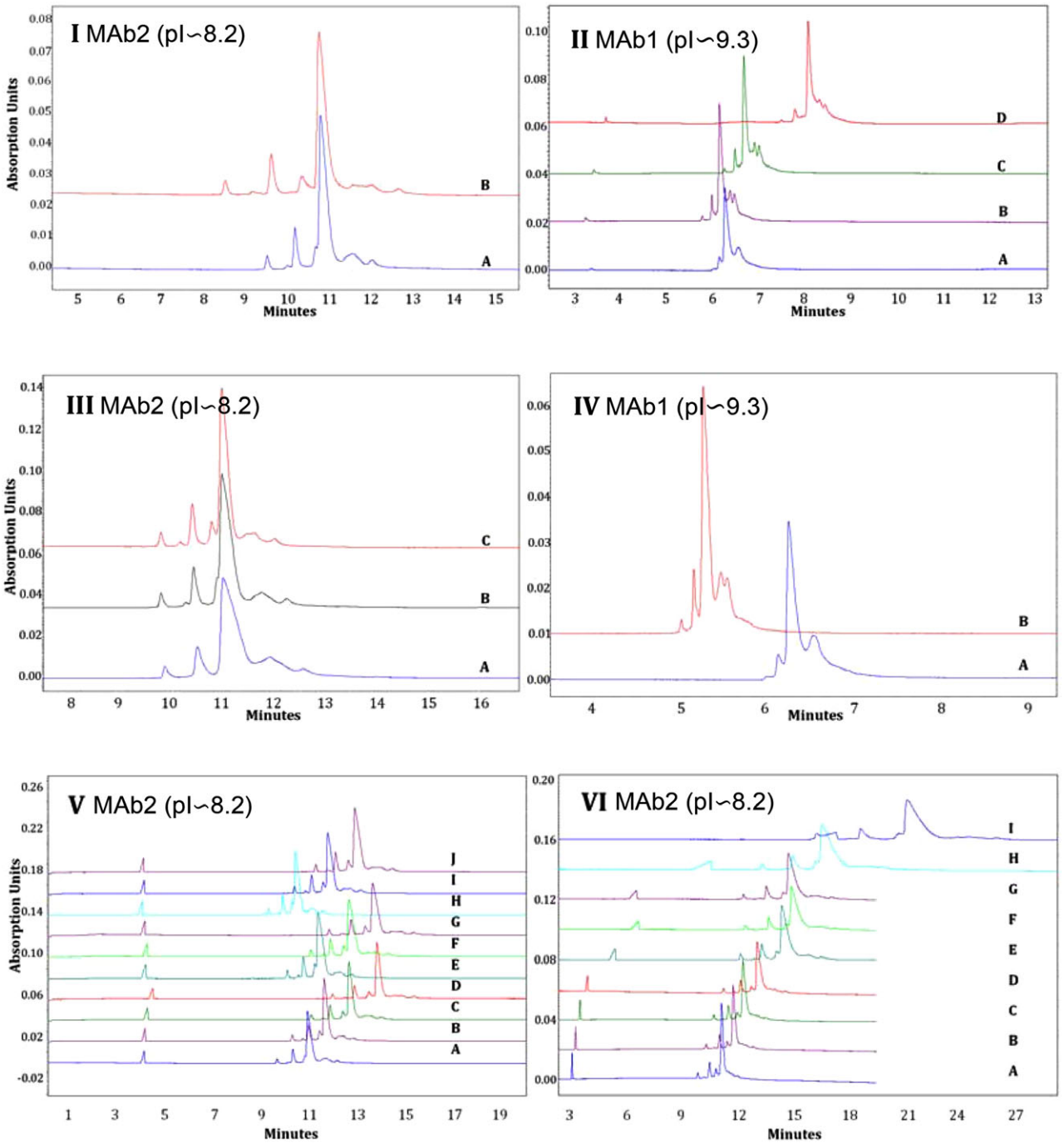


Figure 1. (I) Antibody MAb2 (pI∽8.2) before (A) and after (B) addition of 30 mM Butanolamine; electropherogram B was shifted in order to match main peaks and to focus on differences in resolution. (II) CZE separation of MAb1 (pI∽9.3) in the presence of different levels of acetonitrile [0% (A), 5% (B), 10% (C) and 20% (D)] in the separation buffer. (III) Electropherogram of antibody MAb2 (pI∽8.2) at different pH values of the separation buffer; pH value of 6.4 (A), 5.7 (B) and 5.4 (C). Electropherograms B and C were shifted in order to match main peaks and to focus on differences in resolution. (IV) Electropherogram of antibody MAb1 (pI ∽9.3) before (A) and after (B) replacement of 0.05% HPMC by 0.05% HPC. (V) Separation of MAb2 (pI∽8.2) with different charged additives. From the bottom to top: published method (He et al., 2011) [A], addition of 10 mM, 20 mM and 30 mM of butanolamine [B,C,D], sodium chloride [E, F, G] and ammonium chloride [H, I, J]. (VI) MAb2 (pI∽8.2), overlay of nine chromatograms obtained with separation buffer at different pH value but same composition. From the bottom, the pH was risen as follows: 5.1 – 5.3 – 5.5 – 5.7 – 6.1 – 6.3 – 6.5 – 6.9 – 7.3.

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Table 3. Overview on response factors for MAb1: The weighting factors, acceptable ranges for mathematical CZE parameter optimization (see also Fig. 3) and ranges predicted by the obtained model (see also Fig. 2) are shown. (A) parameter weighting for the 'compromise method'; (B) parameter weighting for the 'highest number of peaks method'

response	weight		acceptable range			predicted by the model	
	A	B	min	target	max	min	max
number of peaks	1	1	6	9	-	1	9
number of acidic peaks	1	1	3	5	-	0	6
number of basic peaks	1	1	2	3	-	0	3
sum of USP resolutions	1	0	5.1	6.8	-	-0.4	6.6
width of the main peak	0.4	0.1	-	0.2	0.3	0.2	0.6
overall qualitative response	1	0.1	3.5	4.3	-	0.9	4.2

peak width of MAb1 separations. As could be expected from the response with regard to the total number of peaks, an increasing pH also leads to an increase in the number of basic and acidic peaks. In the number of peak plots, the resolution plots and the qualitative response plots, the highest values (i.e. red areas) are considered to be optimal. On the other hand, in the peak width plots the lowest values (blue areas) are preferred. Accordingly, TETA/butanolamine concentration combinations in red or blue areas are considered to be optimal.

The combination of parameters for the '*compromise method*' (black stars in the contour plots) are in the preferred area for all responses. The black star is located near the maximum for total '*number of peaks*', '*qualitative response*' and '*resolution*' and around the minimum of the '*peak width*'. It is clear, that a most optimal position for all responses is not possible at the same time.

The combination of parameters for the '*highest number of peak method*' (white stars) is closer to the optima for the total '*number of peaks*', the '*number of acidic peaks*' and the '*number of basic peaks*'. This was expected because optimization was focused on these parameters.

MODDE® software (MKS Umetrics; Umeå, Sweden) does also allow an evaluation of the whole set of responses in parallel. The result is shown graphically by a 4D Sweet Plot (Fig. 3) that shows how many responses are within the acceptable range (Table 3) at different parameter combinations. This plot demonstrates that the '*compromise method*' (black star, located within the green spot) approaches preferred settings of all response parameters. However, due to explicit focus on the number of peaks the white star of the '*Highest number of peaks method*' is outside the green sweet spot.

The '*compromise method*' and the '*highest number of peaks method*' were verified for MAb1 and several other antibodies or related formats; 13 out of 16 antibodies showed an evident improvement of the separation profile. This is exemplarily shown by an overlay of the new methods and the originally published method from He et al. [11] for MAb1 (Fig. 4) and MAb3 (Fig. 5).

With the '*compromise*' and '*highest number of peaks method*' there is an evident enhancement in both, the resolution and the number of separated peaks. As expected, most peaks are resolved with the '*highest number of peaks method*'. Therefore, at a first glance, the '*highest number of peak method*' may be optimal. However, the presence of tiny and presumably fluctuating peaks could decrease robustness. Methods that are applied for routine release and stability testing have to be very reproducible, precise and accurate. Obtained results have to be comparable over many years. Therefore, also validation considerations are very important and should be taken into account for final choice of the method.

In summary, the derived DoE model could be verified. In addition, a generic applicability of the optimized methods was shown. However, for MAbs that were not used in this first DoE study, some more improvements may be obtained by molecule specific DoE. This fact motivated a second DoE study for a more acidic antibody (MAb2) that exhibited the slowest CZE migration of all available antibodies.

As for the DoE study for MAb1, the pH value and the acetonitrile, butanolamine and TETA concentrations were varied in parallel. The coefficient plots for the number of peaks, the sum of resolutions, the width of the main peak and the qualitative evaluation showed the influence of different parameters (pH, butanolamine-concentration, TETA-concentration and their interaction) on the responses (data not shown). The obtained model was optimized for all responses with the number of peaks and the resolution as focal points. This resulted in a '*MAb2 specific compromise method*': 40 mM EACA, 2 mM of TETA, 0.05% HPMC, 54 mM of butanolamine and 4% of acetonitrile at a final pH of 6.0.

This method is close to the relative maximum for the number of peaks and that for resolution as well (respective contour plots are not shown here). As expected, the '*MAb2 specific compromise method*' shows a better separation of MAb2 than the '*compromise method*' for MAb1 and the originally published method [11] (Fig. 6). This demonstrated that in spite of some generic applicability of the methods, molecule specific DoEs can result in further improvement.

4 Concluding remarks

Enhanced method development principles were applied in order to investigate possibilities for the optimization of charge heterogeneity testing by CZE. The originally published

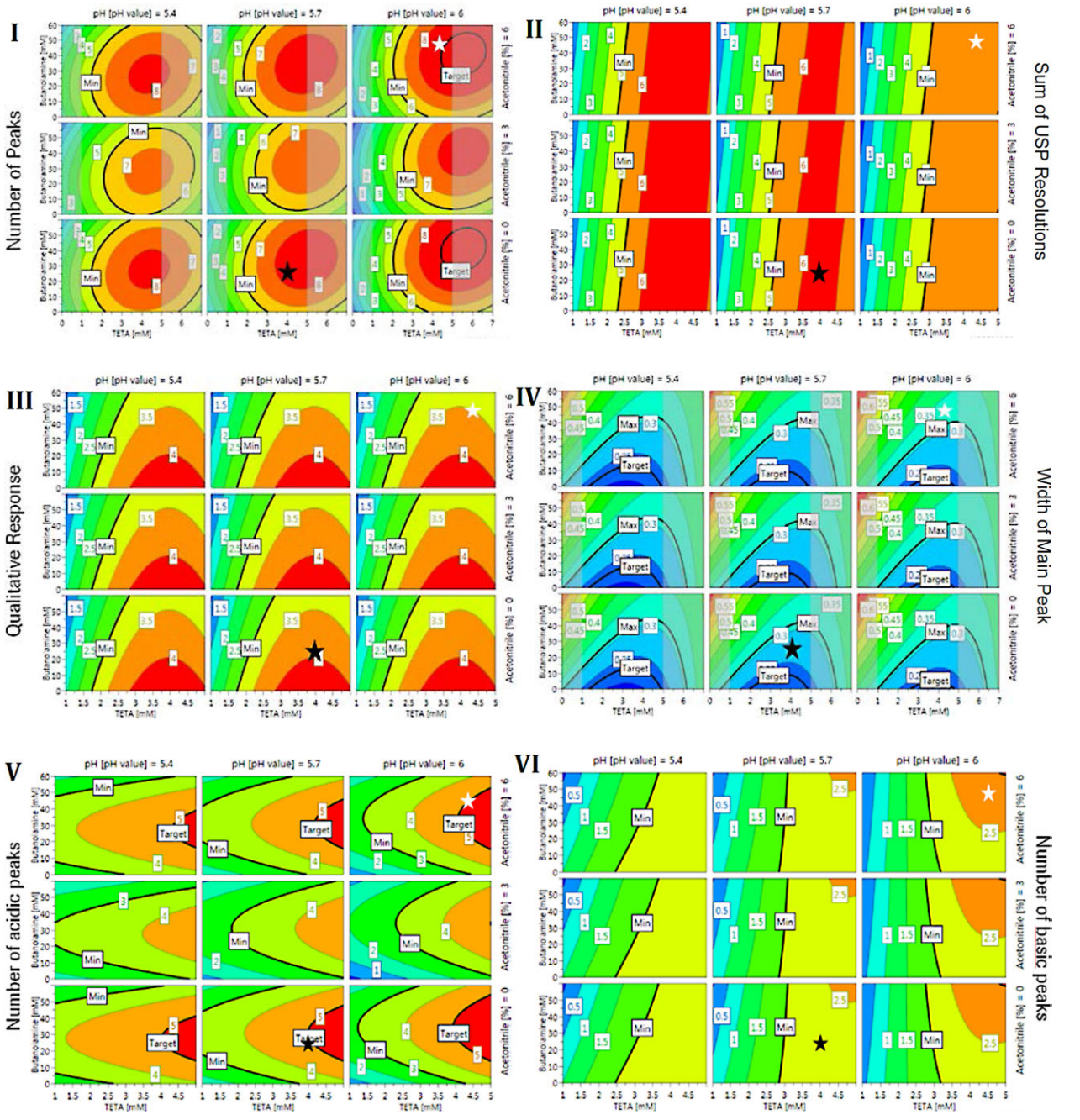


Figure 2. Response 4D Contour Plot of some important responses for MAb1 (MODDE, version 11.0.1, MKS Umetrics; Umeå, Sweden); (I) number of peaks, (II) sum of USP resolutions, (III) qualitative response (resolution), (IV) width of main peak, (V) number of acidic peaks, (VI) number of basic peaks. The colors are representing quantity of each response (third dimension) whereby red is the highest number and blue is the lowest. The gray shaded range of the plots including TETA are actually out of the investigated range and extrapolated. The black stars indicate the position of the 'compromise method' while the white stars indicate the position of the 'highest number of peaks method'.

method from He et al. [11] was found to be an excellent starting point for further molecule specific optimization.

Parameter selection yielded important parameters for CZE optimization: TETA concentration, pH value, polymer additive (HPC versus HPMC) and other additives (butanolamine and acetonitrile). Interaction of these parameters was investigated in several molecule specific DoE studies. Best fit for different weighting of important response parameters of a basic antibody (MAb1) and a more acidic antibody (MAb2) resulted in a significant improvement of CZE separation quality. A compromise between important responses may not deliver a maximum number of peaks

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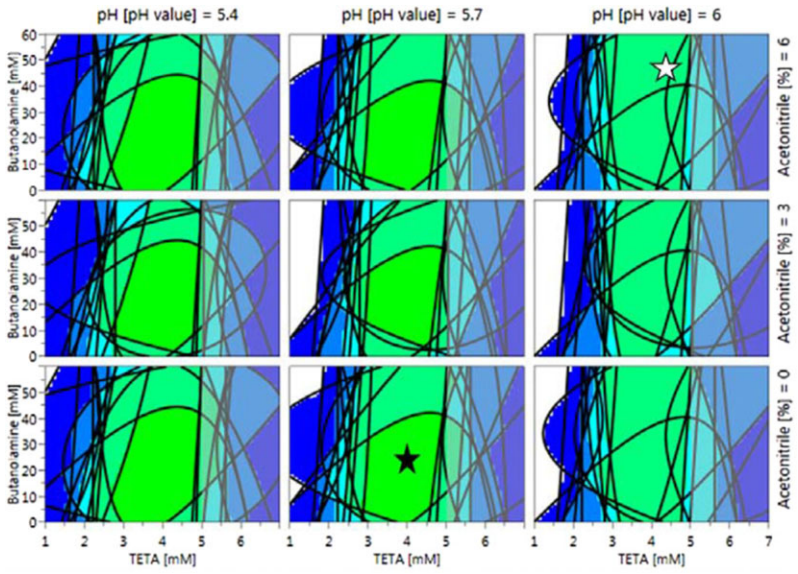


Figure 3. 4D Sweet spot for 14 responses (MODDE, version 11.0.1, MKS Umetrics; Umeå, Sweden): the four parameters are displayed in the same order as in the 4D Contour Plot, the colors represent the number of acceptable responses (see also Table 3); green is where all the 14 criteria are met (sweet spot) while other colors indicate that less criteria are optimal (■ Sweet spot; ■ 10–12 of 14 criteria met; ■ 7–9 of 14 criteria met; ■ 4–6 of 14 criteria met; ■ 1–3 of 14 criteria met). The position of the 'compromise method' (black star) and that of the 'highest number of peaks method' (white star) are shown.

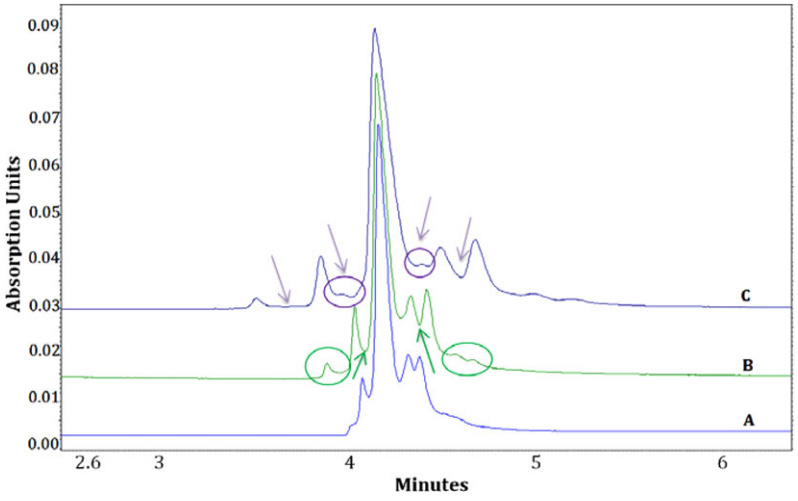


Figure 4. CZE separation of MAb1: published method according to He et al. [11], 2011 (A); '*compromise method*' (B) and '*highest number of peaks method*' (C). Circles indicate new peaks revealed with the optimized methods and arrows indicate improved resolution.

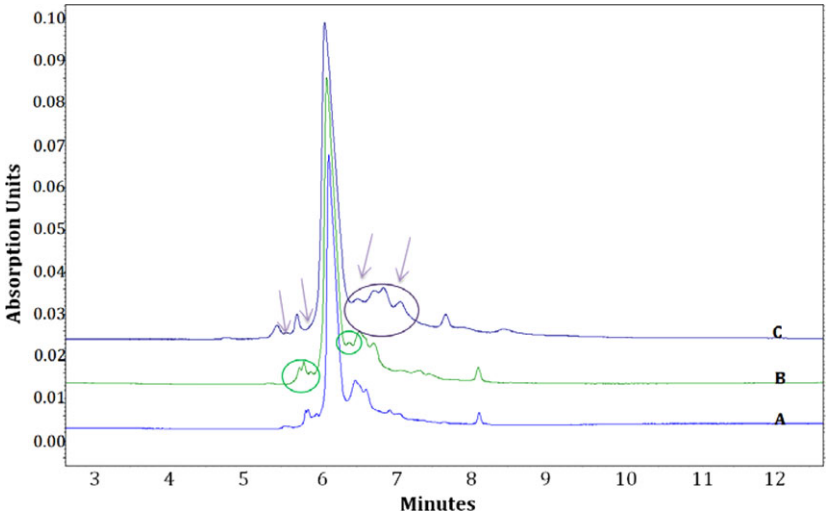


Figure 5. CZE separation of MAb3: published method according to He et al., 2011(A); 'compromise method' (B) and 'highest number of peaks method' (C). Circles indicate new peaks revealed with the optimized methods and arrows indicate improved resolution.

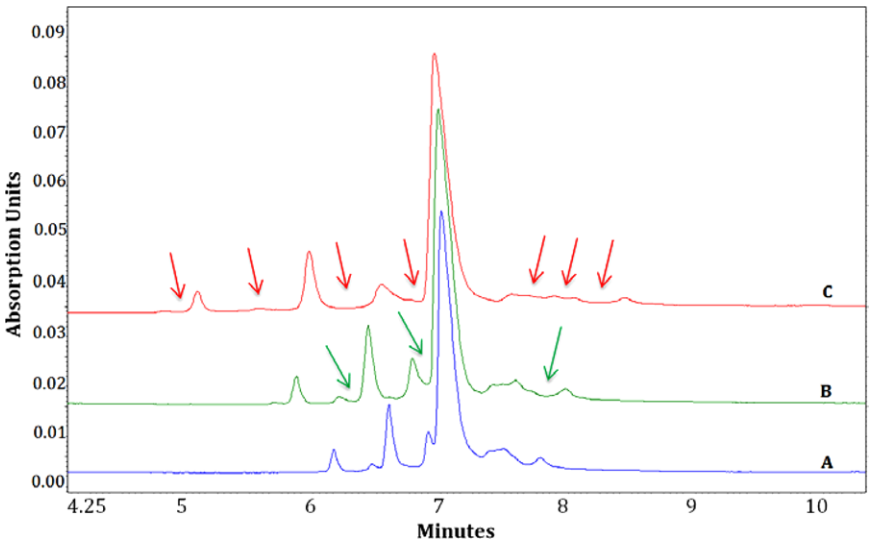


Figure 6. CZE separation of MAb2: published method according to He et al., 2011(A); 'compromise method' for MAb1 (B) and MAb2 specific 'compromise' method (C). Arrows indicate improved resolution.

but may be the better choice for higher precision and reproducibility that is very important for long-term stability and consistency trending of pharmaceuticals. Weighting of different response parameters has to be decided on a case-by-case basis and should always account for validation characteristics and molecule specific criticality of involved charge variants.

For the basic antibody MAb1 a '*compromise method*' and a '*highest number of peaks method*' were developed. The '*compromise method*' accounts for 14 different responses, whereas the '*highest number of peaks method*' mainly accounts only for the number of resolved peaks. As expected more peaks can be resolved with the latter method, however, reproducibility of this method may be lower since some peaks (especially at the main peak) are only poorly resolved and may not always be separated. In this case, validation of precision, accuracy and specificity may be more difficult.

In spite of previous product specific DoE optimization, combination of parameters resulted in significant method improvements for 13 out of 16 different antibodies and product formats (p*I* 7.5–9.5). In conclusion, a CZE method that was developed for one single product may likely be successfully transferred to other products. This generic applicability allows for a reduction of development effort. For some products further optimization by additional DoEs may be required. For the more acidic antibody MAb2 DoE optimization resulted in a '*MAb2 specific compromise method*' that was tailored to this antibody.

The set screws, that were evaluated in this study and elsewhere [11, 12], are well suited for successful development of CZE for protein pharmaceuticals (e.g. antibodies and new formats). Since important parameters and their working ranges are known, the development of new methods for other pharmaceuticals is expected to become faster by our work. However, the development effort should always depend on the fulfillment of acceptance criteria for relevant validation items and on the resolution of critical molecule specific charge species.

The authors would like to thank Nadine Holzmann, Marion Krafft, Zoltan Kranitz, Katja Kolb, Carole Wofner, Sonja Gisin, Margarete Jehmlich, Chantal Roger, Rolf Ketterer and Christiane Ancel for their valuable contributions to this study.

The authors have declared no conflict of interest.

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