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Evaluation of capillary zone electrophoresis for charge heterogeneity testing of monoclonal antibodies

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ABSTRACT

Within pharmaceutical industry charge heterogeneity testing of biopharmaceuticals has to be reproducible and fast. It should pass method validation according to ICH Q2. Classical approaches for the analysis of the charge heterogeneity of biopharmaceuticals are ion exchange chromatography (IEC) and isoelectric focusing (IEF). As an alternative approach, also capillary zone electrophoresis (CZE) was expected to allow reliable charge heterogeneity profiling by separation according to the analyte's net charge and hydrodynamic radius.

Aim of this study was to assess if CZE possesses all of the required features. Therefore, beside lab internal validation of this method also an international cross company study was organized.

It was shown that CZE is applicable across a broad p*I* range between 7.4 and 9.5. The coefficient of correlation was above 0.99 which demonstrated linearity. Precision by repeatability was around 1% (maximum relative standard deviation per level) and accuracy by recovery was around 100% (mean recovery per level). Accuracy was further verified by direct comparison of IEC, IEF and CZE, which in this case showed comparable %CPA results for all three methods. However, best resolution for the investigated MAb was obtained with CZE. In dependence on sample concentration the detection limit was between 1 and 3%.

Within the intercompany study for CZE the same stressed and non-stressed samples were analyzed in each of the 11 participating labs. The finally obtained dataset contained more than 1000 separations which provided an extended dataset for further statistical evaluation. Among the different labs no significant differences between the peak profiles were observed. Mean driver for dropouts in quantitative evaluation was linked to the performance of some participating labs while the impact of the method performance was negligible. In comparison to a 50 cm capillary there was a slightly better separation of impurities and drug substance related compounds with a 30 cm capillary which demonstrates that an increased

Abbreviations: (HPMC), hydroxypropyl methyl cellulose; (EACA), epsilon amino caproic acid; (TETA), triethylenetetramine; (imaged cIEF), imaged capillary isoelectric focusing; (MAbs), monoclonal antibodies; (EOF), electro-osmotic flow; (ANOVA), analysis of variance; (IEC), ion exchange chromatography; (CPA), corrected peak area; (TOST), two one-sided tests; (ID), inner diameter.

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stability indicating potential can be combined with the increased separation velocity and high throughput capability of a shorter capillary. Separation can be performed in as little as approx. 3 min allowing high throughput applications. The intercompany study delivered precise results without explicit training of the participating labs in the method prior to the study (standard deviations in the range of 1%).
It was demonstrated that CZE is an alternative platform technology for the charge heterogeneity testing of antibodies in the pharmaceutical industry.

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1. Introduction

There are different groups of modifications that influence the charge heterogeneity profile of proteins like monoclonal antibodies [1]. One of these groups causes a shift of the charge profile towards basic forms, e.g. by succinimide formation [2] or C-terminal lysine heterogeneity [1]. Another shifts the profile towards the acidic forms e.g. by deamidation [2], sialylation [3], glycation [4,5], or pyroglutamate formation [6]. In addition, fragmentation may cause a shift in both directions. Modifications like Asp isomerization do not change the net charge but can have an influence on the surface charge distribution [7]. Some of these modifications may influence potency [8]. Therefore, charge heterogeneity testing is very important for the QC testing of protein based pharmaceuticals.

Within pharmaceutical industry charge heterogeneity testing of biopharmaceutical drug products is important for control of the production process, for technical development and for release and stability testing. Therefore, the applied methodology has to be reproducible (i.e. comparable results over years) and fast (i.e. enabling of high throughput analyses). For fulfillment of underlying GMP requirements it has to pass method validation according to ICH Q2. Furthermore it should be stability indicating and be able to address compounds linked to potential critical quality attributes (CQAs).

The most common techniques currently used for charge heterogeneity testing are IEC [9], cIEF [10] and imaged cIEF [11,12]. Each method has complementary separation mechanisms. In case of cIEF and imaged cIEF analytes are focused within a pH gradient according to their apparent p*I* values, whereas in case of IEC the separation occurs by electrostatic interaction of the analytes with cationic or anionic functional groups of the stationary phase of the IEC column.

In 2011 a new CZE method for charge heterogeneity testing was published by He et al. [13]. A similar CZE method was described by Shi et al. [14]. An advantage of this method is the use of bare fused silica capillaries. Additionally, interaction of analytes with the capillary wall and any residual electro-osmotic flow (EOF) are suppressed by dynamic coating with Triethylenetetramine (TETA) and by an elevated concentration of epsilon amino caproic acid (EACA). UV detection at 214 nm enables high sensitivity. In CZE, separation is achieved based on molecular charge-to-hydrodynamic radius ratio. Investigation of capillary zone electrophoresis as an alternative approach was performed since many pharmaceutical companies consider CZE as a fast, robust, and reliable methodology for daily routine applications.

IEC, IEF and CZE have different pros and cons for different application areas or analytes. IEC which allows MS characterization of different charge species after desalting of single fractions by dialysis and/or RP-HPLC separation [15–17] is currently the most important method which is preferred for biopharmaceuticals during late stage and market supply. Due to very small amounts and MS incompatible compounds like ampholytes or running buffer components identification of CE fractions is more difficult [18] and requires indirect approaches like Rotofor [19], free flow electrophoresis (FFE) [20] offgel-fractionation [21] or complex online coupling technology [18]. In case of charge differences that are buried within the three dimensional structure IEF and CZE are expected to be advantageous whereas in case of changes of the surface charge pattern or changes without influence on the net charge IEC may be preferred [7]. Method selection has to be decided on a case by case basis.

In order to demonstrate required features a lab internal method assessment (range, precision, accuracy, sensitivity, stability indicating properties and high throughput capabilities) and an intercompany study were performed for the analysis of monoclonal antibodies by CZE. An international team of 11 laboratories from 9 independent companies/organizations in the US, Switzerland and Germany was formed to contribute to this study. As already demonstrated for capillary IEF [22] and imaged capillary IEF [23] the aim of the present CZE study was to show applicability in this context.

2. Materials and methods

2.1. CZE separation

All separations were performed on a capillary electrophoresis system capable of capillary temperature control at 20 ± 2 °C and UV detection at 210 – 220nm. Therefor a Beckman Coulter PA 800 Pharmaceutical Analysis System (PA 800 *plus* or *Enhanced*), equipped with UV detector and 214 nm filter (aperture: 800 μm, data rate: 8 Hz) was used. Separations were performed in two different bare fused silica capillaries with capillary length to detector/total length 40 cm/50 cm (ID: 50 μm; Beckman Coulter cat. no.: 338451) [variant A] and 20 cm/30 cm (ID: 40 μm; Polymicro Technologies cat. no.: TST040375) [variant B]. Before each run capillaries were rinsed with 0.1 M HCl (*acidic wash solution*) for 5 min [variant A] or for 4 min [variant B] (60 psi each). The provided MAb samples were diluted to 1 mg/ml with purified water. At least 80 μl of each sample were carefully transferred into PCR vials. After pre flushing with *separation buffer* (400 mM EACA, 2 mM TETA, 0.05% HPMC) for 10 min [variant A] or 9 min [variant B] (50 psi each) samples were injected using a pressure of 0.5 psi (10 s). Polarity was positive (capillary inlet) to negative (capillary outlet). The applied separation voltage was 30 kV. The capillary temperature was 20 °C. Separation time was 30 min [variant A] or 10 min [variant B]. Column storage for long term use: 0.1 M HCl in capillary with two ends dipped in water.

For lab internal investigation of validation characteristics same conditions as before with exception of the following points were applied: rinse with 0.1 M HCl for 1 min (60 psi); pre-flush with separation buffer for 1 min (50 psi); separation voltage: 20 kV.

2.2. Imaged cIEF and IEC

Imaged cIEF of MAb2 (p*I* approx. 8.5): after dilution sample was mixed with Pharmalytes pH 3–10 and separated with an iCE280 system (Protein Simple, Santa Clara, USA). IEC of MAb2: separation was performed in a Dionex ProPac WCX-10 column with a

Table 1
Intercompany study: summary of the performed separations of MAb1. *Scenario 1:* all participants were centrally provided with the same buffers; *Scenario 2*: each group used its own buffers. Separation of stressed and non-stressed samples of MAb1 (us = unstressed; s = stressed). Capillary length: 30 cm capillary (separation length from inlet to detection window 20 cm; ID 40 μm)/50 cm capillary (separation length from inlet to detection window 40 cm; ID 50 μm). Each sample preparation was performed in duplicates (A&B; prep. = preparation).

	Scenario 1								Scenario 2							
Length	30 cm				50 cm				30 cm				50 cm			
Sample	s		us		s		us		s		us		s		us	
Prep.	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B

sodium chloride gradient after carboxypeptidase B treatment (*eluent A:* 20 mM MES, pH 6.2; *eluent B:* 20 mM MES, 750 mM NaCl, pH 6.2).

2.3. Samples, chemicals and materials

Intercompany study: the 11 participating laboratories were provided with both a stressed (light stress for 7 h at 600 W/m^2) and non-stressed antibody sample called MAb1. There was no collective method training prior to the study. The study was performed according to the design in Table 1.

Samples of the monoclonal MAb1 were distributed to all study participants and analyzed according to two scenarios: in *Scenario 1* each lab used the same buffers that were centrally provided and distributed. In *Scenario 2* each lab used its own buffers. Each sample preparation was performed in duplicates (A&B in Table 1). From each sample preparation six separations were performed. This means that in each group 2 (samples) × 2 (capillaries) × 2 (scenarios) × 2 (sample preparations) × 6 (separations), i.e. 96 CZE analyses were planned. Since 11 groups participated in this study this results in 1056 separations that were planned for this study. For each separation every participant was supposed to evaluate the corrected area percent (%CPA) composition for the *main peak*, the *sum of acidic peaks* and the *sum of basic peaks*.

2.3.1. Scenario 1 and Scenario 2 of the intercompany study

For both scenarios the same stressed and unstressed MAb1 (IgG1) samples were used as centrally provided. Materials required but not provided: deionized water (e.g. Ultra-Pure Water System: Millipore, Milli-Q Plus); Beckman Coulter PA 800 *plus* or *Enhanced*. Accessories with Beckman Coulter part numbers: capillary cartridge (cat. No.144738); vials (cat. no A62251); caps (cat. No A62250); 0.25 ml PCR vials (cat. No. 144709); capillary cartridge coolant (cat. No. 359976). Further accessories: 0.2 μm pore size nylon membrane syringe filters (Corning® Bottle-Top Vacuum Filters, Corning cat. no. CLS430049).

2.3.2. Scenario 1

In case of *Scenario 1* the following consumables/buffers were provided:

Solution 1: 500 mM EACA + 2.5 mM TETA pH 5.7; *solution 2*: 1% HPMC; bare fused silica capillaries and *acidic wash solution*. For details about constituents of *solutions 1* and *2* see subsequent section '*Scenario 2*'. Storage conditions: the IgG samples were stored frozen at −70 to −80 °C. *Solution 1* was stored at 2–8 °C and *Solution 2* at ambient temperature. Also the *acidic wash solution* was stored at ambient temperature. Preparation of CZE *separation buffer*: 2.5 ml of *Solution 2* was added to 40 ml of *Solution 1*. The mixture was brought up to 50 ml with purified water and homogenized. The CZE *separation buffer* was always prepared freshly in each lab.

2.3.3. Scenario 2

In *Scenario 2* each participant prepared his own buffers/solutions: 1 M EACA stock solution (Sigma, cat. no.: A-7824), 1 M TETA stock solution (Sigma, cat. no.: 90460), *acidic wash solution* (0.1 M HCl; Merck, cat. no.: 109973) and 1% (w/v) Hydroxypropylmethylcellulose (HPMC) (Sigma, cat no. H-7509) were prepared. Except for HPMC all solutions were filtered through a 0.2 μm filter. *Separation buffer* (400 mM EACA, 2 mM TETA, 0.05% HPMC) was prepared as follows: 40 ml of 1 M EACA stock solution, 0.2 ml of 1 M TETA stock solution and 40 ml deionized water were mixed in a 100 ml volume flask. pH was adjusted to 5.7 ± 0.1 with acetic acid (Merck, cat. no.: 45730) [approx. 50% (v/v)]. 5 ml of 1% HPMC were added. The mixture was then filled up to 100 ml with deionized water. The volume flask was inverted a few times to mix. Any bubbles present in the HPMC solution were removed by leaving the solution on the bench.

2.4. Statistical evaluation

Statistical data evaluation was performed with Minitab version 15.1.0.0 (State College, PA, USA). Anderson–Darling-tests [24] were applied to test for normal distribution of CZE results. Besides one-way analysis of variance (ANOVA) for normal distributed data also tests according to Kruskal–Wallis (with ties) [25] that do not require normal distributed data were applied in order to test for equal medians. Box plots [26] were used for data visualization.

3. Results and discussion

3.1. pI range and high throughput capabilities

The functional p*I* range and high throughput capability of CZE were investigated for monoclonal antibodies (MAbs). Fig. 1 shows the successful separation of 23 MAbs with p*I* values between 7.4 and 9.5 illustrating that CZE is applicable across a broad p*I* range. For each MAb, a very detailed structure of the charge heterogeneity profile was obtained. The MAb used for the intercompany study (MAb1) is the most acidic MAb in Fig. 1 and therefore represents a worst case example with regard to separation velocity. A subset of MAbs that again spans the same p*I* range was performed by a 30 cm capillary with an inner diameter of 40 μm. The migration times were now in the range between 3.5 and 7.5 min with resolution still comparable to the separations in the longer capillary. This demonstrates that acceleration of charge heterogeneity testing is possible without loss of information. Hence, the shorter capillary was added to the intercompany study.

3.2. Investigation of selected validation characteristics

According to ICH guidance (Q2(R1) 'VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY') methods have to be checked for pre-defined validation characteristics before they can be applied for QC testing of biopharmaceuticals. Therefore, selected validation characteristics were investigated.

For MAb1, linearity, accuracy and precision were demonstrated in a combined experiment [27]. The results are shown in Table 2. Three repetitive measures were performed on nine different concentration levels in the range of 20% (0.2 mg/ml) to 420%

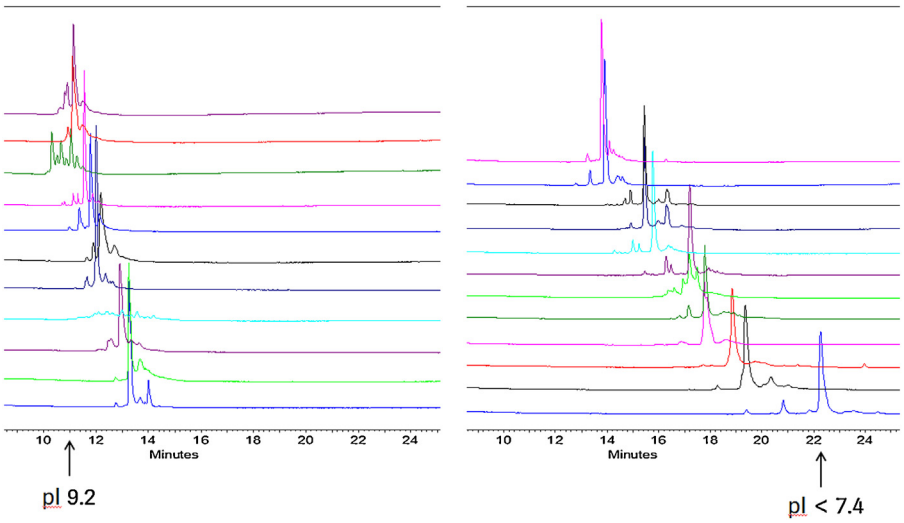


Fig. 1. CZE separations of 23 MAbs with p*I* values between 7.4 and 9.5. Separations have been performed in a 40/50 cm capillary (separation length between capillary inlet and detection window/total length) with an inner diameter of 50 μm.

(4.2 mg/ml) of the applied amount according to method description (100%, 1 mg/ml). The results for linearity were based on absolute CPA, whereas precision and accuracy were based on %CPA. Linearity was demonstrated by the correlation coefficients of the plots of CPA results for the *sum of basic peaks*, the *sum of acidic peaks* and the *main peak* against the concentration levels. Obtained correlation coefficients were always above 0.99 (Table 2). Precision was demonstrated by the maximal relative standard deviation (per level) that was 1.4% CPA. Since RSD is related to the relative amount, the higher SD's for the lower abundant sums of acidic and basic peaks were expected. Accuracy by recovery was determined with the mean value of all 100% (1 mg/ml) separations (defined as expected value). For the *main peak*, the range of mean recovery per level was 99.3–101.4%. Accuracy was further verified by direct comparison of IEF, IEC and CZE separations of the same sample as shown by the quantitative data in Fig. 2. For this example the relative content of the three *main peaks* was comparable. Best resolution was obtained with CZE.

For detection/quantification limit (DL/QL) determination, MAb1 was diluted with water to 0.1 mg/ml. Based on this pre-dilution, a dilution series with 10 concentrations between 0.020 and 0.001 mg/ml was made. The detection limit was derived from the obtained signal to noise ratios. The DL value (%CPA value of peaks whose height is equal to 3-fold baseline noise) depends on the initial sample concentration. It was 1% for 1 mg/ml and 0.3% for 3.5 mg/ml. Respective QL values (%CPA value of peaks whose height is equal to 10-fold baseline noise) were 3.3% for 1 mg/ml and 1% for 3.5 mg/ml.

3.3. Intercompany study

For each separation of the intercompany study the relative contents of the *main peak*, the *sum of acidic peaks* and the *sum of basic peaks* were reported. Not all data were made available which reduced the final data set to 1008 separations. However, this still presented 95% of the initially planned data set of 1056 separations which was a high return rate for this complex study design.

Peak profiles (Fig. 3) and complete %CPA datasets (Figs. 4 and 5) are exemplarily shown for *Scenario 2*, since most variability was expected for *Scenario 2* conditions.

Fig. 3 shows the *Scenario 2* separations with the 40/50 cm capillary (separation length between the inlet and detection window/total length). The results for the unstressed sample are depicted on the left hand side and those of the stressed sample on the right hand side. No significant differences between the peak profiles (resolution and relative peak height) were observed within each data set. The same was found for the 20/30 cm capillary (data not shown) which showed that CZE is robust in this regard.

The detailed course of the obtained %CPA results for the *main peak*, the *sum of acidic peaks* and the *sum of basic peaks* of *Scenario 2* with the 40/50 cm unstressed sample is shown in Fig. 4. The data are uniform. For group 5 the results were not available. In Fig. 5 the same is shown for *Scenario 2* with the 20/30 cm capillary. A few outlier results are circled. Group 7 had a systematic shift in the 20/30 cm datasets affecting 48 separations of this group. Also group 3 had some noticeable results with respect to %CPA results. There is currently no complete explanation for these deviations, which were most likely related to lab specific handling and not to the method performance itself. Omission of outliers for statistical evaluation is discussed below.

The choice of analytical tools for statistical evaluation depends on the distribution function that can be approximated by the obtained dataset. Comparison of mean values by ANOVA requires a normally distributed dataset [25]. Therefore, in a first step data were tested for normal distribution. Deviations from linearity in the probability plot and a *p*-value below 0.05 for the Anderson–Darling

Table 2
This table contains values for linearity, accuracy by recovery and precision by repeatability of MAb1 in the range of 20% (0.2 mg/ml) to 420% (4.2 mg/ml) of the applied amount according to method description (100%, 1 mg/ml). Three repetitive measures were performed on nine different concentration levels between 0.2 and 4.2 mg/ml. Linearity of CPA values was demonstrated by the coefficient of correlation. Accuracy by recovery was determined with the mean %CPA value of all 100% (1 mg/ml) separations (defined as expected %CPA value). The range of mean recoveries [%CPA] per level is reported. The maximum relative standard deviation per level [%CPA] is reported as precision by repeatability.

		Sum of basic peaks	Main peak	Sum of acidic peaks
Linearity	Coefficient of correlation	0.9988	0.9984	0.9977
Accuracy [%CPA]	Range of mean recovery per level	96.1–102.1%	99.3–101.4%	96.5–103.8%
Precision [%CPA]	Maximum relative standard deviation (per level)	4.4%	1.4%	3.7%

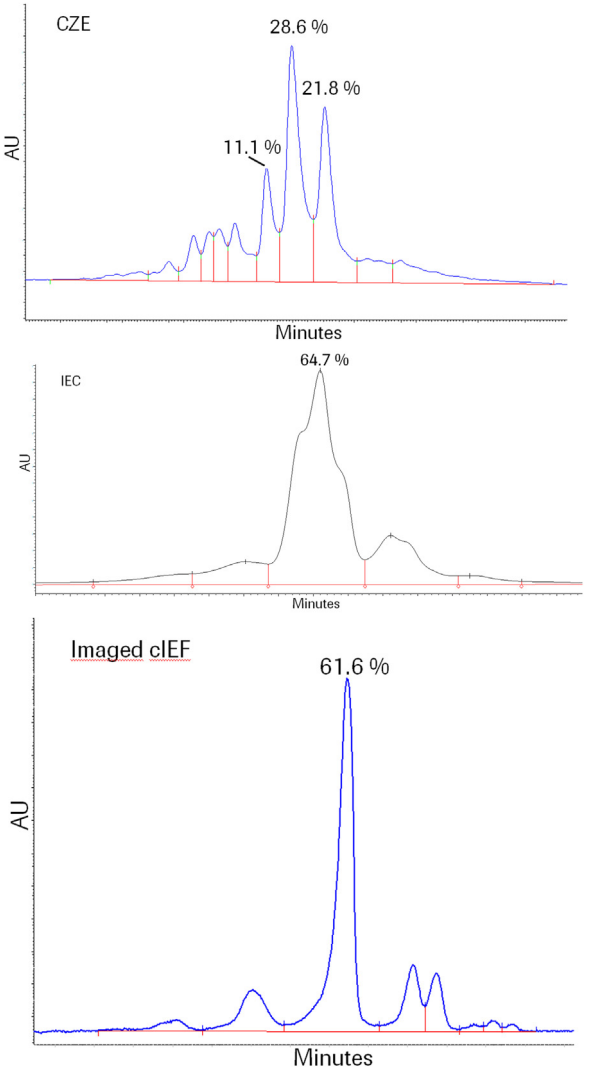


Fig. 2. CZE, IEC and imaged cIEF separations of MAb2. The relative corrected peak areas (%CPA) of the *main peak*(*s*) are reported. Experimental details of imaged cIEF and IEC are described in Section 2.2.

test demonstrated that Gaussian distribution is not applicable for the obtained %CPA datasets. The same was observed for the probability plots and Anderson–Darling tests for normal distribution of CZE-results for the *sum of acidic peaks* and the *sum of basic peaks* (data not shown).

Due to absence of normal distribution, comparison of mean values between different groups by ANOVA might be misleading. Instead, non-parametric tests such as Kruskal–Wallis (with ties) [25] for comparison of medians that do not require normally distributed results were applied. However, in most cases ANOVA and Kruskal–Wallis-test came to the same conclusion e.g. that there is a significant difference between the results obtained by different laboratories. The question whether a significant difference between results is also of practical relevance is more difficult to answer. One approach to test for equivalence of results obtained by two groups is the two one-sided *t*-test TOST [28]. With TOST the confidence interval of the difference between the mean values obtained by two groups is calculated and compared with an acceptance range $[-\theta,\theta]$ which provides a more meaningful outcome than the *p*-value of an ordinary *t*-test. When using TOST the limits of the acceptance range are typically derived from the data subjected to equivalence testing.

However, the limits of the acceptance range may also depend on the context where the analytical method is being applied. Therefore, we decided to present data from the intercompany study by means of descriptive statistics rather than drawing potentially irrelevant conclusions by inferential statistics.

In many labs CZE was performed for the first time without intensive training in advance, which might explain results with a high difference to the overall median results. These results were identified on basis of the absolute distances to the respective median values [$\Delta = I$%CPA value-medianI]. The obtained Δ values were arranged in order of size. 10% of the acidic, basic and main values with the highest Δ values were identified for each dataset. Subsequently, the complete data triplet (i.e. *main peak*, *sum of acidic peaks* and *sum of basic peaks*) was removed from the original dataset if at least one of the parameters belonged to the 10% highest absolute deviation from the median. As a result approx. 20% of the whole dataset was removed, which is a similar proportion of outliers found and removed throughout the evaluation of other methods for determination of charge heterogeneity [23].

Outlier testing and resulting data removal is not unrestrictedly allowed in GMP environment. However, this exploratory approach is deemed to be justified since descriptive statistical evaluation indicates that dropouts are not caused by method performance. The resulting percentaged dropout rates (number of removed outliers/total number of %CPA results within each dataset × 100) are illustrated in Fig. 6. Since the dropouts are located only at a subset of laboratories the dropouts were more likely to be related to the lab performance and not to the method performance, i.e. to the scenario or the capillary length. In case method performance would be the reason for the highest absolute deviations from the median, the affected results (dropouts) would be more or less evenly distributed over all participating labs. But dropouts are predominantly related to some companies. Especially for companies six to nine the dropout rate is significantly increased. This is confirmed by the size and direction of median shifts after omission of dropouts which is also related to single companies (data not shown). On the other hand the dropout rates are very low for seven companies. This shows that this method can easily be transferred from lab to lab. Subsequent statistical evaluations were performed on the dataset without dropouts in order to avoid mixing up method performance with lab performance.

Reasons for the observed dropouts are speculative and may be related to differences in the sample handling, system operation or details of integration. This should be a major focus of further implementation of CZE within a GMP environment. It is expected that an intensive training which was not possible during this large intercompany study but which is standard before the performance of transfer tests will avoid dropouts during future GMP testing. This improved training usually comprises a very thorough exchange of all method details.

Focus of the subsequent data evaluation will be descriptive statistical parameters such as median values and percentiles that are easily illustrated in form of box plots. By projection of data sets (exemplarily shown in Figs. 4 and 5 for *Scenario 2* separations) on a single bar all data for different capillary lengths, samples and scenarios for the same peak or peak group can be shown in one single diagram which allows a direct comparison. In Fig. 7 the box plots for the *main peak* are shown (unstressed and stressed samples for both scenarios and capillaries). As expected the *main peak* is lower in the stressed sample. The interquartile ranges always contain 50% of the data. Within the boxes the median values are shown. The whiskers go to the last value that is still within the 1.5-fold interquartile range. There are still some noticeable results which are depicted as stars. But in the reduced datasets they are not far away from the 1.5-fold interquartile range. As expected the box plots for the

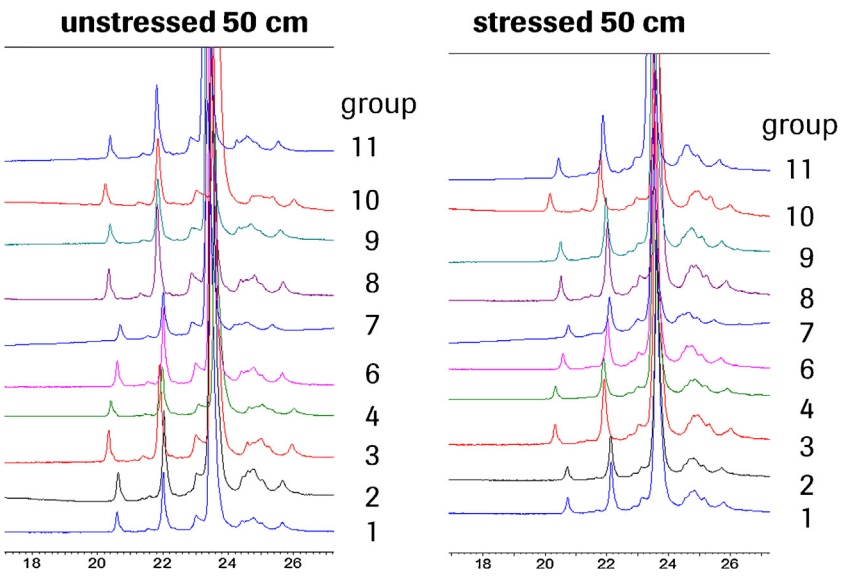


Fig. 3. Overlay of all *Scenario 2* separations with the 40/50 cm capillary.

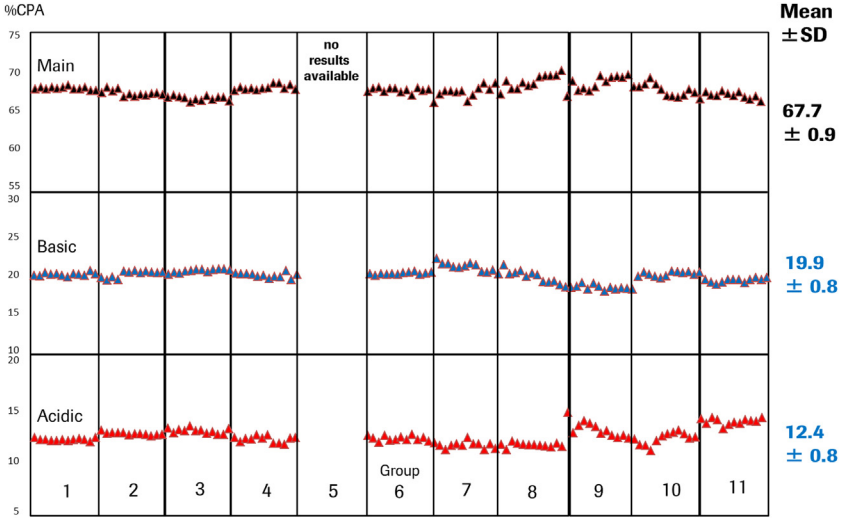


Fig. 4. %CPA results for all *Scenario 2* unstressed sample with the 40/50 cm capillary.

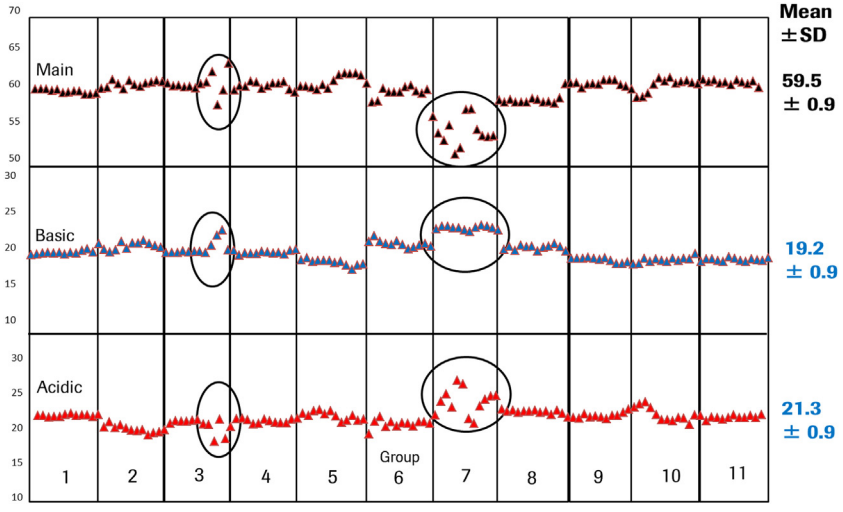


Fig. 5. %CPA results for all *Scenario 2* stressed sample with the 20/30 cm capillary.

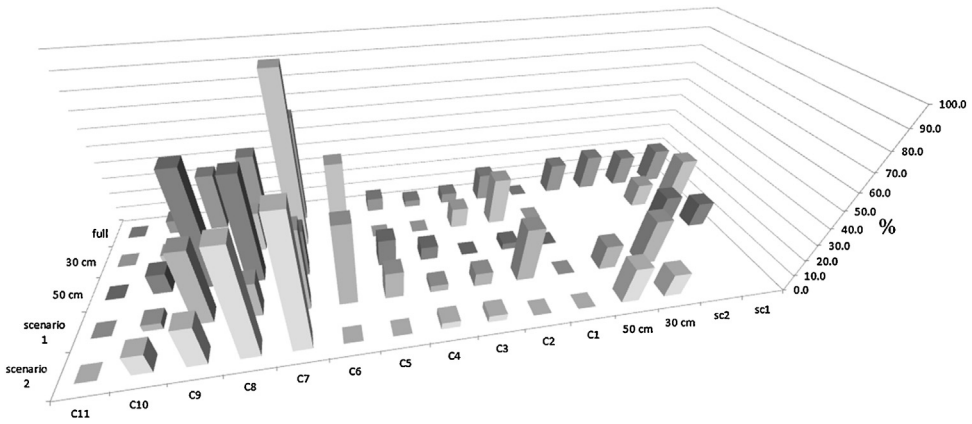


Fig. 6. Dropout rates [%] for the unstressed sample in dependence on company, capillary length and scenario *Abbreviations*: C1–C11: company 1–11; sc1: *Scenario 1*; sc2: *Scenario 2*; 50 cm: total capillary length 50 cm/ID 50 μm; 30 cm: total capillary length 30 cm/ID 40 μm.

complete datasets show a wider interquartile range than those for the reduced datasets.

There is a strong overlap of the datasets, which means that there is no really eye catching difference between the different scenarios and capillaries. There seems to be a slight trend towards lower *main peak* purity for the 30 cm capillary.

The same applies for the *sum of acidic peaks* and for the *sum of basic peaks* (data not shown). The relative content of the *sum of acidic peaks* is increased during light stressing. The relative content of the *sum of basic peaks* has not changed during light stressing. Again the interquartile ranges are very narrow for the reduced datasets. There are still some noticeable results (stars in the box

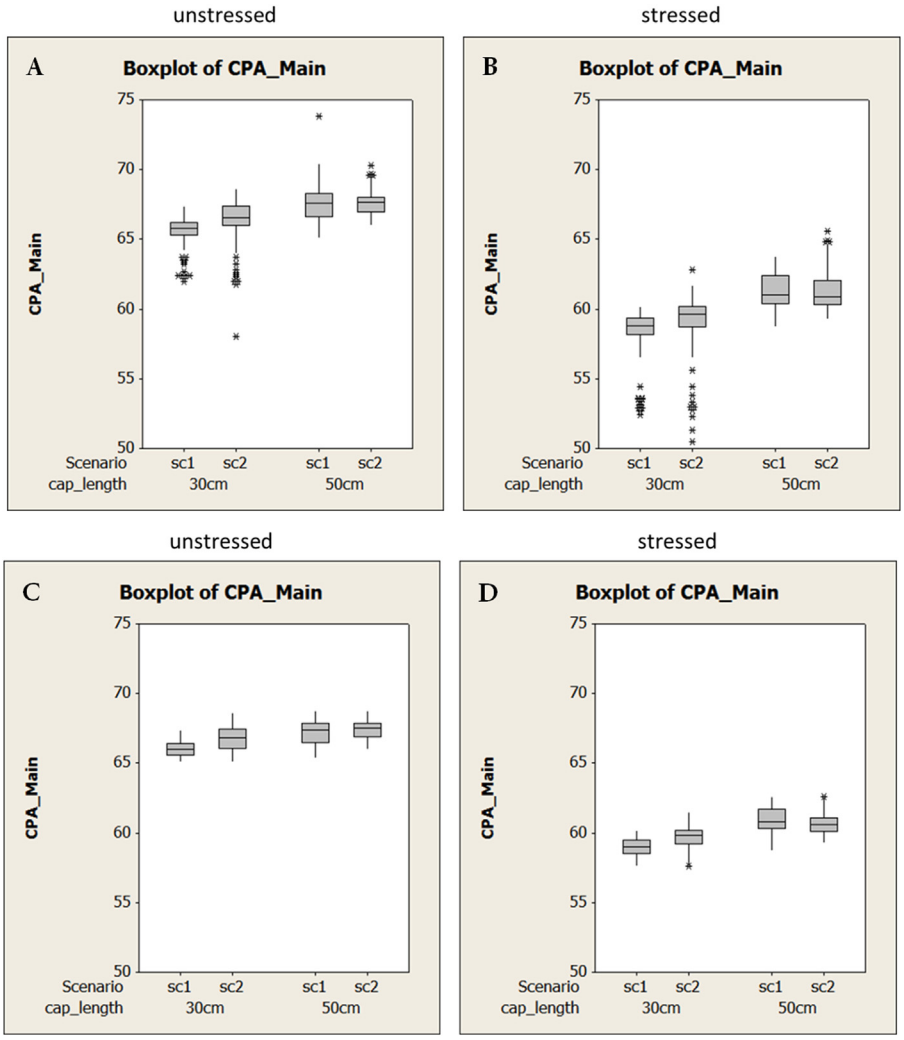


Fig. 7. Intercompany study: box plots of %CPA results for the *main peak*. A and B: full datasets; C and D: reduced datasets. *Abbreviations*: sc1: *Scenario 1*; sc2: *Scenario 2*; 50 cm: capillary length 50 cm/diameter 50 μm; 30 cm: capillary length 30 cm/diameter 40 μm.

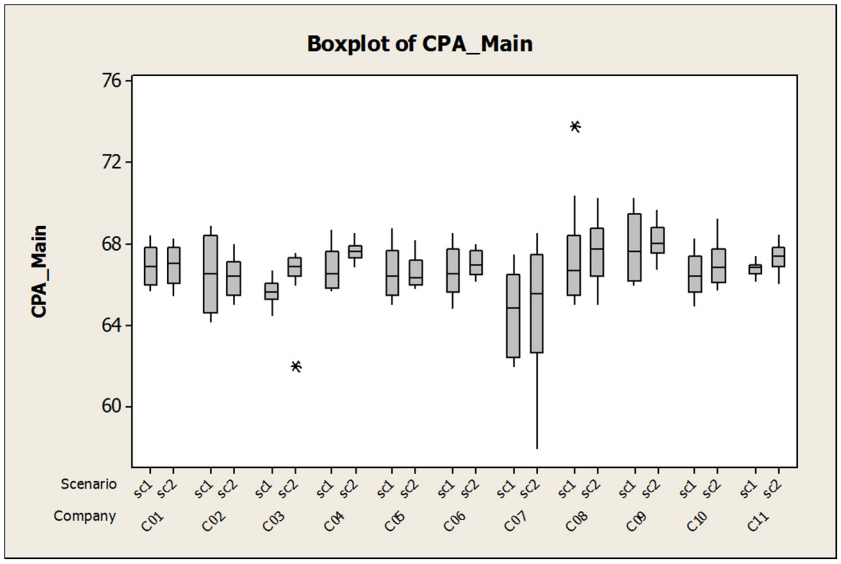


Fig. 8. Intercompany study: Boxplots of %CPA results for the *main peak* (unstressed sample) in dependence on the companies and scenario (full dataset). *Abbreviations: C01, C02. . .*: company 1, company 2. . .; *sc1*: *Scenario 1*; *sc2*: *Scenario 2*.

plots). But as for the *main peak* they are not very far away and the interquartile ranges which are always very narrow.

Only the full datasets for the stressed and unstressed samples were evaluated for overall inter laboratory precision. From the uneven distribution of dropouts between the companies, it is expected that there is higher fluctuation among them (e.g. due to training of the personnel) which can cover the influence of the scenario or the capillary length, when looking at these parameters. Therefore, the influences of the capillary length and the scenario were evaluated separately for each company and were visualized via box-plots (Fig. 8 for *Scenario 1*/*2* and Fig. 9 for 30/50 cm). The interaction between the capillary length and the scenario is regarded as being low. Hence, different scenarios are not split in different capillary lengths and vice versa. In general the differences between length and scenario are significantly smaller than those between the companies. This means that the influence of different scenarios and capillary lengths is less pronounced.

- Comparison of *Scenario 1* and *2*: Median and interquartile range are often comparable within the same company (with a trend towards smaller interquartile range for *Scenario 2*). This means that consumables are not critical for the applied CZE methodology and that the availability of centrally provided CZE kit consumables is not necessarily required. However, availability of commercially available kit materials may be beneficial for cost saving.
- Comparison of 30 and 50 cm: There is a trend towards higher purity values for the 50 cm capillary, whereas the fluctuation of interquartile ranges is comparable. That means that there is a better separation of impurities and drug substance related compounds with the 30 cm capillary that is most likely caused by longer diffusion in the 50 cm capillary. This is a very beneficial result since an increased stability indicating potential can be combined with the increased separation velocity and high throughput capability of the shorter capillary.

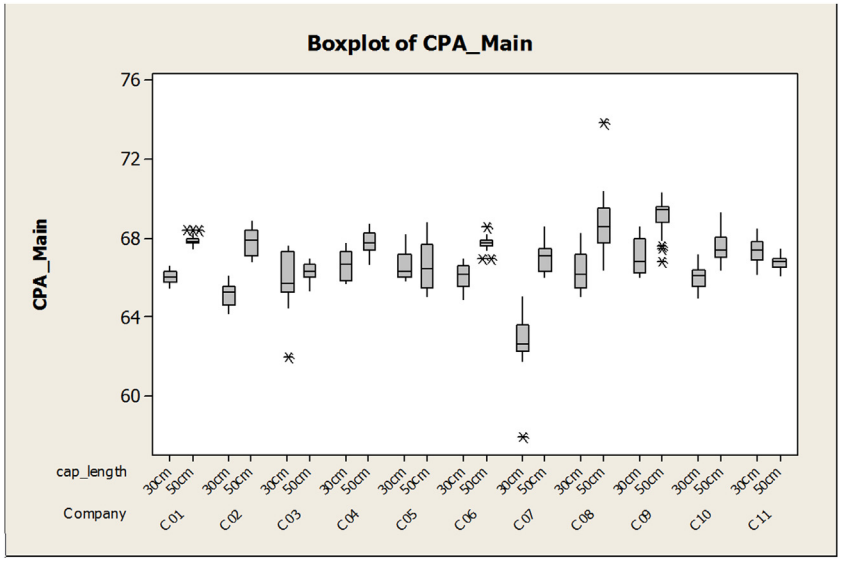


Fig. 9. Intercompany study: Boxplots of %CPA results for the *main peak* (unstressed sample) in dependence on the companies and capillary length (full dataset). *Abbreviations: C01, C02. . .*: company 1, company 2. . .; 30 cm: capillary 20 cm/30 cm (distance from inlet to detector window/total length, inner diameter 40 μm); 50 cm: capillary 40 cm/50 cm (distance from inlet to detector window/total length, inner diameter 50 μm).

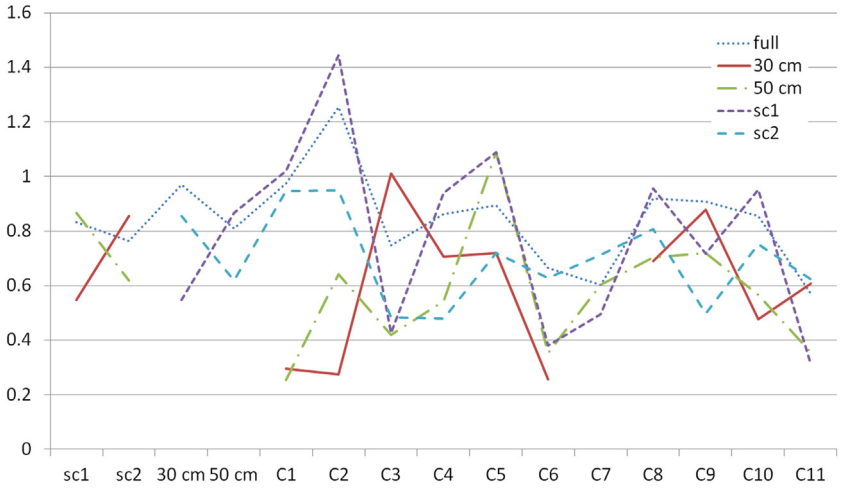


Fig. 10. Standard deviations for %CPA values (*y*-axis) of the *main peak* after removal of outliers *Abbreviations: C1, C2. . .*: company 1, company 2. . .; 30 cm: capillary 20 cm/30 cm (distance from inlet to detector window/total length, inner diameter 40 μm); 50 cm: capillary 40 cm/50 cm (distance from inlet to detector window/total length, inner diameter 50 μm).

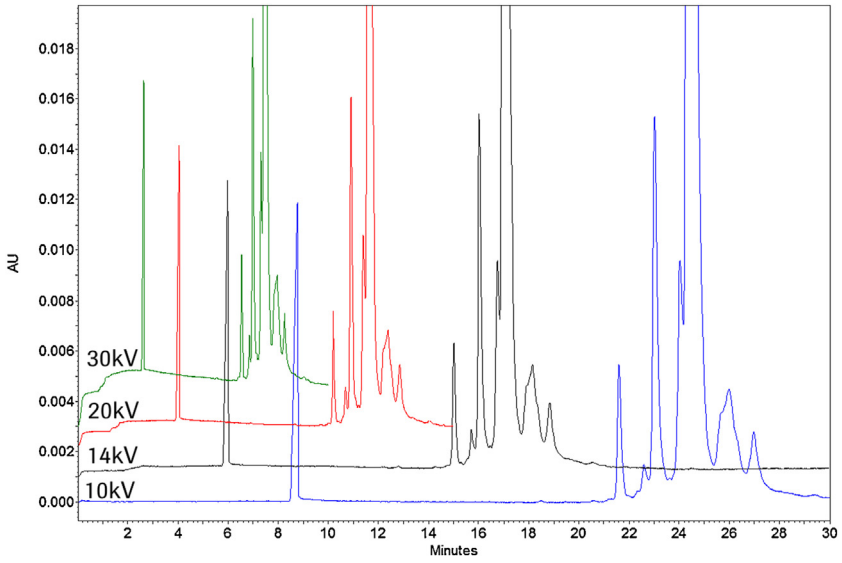


Fig. 11. CZE separations of MAb1 with different separation voltages (10, 14, 20, 30 kV).

For the reduced datasets the overall method performance is shown by the standard deviations for the *main peak* (Fig. 10). In order to reveal potential differences between scenarios, capillary lengths and companies, the standard deviations are also shown separately. The obtained values are in the range of 0.6–1.4%. They are not higher than the intra-laboratory results for different injection amounts. Except for single companies the standard deviations are even below 1%. Since that was the first worldwide attempt without prior training this demonstrates the ease of implementation and the excellent precision of CZE for charge heterogeneity testing.

3.4. Further method optimization

Subsequently further optimization of the applied CZE methodology could be achieved: Separation becomes faster with increasing voltage. However, baseline drift increases which may lower method precision (Fig. 11). Therefore, it is recommended to reduce the separation voltage to approx. 20 kV (30 kV was applied for the intercompany study).

A further acceleration was obtained when choosing the 10 cm separation direction of the 30 cm capillary. That was already described in the original publication [13]. However, in this case the resolution is slightly lower and therefore this strategy was not applied for the intercompany study or for the additional assessment of some validation characteristics. A reduction of the flushing time from 14 to 2 min had no influence on the profile.

In summary these investigations showed that the high precision and high throughput capabilities of CZE can be further increased by simple modifications of the method.

4. Conclusions

CZE is a very efficient and robust platform method for charge heterogeneity testing of biopharmaceuticals. The implementation of CZE for different products is very easy. Sample preparation and separation are fast and allow high throughput applications. The intercompany study performed and described here delivered precise and robust results without the need for prior method training. CZE for charge heterogeneity profiling of MAbs is stability indicating, precise, accurate, robust, linear and sensitive. This

demonstrates that it is suitable for use in a GMP environment. The method has a broad range of applicable analyte p*I* and therefore is generically applicable for MAbs. CZE appears as a new powerful platform technology for the charge heterogeneity testing of antibodies in the pharmaceutical industry.

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