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Research Article

Interference-free mass spectrometric detection of capillary isoelectric focused proteins, including charge variants of a model monoclonal antibody

CIEF represents an elegant technique especially for the separation of structural similar analytes, whereas MS is a state-of-the-art instrumentation for the identification and characterization of biomolecules. The combination of both techniques can be realized by hyphenating CIEF with CZE-ESI-MS applying a mechanical valve. During the CZE step, the remaining ESI-interfering components of the CIEF electrolyte are separated from the analytes prior to MS detection. In this work, a multiple heart-cut approach is presented expanding our previous single heart-cut concept resulting in a dramatical reduction of analysis time. Moreover, different sample transfer loop volumes are systematically compared and discussed in regard to peak width and transfer efficiency. With this major enhancement, model proteins (1.63–9.75 mg/L), covering a wide pI range (5–10), and charge variants from a deglycosylated model antibody were analyzed on intact level. The promising CIEF-CZE-MS setup is expected to be applicable in different bioanalytical fields, e.g. for the fast and information rich characterization of therapeutic antibodies.

Keywords:
Isoelectric focusing / Mass spectrometry / Monoclonal antibody / Online hyphenation
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1 Introduction

IEF combined with SDS PAGE represents a common technique for the characterization of proteins [1]. With this approach, impressive results were achieved based on the orthogonality of pI value and molecule mass. However, the mass accuracy applying SDS PAGE is low and in comparison to MS no additional information by MS/MS experiments can be collected. CIEF, developed by Hjerten and Zhu [2], is a method with reasonable success for the separation and analysis of proteins and peptides [3], providing the pI as an important identification feature. Moreover, CIEF shows a higher accuracy, improved reproducibility and the possibility for quantification and simple automation compared with classical IEF [4]. In order to bring the combination of separation according to the pI and characterization by mass to the next level, a combination of CIEF and MS is aspired unifying the best possible results of both techniques.

Up to date, the most common approach for the hyphenation of CIEF and MS represents the direct coupling via an ESI interface, which was first published by Tang et al. [5] and further developed by Zhong et al. [6]. Following this straightforward arrangement promising results have been achieved, e.g. the analysis of bacteria cell lysates [7] or the characterization of highly basic cytokine human interferon-gamma [9]. Nevertheless, in this way no separation of analytes and highly ESI-interfering ampholytes is achieved prior to MS detection, which inevitably results in considerable signal suppression and loss of data quality. In order to counteract these effects lower concentrations of ampholytes are commonly applied [8]. However, this approach is associated with the deterioration of the CIEF separation efficiency. On the other hand, a variety of applications were performed implementing an HPLC system between CIEF separation and MS detection, e.g. a protein profiling method including an online digestion [10]. In this way, it is possible to fully separate analytes and ampholytes prior to MS detection. However, this

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Abbreviations: IDA, iminodiacetic acid; **PEEK**, polyether ether ketone

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hyphenation technique is often associated with a complex and expensive instrument setup, dilution effects in the HPLC, a loss of resolution caused by implemented trap columns, excessive sample loop volumes and long analysis times. Besides the aforementioned limitations both, the direct hyphenation and the application of an HPLC intersystem, are fully comprehensive. Further important CIEF-MS hyphenation techniques have been reviewed recently [11].

MAbs represent the most rapidly growing class of therapeutics in recent years [12]. A variety of translation and stress-induced variants possibly occur during manufacturing and storage, which can have a significant influence on the mAb bioactivity [13]. For this reason, there is a high demand for the analysis of mAbs regarding purity and stability [14]. Traditional analysis technique includes i.a. gel-based IEF and IEC. Analyses based on IEC suffer from long method development times and low resolution, whilst IEF is associated with low throughput and difficulties regarding quantitative information [15]. In contrast, CIEF separations feature high resolution, short method development and analysis times, minimal solvent consumption and concentration factors of up to 500 [3, 12, 16]. However, CIEF methods have to fulfill certain criteria to be considered valid for mAb analysis, including i.a. a minimal EOF combined with a reproducible system [14]. Nevertheless, CIEF alone cannot be applied for complete identification of mAb variants, due to the lack of information associated with the commonly applied optical detection [16]. Therefore, the hyphenation of CIEF and MS would provide a powerful tool for the separation and detailed characterization of mAb charge variants.

In our previous work [17], we have shown a proof of concept study of a single heart-cut CIEF-CZE-MS system using a mechanical valve (20 nL sample loop) for the separation and interference-free MS detection of three model proteins. Here, the pI range of the CIEF dimension was expanded by analyzing a peptide/protein mix comprising six different analytes. The analysis time was significantly reduced by the implementation of a multiple heart-cut analysis of all (six) analytes from one CIEF run. Furthermore, different sample loop sizes were tested and evaluated in order to find appropriate transfer volumes. The applicability of our system to relevant biological samples is demonstrated by the analysis of charge variants of a model mAb.

2 Materials and methods

2.1 Materials

Ultrapure water of an electrical resistivity > 18 MΩcm was supplied by an UltraClear ultra violet (UV) system (Siemens water technologies, Günzburg, Germany) and was used for the preparation of all samples, rinsing solutions, and BGEs. Bare fused silica capillaries (50 μm i.d., 363 μm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). Methanol (LC-MS grade), isopropanol (LC-MS grade), sodium hydroxide (≥ 98%), acetic acid (supra-quality, 100%) and ammonia bicarbonate (≥ 99%, p.a.) were received from Carl Roth (Karlsruhe, Germany). L-arginine, poly(vinyl alcohol) (≥ 99%, average MW 89 000–98 000), myoglobin from equine skeletal muscle (95–100%), glutaraldehyde solution (50% in water, suitable for photographic applications), ß-lactoglobulin A from bovine milk (≥ 90% PAGE), ribonuclease A from bovine pancreas (≥ 60% SDS PAGE), angiotensin I, human (≥ 93%), carboanhydrase A from bovine erythrocytes (≥ 95%) and cytochrome C from equine heart (≥ 95%) were obtained from Sigma Aldrich (Steinheim, Germany). Hydrochloric acid (37% p.a.), phosphoric acid (85% p.a.) and formic acid (99–100% p.a.) were purchased from Häffner GmbH & Co KG (Asberg, Germany). Imminodiacetic acid (i) (98% p.a.) was received from Merck (Darmstadt, Germany). Servalyt™ 3–10 and 6–8 (40% w/v solution in water) were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). *N*-glycosidase F was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The C4N-4354-.02D microinjector four-port-valve including rotors (20, 10 and 4 nL sample loop) and stators out of plastic material (Valcon E®, PTFE/polyether ether ketone (PEEK) composition) were purchased from VICI AG International (Schenkon, Swiss). Nanosep 10 K omega centrifugal devices were obtained from Pall Life Sciences (Ann Arbor, MI, USA).

All used capillaries were neutral precoated with PVA to prevent any protein adsorption at the capillary wall and eliminate the EOF. The model antibody (mAb X) sample was deglycosylated by using *N*-glycosidase F. A detailed description of the capillary coating and mAb deglycosylation procedures can be found in the supporting information.

2.2 CIEF-CZE-MS setup

A scheme of the instrumental setup is shown in Fig. 1. In general, the CIEF-CZE-MS methods can be divided into two parts: First, the CIEF separation until the positioning of the analyte in the sample loop of the valve is performed. After switching the valve, the ESI-interfering components are separated from the analyte in the second dimension (CZE-MS). The most important method parameters are presented in the following two sections. An extensive description of the parameters is illustrated in Supporting Information Table S1.

2.3 Part 1: CIEF dimension and positioning of analytes in sample loop

CIEF with an external UV detector (J+M Analytik AG (Essingen, Germany)) was performed as first dimension utilizing an HP ^{3D}CE electrophoresis instrument from Agilent (Agilent Technologies, Waldbronn, Germany). A 40.0 cm PVA coated capillary was connected from CE1 to junction "S" of the mechanical valve through the external UV detector, which is displayed in Fig. 1. A 20.0 cm PVA capillary was connected from junction "W" to an external outlet vial. Thus, the CIEF separation takes place in a total length of 60.0 cm (plus the

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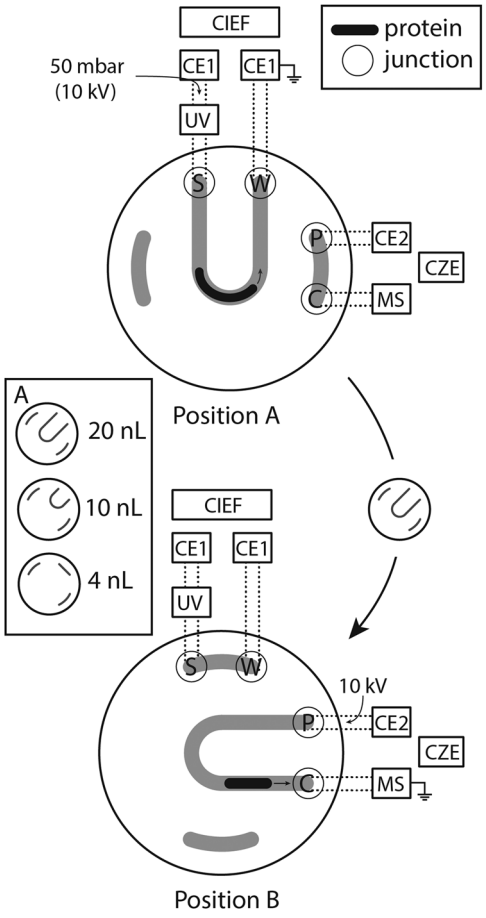


Figure 1. General arrangement of the 2D CIEF-CZE-MS setup. Thereby, CE1 and the UV detector represent the CIEF dimension and CE2 and the MS the CZE dimension. The separation capillaries of the CIEF dimension are connected to junction S and W, whereas the capillaries of the CZE separation are connected to junction P and C. During the mobilization step (with remaining 10 kV to keep the focusing) of the CIEF process the analyte of interest is positioned in the sample loop, subsequently the rotor is switched from position A to position B, connecting the sample loop with the second dimension. Finally, high voltage (10 kV) is applied in the CZE dimension and the analyte migrates to the MS. Inset A shows the appearance of the rotors with the different sample loop volumes which were tested in the here presented work (4, 10 and 20 nL).

distance inside the valve). The focusing distance (prior to UV detection) was 35.5 cm. L-arginine was used to block the distance from UV through the valve to the outlet vial in such a way, that all analytes are focused in front of the external UV detector. Anolyte and catholyte solution vials were kept at the same height to prevent any undesired mobilization by hydrodynamic effects during experiments. The preconditioning procedure prior to every measurement series and run can be found in Supporting Information Table S1. The sample composition is shown in detail in Supporting Information Table S1. Hence, IDA was used to prevent a cathodic drift of the sample, L-arginine was utilized as an anolytic spacer, urea to prevent protein precipitation and Servalyt™ for applying the pH-gradient inside the capillary and the protein, peptide or antibody. Injection was performed with approximately 1.5 bar for 1.5 min. 10 kV were applied for 10 min as focusing step. The mobilization was performed with pressure (50 mbar) and an additional high voltage (+10 kV) to preserve the separation until the signal of interest is positioned inside the sample loop. Therefore, the determination of the flow rate in the 1st dimension prior to each sample injection was required in order to achieve an accurate positioning of the analyte inside the sample loop. Hence, a caffeine standard (1000 mg/L) was injected (50 mbar, 12 sec) and 50 mbar were applied until the caffeine signal appears in the UV detector. Subsequently, the flow rate (50 mbar) was calculated by dividing the volume from the inlet to the UV detector by the required time of caffeine to pass the detector. After the desired analyte was positioned in the sample loop the valve was switched to position B. For the multiple heart-cut approach, 50 mbar were applied for the time distance between the previous and the next analyte in the UV detector, in order to cut and analyze the following analyte species. Cleaning and storage procedures of the capillaries are described in Supporting Information Table S1.

2.4 Part 2: CZE-MS dimension after positioning of the analyte inside the sample loop

CZE-MS was performed utilizing an HP ^{3D}CE electrophoresis instrument (Agilent Technologies, Waldbronn, Germany) and a *compact* quadrupole–quadrupole time of flight (QqTOF) instrument from Bruker Daltonik (Bremen, Germany). Two 40.0 cm long PVA capillaries were connected to the mechanical four-port valve. One was mounted between CE2 and junction "P" and the other between junction "C" and the ESI sheath liquid sprayer of the qTOF MS instrument (see also Fig. 1). Due to the bulkiness of the two CE and the MS instrument, a minimum length of 40 cm was required for the connection of CE2 and junction "P" of the four-port valve. The PVA capillary was positioned in the sheath liquid sprayer in such a way, that about 0.05 mm stuck outside of the sprayer tip. Isopropanol:water (1:1) with 1% (v/v) acetic acid was used as sheath liquid. The preconditioning, cleaning and storage procedures for the capillaries of the CZE dimension is explained in Supporting Information Table S1. The CZE separation was performed in 0.2 M formic acid as BGE applying 10 kV. A detailed description of the MS parameters for each analyte can be found in the supplementary information (Supporting Information Table S1).

3 Results and discussion

3.1 Transferring analyte from CIEF into CZE using a four-port valve

The transfer of the focused analytes toward the CZE-MS dimension represents a crucial step in the 2D system. A scheme

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of the transfer process is illustrated in Fig. 1. In our previous work, the analyte positioning procedure was performed with an external conductivity (C^4D) and UV detector [17]. However, the placement of two detection systems in front of the valve and the detection of the L-arginine zone is technical challenging. Therefore, a simpler, more robust, approach was pursued. Hence, the calculation of the positioning time of the analyte inside the sample loop of the valve is based on the application of one detector (UV) and the determination of the flow rate (50 mbar) prior to each analysis. The flow rate was determined with a caffeine standard (1000 mg/L) and 50 mbar, as explained in detail in the method section. At the point when the analyte reached the external UV detector the mobilization (50 mbar, +10 kV for refocusing) was remained for approximately 1 min, based on the calculated flow rate, to position the analyte inside the sample loop. The moment when the analyte has arrived at the sample loop the mobilization was stopped and the valve was switched from position A to B as illustrated in Fig. 1. By the determination of the flow rate prior to each run, minor changes in the flow conditions, e.g. caused by switching the valve, could be compensated to ensure a precise as possible cutting procedure.

3.2 Selection of optimal sample/transfer loop size

Three different sample loop sizes (4, 10 and 20 nL), as displayed in Fig. 1 were tested in this study. In general, the selection of the sample loop size is heavily dependent on the peak widths and resolution of analytes within the CIEF separation. For instance, the 10 nL sample loop size was found to be appropriated for the analysis of the model proteins (concentration: 6.5 to 39 mg/L), which is substantiated by the electropherogram in Supporting Information Fig. S1A. Here, a peak width of 8 (0.09 min) to 11 nL (0.13 min) was calculated taking the flow rate and the UV electropherogram of the mobilized CIEF separation in consideration. The distance of the closest signals, from peak maximum to maximum, was calculated to be 11 nL (0.13 min). In this regard, the 20 nL sample loop did not allow cuts of individual protein species, due to the excessive volume compared with the resolution of the signals. Applying the 4 nL sample loop a very precise cutting of single peaks was possible, however, a significant loss of signal intensity in the MS was consequently observed. In conclusion, the best choice for this application was the 10 nL sample loop, which allows the individual cutting of the analytes combined with the transfer of a majority of the desired analyte species to the 2^{nd} dimension. Nevertheless, if demanded, it is possible to cut more than one analyte, e.g. angiotensin I and myoglobin (signal 1 and 2 in Supporting Information Fig. S1A). Hence, both signals are transferred to the CZE-MS dimension by switching the valve. Thereby, angiotensin I and myoglobin could be baseline separated in the CZE dimension and thus, analyzed individually in the MS, which is shown in Supporting Information Fig. S1C.

Furthermore, the three rotors were tested for the analysis of a model antibody (mAb X). The results are displayed in Supporting Information Fig. S1B. By applying the 4 nL sample loop and cutting on signal 1 peak areas of $4.2\times10^3 \pm 7.9\%$ ($n = 3$) and peak heights of $5.4\times10^2 \pm 11.1\%$ ($n = 3$) were determined by integrating the base peak electropherogram. Despite the successful detection of a signal for mAb X, the sensitivity was too low for an accurate deconvolution which is essential to achieve precise mass determination. Utilizing the 10 nL sample loop peak areas of $8.1\times10^3 \pm 7.7\%$ ($n = 3$) and a height of $8.9\times10^2 \pm 3.1\%$ ($n = 3$), which was sufficient for deconvolution. The application of the 20 nL sample loop resulted in the highest achieved peak areas of $3.4\times10^4 \pm 6.7\%$ ($n = 3$) and peak heights of $1.5\times10^3 \pm 4.0\%$ ($n = 3$). However, the deconvolution resulted in shifting average masses (data not shown), which can be explained by the cut of more than one mAb variant due to the excessive transfer volume. In conclusion, the 10 nL rotor was also the best choice for the analysis of mAb X and thus, used in all further experiments.

3.3 Multiple heart-cut CIEF-CZE-MS of model proteins

In order to significantly reduce the overall analysis time combined with less sample consumption, the heart-cut procedure, described in our previous work [17], was expanded to a "multiple heart-cut" approach, enabling to cut more than one peak within one CIEF run. Therefore, a standard mixture containing five model proteins and one peptide, covering a broad pI range (5.1–10.25), was analyzed. All six signals were baseline separated in the CIEF-UV dimension, as displayed in Fig. 2. Each analyte, from cytochrome C (pI = 10.25) to ß-lactoglobulin (pI = 5.1), was positioned in the sample loop, transferred to the second dimension and analyzed via MS consecutively. Each protein could be identified by their respective MS spectra (see Fig. 2) and the according deconvoluted mass. A separation of analyte and ampholyte molecules for an interference-free MS detection of the CIEF separated proteins was achieved in the same way as demonstrated in our previous work [17]. For a precise transfer of the proteins, a reproducible mobilization time and moreover, a consistent resolution between the individual analyte signals is crucial. Therefore, four UV runs on two different days ($n = 4$) were performed and compared regarding mobilization time. The results are summarized in Supporting Information Table S2. The RSD values of the mobilization time were $\leq 1.86\%$ for all investigated analyte species, which is sufficient for a precise positioning of the analyte inside the sample loop of the valve. Furthermore, the current from start (10.27 μA ± 5.38%) to the end of the focusing step (5.93 μA ± 5.15%) was comparable for all runs, which indicates a reproducible and stable CIEF system setup. No considerable peak broadening of the focused proteins in the CIEF dimension during the individual CZE analyses was observed, which was tested by inverse mobilization of the proteins back to the front of the external UV detector and comparison of the resulting peak widths. In this way, the precise cutting of more than one protein per run was verified. An unintentional mobilization of the focused

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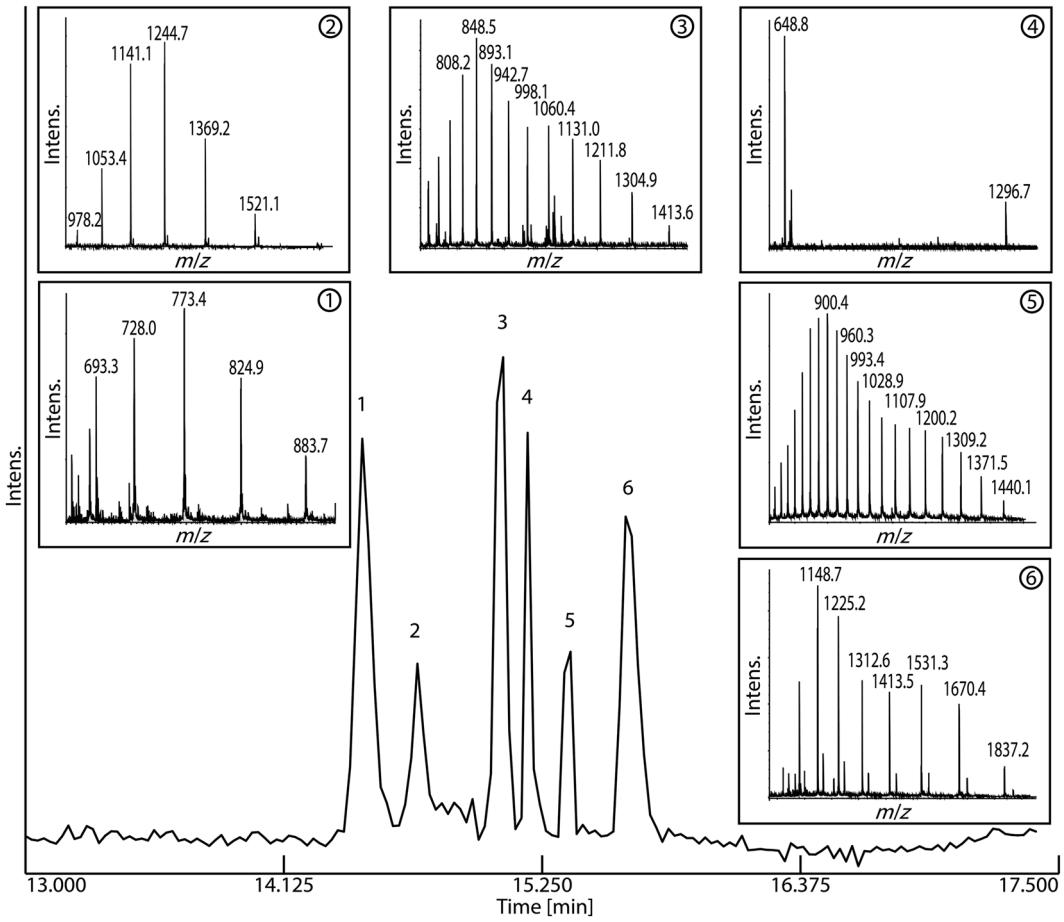


Figure 2. Mobilization of the focused model proteins applying 0.1 M H_3PO_4 as anolyte and 0.2 M NaOH as a catholyte. Focusing was achieved by 10 kV for 10 min. Mobilization was performed utilizing 50 mbar until the first signal is positioned in the four-port valve. The high voltage was remained during the mobilization. UV signals at 280 nm (main Figure) and the MS spectra (inset 1 – 6) at the migration time of the protein in the CZE-ESI-MS dimension are shown: cytochrome C; pI = 10.3 (1), RNAse A; pI = 9.6 (2), angiotensin I; pI = 7.9 (3), myoglobin; pI = 7.2 (4), carboanhydrase A; pI = 6.4 (5) and ß-lactoglobulin; pI = 5.1 (6). The linearity between the mobilization time and the pI value was given ($R^2 = 0.996$).

proteins during the analysis in the second dimension was prevented by adjusting the inlet and outlet vial to the exact same height, avoiding hydrostatic effects. A peak broadening of the focused analytes in the first dimension is noticeable after approximately 10 CZE-MS analyses (~10 min per run). Therefore, a full comprehensive approach was not feasible. Nevertheless, the overall analysis time, characterizing each peak once ($n = 6$), was significantly reduced from >6 h to 2.8 h applying the novel multiple heart-cut approach.

3.4 Multiple heart-cut CIEF-CZE-MS of mAb X

The separation of intact mAb charge variants represents a common application for CIEF, e.g. in product stability testing. Nevertheless, an MS analysis of CIEF separated variants is challenging due to the highly ESI-interfering ampholytes, the closely related structures of these variants and the low volumes in capillary-based separations. Still, the MS analysis of antibody variants is of high interest, especially in the pharmaceutical industry. Therefore, deglycosylated mAb X (solved in formulation buffer) was analyzed as model monoclonal antibody to test the applicability of the 2D system for mAb characterization. Four different signals were observed in the electropherogram of the first dimension after the focusing step, which is displayed in Supporting Information Fig. S2. Signal 1 and 4 were transferred to the second dimension using the multiple heart-cut procedures for further mass spectrometric characterization. The raw and deconvoluted mass spectra of both species can be found in Fig. 3. The deconvolution of signal 1 resulted in an exact mass of 145168.6 ± 0.4 Da ($n = 4$). Signal 4 resulted in an exact mass of 145 170.7 ± 0.6 Da ($n = 4$). A mass difference of 2 Da in combination with a significant shift in the pI value of the variants could be explained by potential deamidation (or amidation) processes. A detailed interpretation of the relative intensity of these variants was not possible due to the occurrence of additional chemical deamidation processes caused

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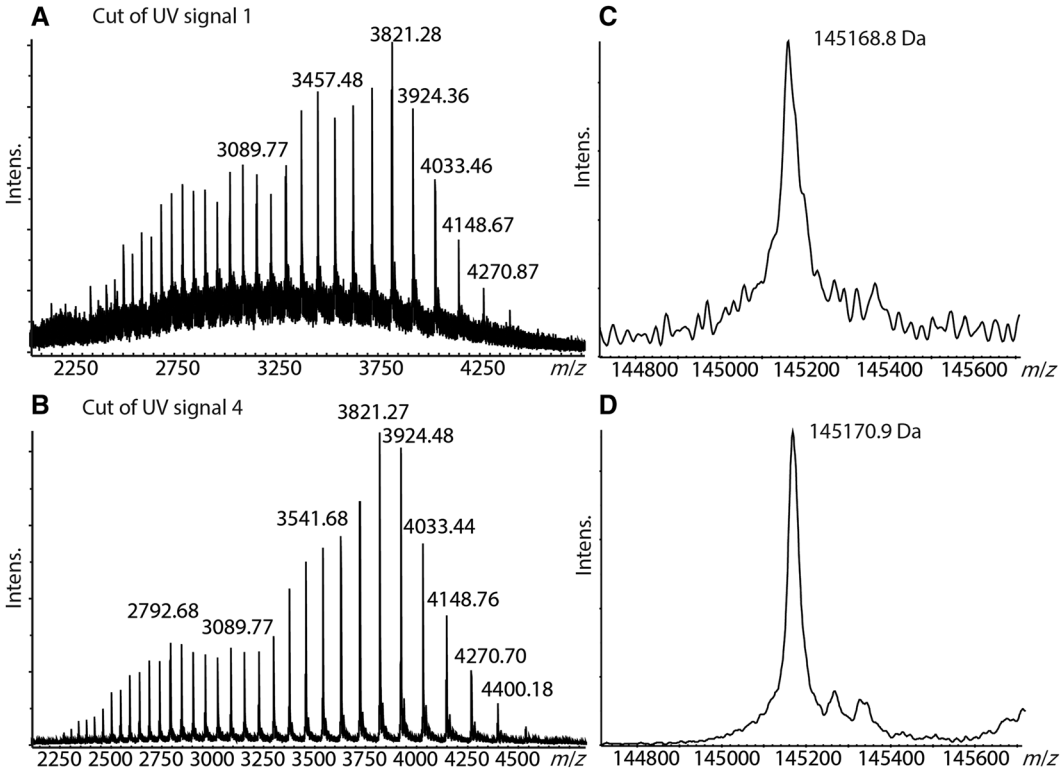


Figure 3. MS identification of variants from CIEF-CZE-MS separation of mAb X. Cuts from focusing of signal 1 (A) and 4 (B) and the corresponding deconvoluted mass spectra (C and D). A difference of approximately 2 Da was observed between the mAb variants.

by the PNGase F treatment [18]. Complementary techniques, such as analyses on peptide level, could support to ensure the occurrence of deamidation products.

3.5 CIEF-CZE-MS limit of detection for proteins

In general, CIEF provides a considerable enrichment factor in comparison to other electromigrative techniques such as CZE, due the IEF separation mechanism and thus, represents an excellent technique for the analysis of low concentrated samples [19]. Therefore, the LOD (S/N 3:1) for the 2D CIEF-CZE-MS approach was estimated. In the case of the low mass (10–50 kDa) protein/peptide mixture the external UV detector proved to be the limiting factor. The LOD of the UV detection (280 nm wavelength) was between 9.75 (RNAse A) and 1.63 mg/L (myoglobin). The UV signal of RNAse A (1) and myoglobin (2) at the LOD is shown in Fig. 4. Obviously, a lower concentration would result in difficulties regarding the clear identification of the protein signals and precise cutting of the individual analytes species. Nevertheless, the lowest concentration (9.75 mg/L for RNAse A and 1.63 mg/L for myoglobin) lead to mass spectra with sufficient intensity for deconvolution. Moreover, the LOD in the MS was estimated to be 0.24 mg/L for RNAse A and 0.20 mg/L for myoglobin. The respective mass spectra of RNAse A and myoglobin at the LOD level of the UV detector are displayed in 4 and C. Reproducibility data of three different concentration levels and two different proteins (RNAse A and myoglobin) are shown in Supporting Information Table S3. The concentrations were selected to cover a range from the model protein experiment (see above) down to the LOD of the protein in the UV detection. As an indicator for a precise cutting, the peak area values of the extracted ion electropherogram of the most abundant charge variant of each protein were considered. As expected, the RSD increased from the highest (5.5–7.9%) to the lowest concentration (17.3–21.6%) due to less signal intensity and difficulties during the identification of the peak maximum in UV for the cutting. Still, a more sensitive detection in the first dimension would enable to analyze even lower concentrated proteins. Nevertheless, the MS identification of proteins in the 1–10 mg/L range represents a promising result.

In the case of the model antibody mAb X, the MS showed to be the limiting factor regarding sensitivity. Appropriate raw mass spectra were achieved for a sample containing 375 mg/L. Still, in order to achieve sufficient signal quality for an accurate deconvolution, a concentration of 1500 mg/L mAb X was required. This rather high LOD can be explained by the low ionization efficiency of high-molecular-weight species such as the analyzed model antibody. Nevertheless, 1500 mg/L for an MS analysis represents an excellent value compared to previous works regarding CZE-MS analysis of antibodies. Exemplarily, Biacchi et al., 2015 demonstrated a glycoform separation and characterization by an offline coupling of CZE-UV with ESI-MS with a sample concentration of 5000 mg/L [20]. Another common top-down analysis

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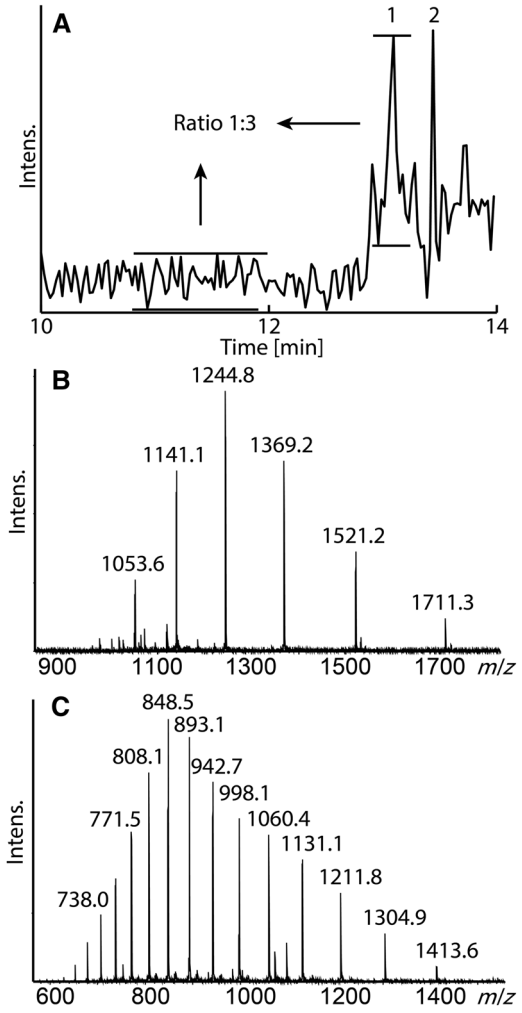


Figure 4. (A) UV signal of CIEF mobilization of a sample containing RNAse A and myoglobin at the LOD (S/N 3:1) level of UV detection (9.75 and 1.63 mg/L, respectively). Noise was defined as baseline noise in proximity to the protein signals. B and C illustrate the respective mass spectra of RNAse A and myoglobin.

technique for mAbs is provided by ultra-high-performance liquid chromatography (UHPLC)-MS. Applying this approach for the analysis of free thiol variants of a recombinant mAb, Liu et al., 2015 analyzed a sample containing 2000 mg/L [21]. Performing an in-house comparison of our CIEF-CZE-MS setup including the 10 nL transfer loop with a CZE-MS system utilizing 10 nL as injection volume, areas of 1.1×10^5 (1500 mg/L) and 0.9×10^5 (30 000 mg/L) were found, respectively. Taking the different concentrations into account a factor of 23 higher sensitivity for the CIEF-CZE-MS setup was determined.

4 Concluding remarks

The high resolution and enrichment factor of CIEF combined with interference-free, high accuracy mass detection represents a promising approach for the analysis of different bioanalytical and biopharmaceutical samples. Compared to our previous work, a "multiple heart-cut" procedure was established considerably reducing analysis time. Up to six analytes, covering a wide pI range of 5.1–10.3, were transferred from a single CIEF run and analyzed via CZE-MS in less than 3 h. In addition, it was proven that it is possible to separate proteins with similar pI values (angiotensin I and myoglobin) in the CZE dimension prior to MS detection. In the case of low to medium molecular weight proteins (10–50 kDa), the external UV detection represents the limiting factor regarding LODs. Nevertheless, it was possible to detect these peptides/proteins down to a concentration of 1–10 mg/L with more than sufficient signal intensity in the MS. Moreover, a more sensitive external UV detector would improve the LOD values of the complete 2D system for these analyte species. Nevertheless, the LOD values indicate the great benefit of the CIEF-CZE-MS approach compared with the classical CZE-MS analysis of proteins. A promising application of the CIEF-CZE-MS system is provided by the mass spectrometric characterization of intact mAb charge variants. It was possible to detect a mass difference of approximately 2 Da between two variants of mAb X. In contrast to the MS analysis of antibody variants by CIEF followed by fraction collection combined with HPLC-MS, the here presented system represents a straight forward and fast approach. The CIEF-CZE-MS setup using a mechanical valve embodies a highly promising system, which can potentially be applied in many different fields of pharmaceutical and biochemical analysis, such as the demonstrated characterization of mAb charge variants.

The authors have declared no conflict of interest.

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