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Research Article

Capillary zone electrophoresis tandem mass spectrometry detects low concentration host cell impurities in monoclonal antibodies

We have evaluated CZE-ESI-MS/MS for detection of trace amounts of host cell protein impurities in recombinant therapeutics. Compared to previously published procedures, we have optimized the buffer pH used in the formation of a pH junction to increase injection volume. We also prepared a 5-point calibration curve by spiking 12 standard proteins into a solution of a human mAb. A custom CZE-MS/MS system was used to analyze the tryptic digest of this mixture without depletion of the antibody. CZE generated a ~70-min separation window (~90-min total analysis duration) and ~300-peak capacity. We also analyzed the sample using ultra-performance LC-MS/MS. CZE-MS/MS generated approximately five times higher base peak intensity and more peptide identifications for low-level spiked proteins. Both methods detected all proteins spiked at ~100 ppm level with respect to the antibody.

Keywords:
Bottom-up proteomics / CZE / Host cell proteins DOI 10.1002/elps.201500301

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1 Introduction

Recombinant human monoclonal antibodies are commonly used in research, diagnostics, and therapy. Recombinant antibodies are produced by expressing the desired gene in a host cell. After culture, the target protein is purified through multiple chromatographic steps to remove, among other contaminants, the vast majority of host cell proteins (HCPs), resulting in a highly pure product [1, 2]. During this process, some HCPs potentially copurify with the target protein. Residual HCPs present a safety risk in biopharmaceutical products [3]. Therefore, identification and quantification of these HCPs are important in determining potential clinical safety risks.

Traditional methods for measuring and detecting HCPs either use an immunoassay with a polyclonal antibody directed against proteins from the host cell organism, which is suitable to estimate the total HCP levels but not for comprehensive identification, or SDS-PAGE, which provides limited quantitative information [4–6]. Alternative HCP analyses utilize MS. Typically, MS is coupled to a separation method such as RPLC. Several reports describe the use of 2D-LC-MS/MS for identification of HCPs from purified recombinant proteins [7, 8]. With depletion of mAb by using a Protein A column, HCPs can be identified at the 10–1000 ppm range [8]. HCP impurities in the 20- to 400-ppm level were quantified from *Escherichia coli* cultures expressing peptibodies [9]. 2D-LC-MS/MS allow the first dimension column to be overloaded, resulting in the overall dynamic range of the analysis to be increased compared to 1D LC-MS/MS. However, some hydrophilic peptides that are poorly retained on column and super hydrophobic peptides that are difficult to elute from column will be lost during analysis. Also, the carry-over of LC-MS makes it difficult to analyze trace-level HCPs.

CZE can provide rapid and efficient separation of biological molecules. Compared with LC, CZE provides very low carry-over, relatively fast separation, and inexpensive instrumentation. The development of sensitive and robust CZE-MS interfaces has led to a reinvestigation of CZE-MS as an alternative to RPLC for proteomics research [10–18].

In 2007, a sheathless interface employed a porous capillary tip as the nanospray emitter was reported [19] Several research groups has used this interface for bottom-up (shot-gun) and top-down proteomics [11, 12, 20]. In 2010,

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Abbreviations: FA, formic acid; **HCP**, host cell protein; **HF**, hydrofluoric acid; **IAA**, iodoacetamide; **LB**, lysogeny broth; **LPA**, linear polyacrylamide; **TPCK**, tosyl phenylalanyl cloromethyl ketone; **UPLC**, ultra-performance LC

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Chen's group reported a flow through microvial interface [21], which has been applied for metabolite [22], glycan [23], and intact protein [24] analysis. Tang's group reported a sheathless CE-MS interface combining a large inner diameter separation capillary and a detachable small inner diameter porous ESI emitter [25].

We have previously described three generations of an electrokinetically pumped sheath-flow electrospray interface to couple CZE with MS [10, 16, 18]. Electroosmosis at the emitter surface generated stable sheath flow at nanoliter per minute rates, resulting in nanospray operation.

Previously, we reported the use of CIEF-MS/MS with our first generation interface for identification of HCPs in a recombinant humanized mAb [26]. The mAb first was depleted from the sample using Protein A and Protein L chromatography; this depletion reduced interference from the mAb. This analysis identified 37 HCPs, but provided no determination of the abundance of those proteins [26]. We then reported the use of CZE for quantification of three HCPs in mAb [27]. In that report, three peptides were synthesized containing isotopically labeled lysine or arginine residues. These labeled peptides were spiked in the HCP digests at known concentrations, and the spiked HCP digests were analyzed by CZE-ESI-MS/MS. By this method, we were able to estimate the absolute amount of HCPs.

In these previous studies, Protein A and Protein L affinity chromatography was used to deplete the mAb from the sample prior to analysis. This depletion was done to reduce the background signal from the mAb itself. However, protein depletion prior to analysis extends the amount of time required for sample evaluation, and there is concern that some HCPs, such as hitchhiker proteins [28], may be lost in the depletion step. In this report, we generated synthetic HCP samples by spiking five concentration levels of 12 different protein standards in a recombinant mAb. We eliminated the use of Protein A/L depletion, and, instead, analyzed samples containing the target protein. This step dramatically simplifies the workflow and eliminates loss of HCPs during the depletion step. CZE-MS/MS has been suggested to be an alternative to RPLC in shotgun proteomics. According to published reports, CZE-MS/MS can generate superior performance to RPLC-MS/MS for trace peptide analysis [12, 14].In this report, after trypsin-based protein digestion, CZE- and RPLC-MS/MS were used to analyze the spiked protein digests. Both methods detected all 12 proteins present at an estimated ~100 ppm level with respect to the antibody, and one was detected at ~10 ppm level. Both CZE- and RPLC-MS/MS appear to be useful for the detection of trace impurities in undepleted recombinant antibodies. CZE consistently detected more peptides than RPLC for almost all proteins at all concentrations.

2 Materials and methods

2.1 Reagents

Acrylamide, ammonium persulfate, bovine pancreas TPCK-treated trypsin, urea, ammonium bicarbonate (NH_4HCO_3), DTT, and iodoacetamide (IAA), and 3-(trimethoxysilyl)propyl methacrylate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (FA) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Methanol and water were purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). Capillary (50 μm id/365 μm od) was purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2 Preparation of capillary

A fused silica capillary (50 μm id/365 μm od) was flushed with 1 M hydrochloric acid for 30 min, water for 10 min, 1 M sodium hydroxide for 30 min, water for 10 min, and finally methanol for 30 min. The capillary was then dried under nitrogen gas at room temperature for 4 h. After that, 50% v/v 3-(trimethoxysilyl) propyl methacrylate in methanol was flushed through the capillary for 10 min. Both ends of the capillary were sealed and the capillary was incubated at room temperature for 24 h. Finally, the capillary was rinsed with methanol for 20 min and dried under nitrogen. The pretreated capillary was stored at room temperature before use.

2.3 Linear polyacrylamide (LPA) coating preparation

The LPA coating preparation has been described elsewhere [29]. Briefly, 2 μL of 5% w/v ammonium persulfate was added to 500 μL of 4% w/v acrylamide solution. The mixture was vortexed for 30 s and degassed for 5 min using nitrogen. Then, the mixture was introduced into a 100-cm long piece of pretreated capillary by vacuum. With both ends sealed, the capillary was put into 50°C water bath for 30 min. After incubation, unreacted material was flushed from the capillary. Finally, the distal end of capillary was etched with HF for 90 min to an outer diameter of about 70 μm [15–17].

2.4 Preparation of samples

Escherichia coli digests were prepared as reported previously [30]. Briefly, *E. coli* was cultured in LB medium and lysed by sonication. After cell culture and cell lysis, 900 μg of *E. coli* homogenate was precipitated by acetone and then dried at room temperature. The dried sample was dissolved in 300 μL of 100 mM NH4HCO3 (pH 8.5) containing 8 M urea. After denaturing at 37°C for 60 min, the proteins were reduced by 8 mM of DTT (final concentration) at 60°C for 1 h and alkylated by 20 mM of IAA (final concentration) at room temperature for 30 min in the dark, followed by dilution to 1.5 mL with 100 mM NH4HCO3 (pH 8.5) to reduce the urea concentration less than 2 M. An aliquot of 120 μg of treated proteins was digested by trypsin at a trypsin/protein ratio of 1/30 w/w for 4 h at 37°C.

Twelve standard proteins (TRFE_BOVIN, TPIS_YEAST, ENO1_YEAST, IPYR_YEAST, HXKB_YEAST, ALBU_BOVIN, G6PD_YEAST, CAH2_BOVIN, G3I3Y6_CRIGR, LALBA_BOVIN, G6PI_YEAST, and ADH1_YEAST

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Table 1. Concentration of spiked proteins (ng/mL or ppm) added to 1 mg/mL mAb-1

Spiked protein	ID	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
TRFE_BOVIN	1	1800	590	200	66	22
TPIS_YEAST	2	620	210	70	23	8
ENO1_YEAST	3	860	290	95	32	10
IPYR_YEAST	4	530	180	60	19	6
HXKB_YEAST	5	710	240	80	26	9
ALBU_BOVIN	6	930	310	105	35	13
G6PD_YEAST	7	640	210	70	24	8
CAH2_BOVIN	8	1000	330	110	37	12
G3I3Y6_CRIGR	9	1000	330	110	37	12
LALBA_BOVIN	10	1000	330	110	37	12
G6PI_YEAST	11	85	28	9.4	3	1
ADH1_YEAST	12	830	280	90	31	10

from Sigma-Aldrich) were spiked in 1 mg/mL of a human mAb (mAb-1; produced as described in [31] obtained from MedImmune [Gaithersburg, MD, USA]) with five levels of spiked proteins concentration relative to mAb-1 (Table 1). hundred microliters aliquots of the five samples were lyophilized and dissolved in 100 mM NH_4HCO_3 containing 1 M urea. Proteins were denatured at 90°C for 10 min, followed by standard reduction and alkylation with DTT and IAA, respectively. Digestion was performed for 4 h at 37°C with trypsin at a trypsin/protein ratio of 1/30 w/w. The digests were desalted with a C18 SepPak column (Waters, Milford, MA, USA), followed by lyophilization with a vacuum concentrator (Thermo Fisher Scientific, Marietta, OH, USA). The dried samples were stored at −20°C before use. Before analysis, samples were resuspended in 10 mM NH_4HCO_3.

2.5 CZE-MS/MS

A PrinCE autosampler (Prince Technologies B.V., The Netherlands) was used for automated sample injection and control of the separation voltage. An electrokinetically driven sheath-flow CE-MS nanospray interface was employed to couple the capillary with the mass spectrometer as described [18]. Briefly, 5% acetic acid was used as the separation buffer and 0.5% formic acid with 10% methanol was used as the electrospray sheath buffer. The nanospray high voltage was supplied by a Spellman CZE 1000R power supply. The emitter was pulled in a P-1000 Sutter pipette puller to a 25 μm od The nanospray voltage was ∼2 kV. The separation was performed at 25 000 V across 1 m of LPA-coated capillary. Hydrodynamic sample injection was performed at 500 mbar pressure for 0.2 min (injection volume 100 nL, 100 ng). Triplicate runs were performed for each sample.

For optimization of pH junction injection, the LPA-coated capillary was coupled to an LTQ mass spectrometer (Thermo Fisher Scientific). Three pH values of sample matrix buffer were evaluated: 10 mM ammonium acetate at pH 6.5, 10 mM NH_4HCO_3 at pH 8.5, and 0.1% NH3H2O at pH 10.0. In total, 0.1% formic acid was used as BGE. Hydrodynamic sample injection was performed at 500 mbar pressure for 1 min (injection volume 250 nL, 100 ng). Full MS scans were acquired over the 395−1900 m/z range. The ten most intense peaks were selected for fragmentation in the ion trap with normalized collision energy of 35%, activation $q = 0.25$, activation time of 30 ms, and one microscan. Peaks selected for fragmentation more than once within 45 s were excluded from selection for 60 s.

For analysis of the spiked protein samples, the LPA-coated capillary was coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Full MS scans were acquired in the Orbitrap mass analyzer over the m/z 350−1500 range with a resolution of 60 000 (at 400 m/z). The 20 most intense peaks with a charge state ≥2 were fragmented in the ion trap with normalized collision energy of 35%, activation $q = 0.25$, activation time of 10 ms, and one microscan. Peaks selected for fragmentation more than once within 20 s were excluded from selection for 60 s.

2.6 LC-MS/MS

For peptide separation and identification, a nanoACQUITY UPLC system with a UPLC BEH 130 C18 column (Waters, 100 μm id × 100 mm length, 1.7 μm particles, column temperature 40°C) was coupled to an LTQ Orbitrap Velos instrument via a pulled capillary tip as the electrospray tip. The RPLC gradient (A, 0.1% v/v FA in water; B, 0.1% v/v FA in ACN) was as follows: 0−10 min, 2% B; 10−11 min, 2−8% B; 11−71 min, 8−30% B; 71−72 min, 30−80% B; 72−77 min, 80% B; 77−78 min, 80−2% B; 78−90 min, 2% B. The flow rate was 1 μL/min. The sample injection volume was 1 μL (100 ng). The electrospray voltage was 1.5 kV, and MS parameters were the same as that used for CZE-ESI-MS/MS analysis.

2.7 Data analysis

Raw MS files were analyzed by MaxQuant [32] version 1.3.0.5. MS/MS spectra were searched by the Andromeda search engine [33] against the CHO database plus 12 spiked protein sequences. The database also included common contaminants. MaxQuant analysis included an initial search with a precursor mass tolerance of 20 ppm, main search precursor mass tolerance of 6 ppm, and fragment mass tolerance of 0.5 Da. The search included enzyme as trypsin, variable modifications of oxidation (M), acetylation (K and protein N-terminal) and deamidation (NQ), and fixed modification of carbamidomethyl cysteine. Minimal peptide length was set to six amino acids and the maximum number of missed cleavages was set to two. The false discovery rate was set to 0.01 for both peptide and protein identifications. The protein intensity of 12 spiked protein was picked up for drawing label-free quantification curve and absolute quantification curve.

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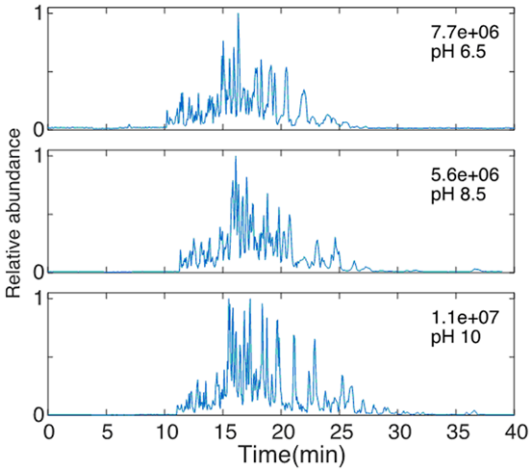


Figure 1. Base peak electropherograms of *Escherichia coli* digests by CZE-MS coupled with pH junction injection with different pH sample matrix (pH 6.5, pH 8.5, and pH 10). Maximum normalized level noted for each electropherogram.

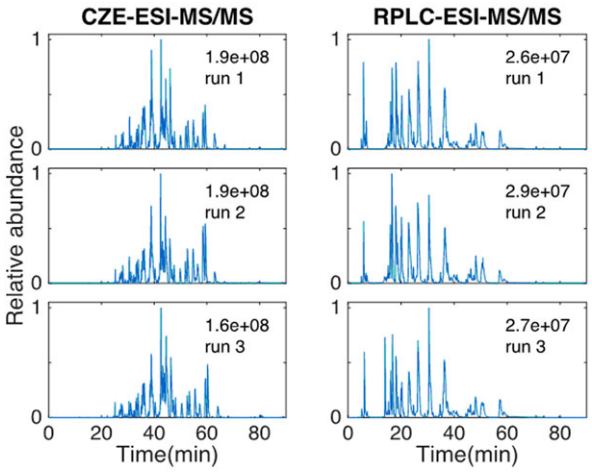


Figure 2. Base peak electropherograms/chromatograms of Sample 4 digest for triplicate CZE-MS and LC-MS runs. Maximum normalized level noted with each separation.

3 Results and discussion

3.1 Optimization of sample matrix in pH junction injection

A pH junction injection was employed with CZE-MS to increase the sample injection volume [24]. We first optimized the pH value of sample matrix. Three pH values of sample matrix buffer were evaluated: 10 mM ammonium acetate at pH 6.5, 10 mM NH_4HCO_3 at pH 8.5, and 0.1% NH_3H_2O at pH 10.0. Figure 1 shows the electropherograms generated from 0.1 mg/mL *E. coli* digests at these pH values. An increase in the pH of the resuspension buffer resulted in a sharpening of late migrating peptides, with the pH 10 sample matrix showing the best performance. A late migrating peak with m/z 870.3857 and +2 charge was extracted from the electropherograms (Supporting Information Fig. 1). The peak width decreases with increasing pH of the sample matrix. However, high pH buffers hydrolyze the silica walls, which lead to degradation of the capillary coating. As a result, the pH 8 NH_4HCO_3 buffer was selected as the sample matrix to perform pH junction injection for label-free quantification analysis with minimal damage to the capillary coating.

3.2 Separation and identification of 12 spiked proteins

RPLC-MS/MS and CZE-MS/MS were performed for analysis of five samples. Figure 2 shows triplicate base peak chromatogram and electropherograms of Sample 4 by RPLC-MS/MS and CZE-MS/MS, respectively. Sample 4 contained relatively low levels of spiked proteins, and the electropherograms are dominated by peptides generated from the digested antibody. The CZE separation window is ~70 min with a 1-m long capillary, which is comparable to the RPLC separation with 90 min gradient. The separation generated ~300-peak capacity. The wide separation window results from the high-quality LPA coating performance using a thermally initiated free radical polymerization method [29]. The separation windows in CZE-MS are comparable to RPLC-MS. However, CZE-MS/MS produces approximately five times higher base peak intensities and narrower peak widths than RPLC-MS/MS. These results agree well with previous reports in which CE-MS shows higher sensitivity compared to LC-MS when equal sample amounts were injected [12, 14].

Database searching was performed with MaxQuant software against CHO plus the 12-spiked protein sequence database. Table 2 shows the protein identification based on tandem MS spectra matching. CZE consistently detected all spike impurity proteins present at ~100 ppm with respect to the antibody, and at the 10 ppm level in favorable cases. CZE consistently detected more peptides than RPLC for almost all proteins at all concentrations; the increased identifications for low abundance spiked impurities using CZE is likely due to the higher peak intensity compared to RPLC-MS/MS.

3.3 Label-free quantitation

We plotted the MaxQuant estimate of protein intensity (Supporting Information Table 1) versus the amount of spiked protein for both CZE-MS/MS and RPLC-MS/MS (Fig. 3). With the exception of ALBU_BOVIN ($r = 0.65$), the correlation coefficients were greater than 0.92 for both the CZE-MS/MS and RPLC-MS/MS data. The average slope of the log–log calibration curve for CZE-MS/MS, 1.6 ± 0.4, is indistinguishable from the RPLC-MS/MS slope, 1.5 ± 0.2.

Note that Fig. 3 and Supporting Information Table 1 include several cases where the peptide was not identified by tandem mass spectral matching, and those peptides are not

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Table 2. Number of unique peptide identifications by MS/MS spectra matching in CZE-MS/MS and UPLC-MS/MS

Spiked protein	CZE-MS/MS					UPLC-MS/MS				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
TRFE_BOVIN	40	16	1	1	0	31	23	6	1	0
TPIS_YEAST	17	7	1	0	0	7	4	1	0	0
ENO1_YEAST	16	6	0	0	0	10	5	0	0	0
IPYR_YEAST	14	10	3	0	0	6	4	1	0	0
HXKB_YEAST	19	11	1	0	0	13	4	2	0	0
ALBU_BOVIN	42	35	41	37	29	16	8	9	6	5
G6PD_YEAST	19	7	1	0	0	11	8	1	0	0
CAH2_BOVIN	11	6	4	0	0	6	4	1	0	0
G3I3Y6_CRIGR	9	4	1	0	0	8	4	2	0	0
LALBA_BOVIN	8	3	2	1	0	4	1	0	0	0
G6PI_YEAST	15	3	0	0	0	7	1	0	0	0
ADH1_YEAST	9	3	2	0	0	4	3	2	0	0

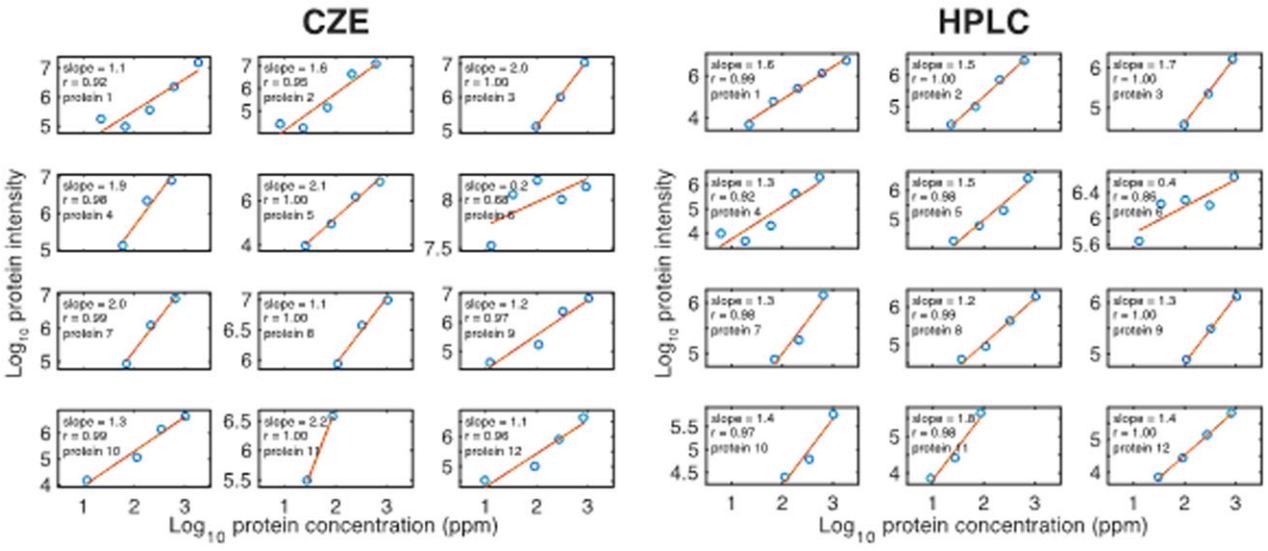


Figure 3. Log–log calibration curves for 12 proteins. Intensity is estimated by MaxQuant analysis of the data. The CZE data are on the left, HPLC data on the right. Protein identification number is given in Table 1.

included in Table 2. During database searching by MaxQuant, the "match between runs" option was chosen, which can transfer peptide identifications to unsequenced or unidentified peptides by matching their mass and retention times [36]. By using this approach, we can increase the number of peptides that can be used for quantification beyond those that have been sequenced and identified by an MS/MS database search engine.

ALBU_BOVIN generated calibration curves with poor linearity. The signal intensity was much higher for this protein than the others, and we likely began to saturate the mass spectrometer's response, particularly for the higher concentration samples. Impurities in the other spiked-in proteins may have contributed to the Albu_Bovine signal. As expected from a comparison of the base peak intensities observed in Fig. 2, the average ratio of CZE to RPLC intensities for 11 proteins was 5 ± 2, neglecting ALBU_BOVIN. That protein generated a 30-fold higher intensity using CZE.

Compared with LC-MS, CZE-MS has low carry-over, so no column wash step is needed between different samples. CZE-MS can generate lower flow rates, which will contribute to the higher sensitivity. The big disadvantage of CZE-MS is low sample loading volume capacity, which limits the performance of CZE-MS for low concentration samples. However, efforts have been made to increase sample loading in CZE-MS. For example a solid-phase microextraction column was attached at the proximal end of the capillary to allow quite large volume sample injection [12, 35, 37].

3.4 Absolute quantification curve

The absolute amount of impurities is important to evaluate the quality of biopharmaceutical products and understand potential clinical safety risks of HCPs. Typical absolute quantification is using isotope-labeled reference peptides as

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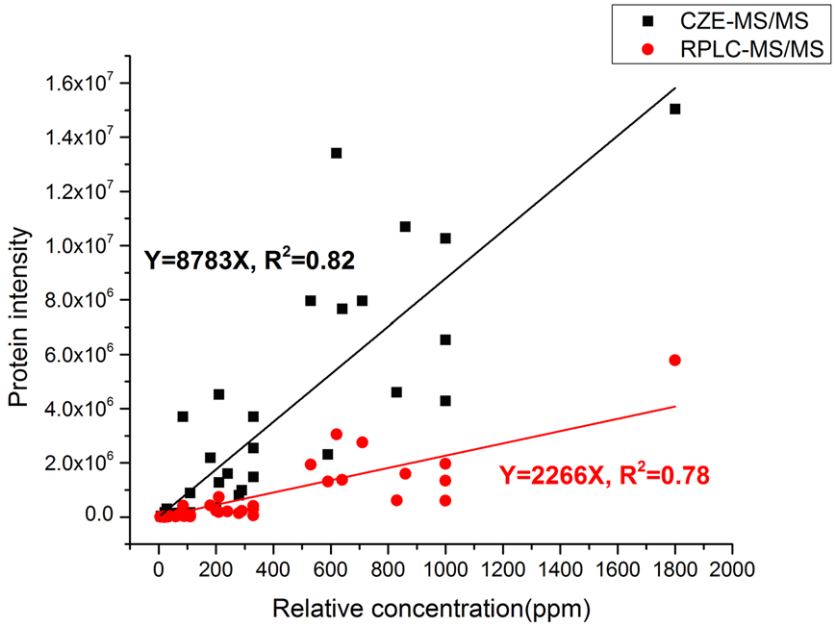


Figure 4. Calibration curves for absolute quantification by CZE-MS and RPLC-MS.

internal standards to mimic native peptides formed by proteolysis [38]. However, the absolute quantification is limited to the availability of synthesis of peptide. In 2009, Aebersold et al. reported an MS-based comprehensive absolute quantification strategy [39] by using 19 proteins in the sample to generate a calibration curve of protein abundance and protein spectra counts. In 2014, Smits et Al. spiked a range of proteins into the sample for global absolute quantification of protein amount in single *Xenopus* eggs [40]. In our work, 40 concentration points of 11 spiked proteins act as calibration points to draw a calibration curve by using spiked protein amount and protein label-free quantification intensity obtained based on MaxQuant database searching (Supporting Information Table 1) to evaluate the potential of absolute quantification of HCPs by using CZE-MS/MS with current spiked-in samples. Figure 4 shows two calibration curves of 11 spiked proteins (except BSA) by RPLS-MS/MS and CZE-MS/MS methods. Both curves have acceptable linearity with R value of 0.894 (UPLC-MS/MS) and 0.911 (CZE-MS/MS). The calibration curve of CZE-MS/MS has higher slope due to higher peptide signal intensity.

4 Concluding remarks

Both CZE-MS/MS and RPLC-MS/MS consistently detect peptides from spiked impurity proteins present at the 100 ppm level in the presence of 1 mg/mL recombinant antibody, without the need for antibody depletion. The sample preparation is simple, requiring denaturation, reduction, alkylation, digestion, and desalting prior to analysis. The total analysis time, including sample preparation and triplicate analysis, requires roughly 20 h. Tryptic digestion in the sample preparation procedure required 4 h. The use of immobilized trypsin can reduce digestion time to a few minutes, which will reduce the total analysis time to ~15 h [34].

The intensities provided by MaxQuant produce acceptable linearity across the tested concentrations, which suggests that MaxQuant intensities can be used to compare HCP abundances between samples during the optimization of protein purification and for comparison between sample lots.

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