Research Article

A comparative study of CE-SDS, SDS-PAGE, and Simple Western: Influences of sample preparation on molecular weight determination of proteins

The development of capillary electrophoresis, especially CE-SDS devices, has led CE-SDS to become an established tool in a wide range of applications in the analysis of biopharmaceuticals and is increasingly replacing its method of origin, SDS-PAGE. The goal of this study was to evaluate the comparability of molecular weight (MW) determination especially by CE-SDS and SDS-PAGE. For ensuring comparability, model proteins that have little or no posttranslational modifications and an IgG antibody were used. Only a minor influence of sample preparation conditions, including sample buffer, temperature conditions, and different reducing agents on the MW determination were found. In contrast, the selection of the MW marker plays a decisive role in determining the accurate apparent MW of a protein. When using different MW markers, the deviation in MW determination can exceed 10%. Interestingly, CE-SDS and 10% SDS-PAGE hardly differ in their trueness of MW determination. The trueness in relation to the reference MW for each protein was calculated. Although the trueness values for the model proteins considered range between 1.00 and 1.11 using CE-SDS, they range between 0.93 and 1.03 on SDS-PAGE, depending on the experimental conditions chosen.

Keywords:
CE-SDS / Method comparison / Molecular weight determination / SDS-PAGE / Simple Western DOI 10.1002/elps.202000199

In the early 1990s, commercial CE instrumentation became available. An established approach of CE is CE-SDS, which can be described as the translation of SDS-PAGE into the capillary format [4]. In contrast to typical polyacrylamide gels used for SDS-PAGE, in CE-SDS non-cross-linked entangled polymer networks are used as separation matrices, e.g., dextran, polyvinyl alcohol, polyethylene oxide, and polyethylene glycol [5–8].

The principle in both techniques, SDS-PAGE and CE-SDS, is the same: during sample preparation, the samples are heated with an excess of SDS to denature the proteins. The addition of reducing agents cleaves disulfide bonds. Upon denaturation, the randomly coiled polypeptide chains open up. The binding of SDS stretches the polypeptides and coats it with negative charges so that similar mass to charge ratios of the denatured protein molecules (1.4 mg SDS binds to 1 mg protein) are obtained [9]. Thus, SDS-protein-complexes migrate in SDS-PAGE mainly on...
their size and no longer in a more complex way that includes the size, charge, and quaternary structure of the protein. This is the main advantage of SDS electrophoresis; the charge density becomes approximately constant for every protein such that, in the molecular sieving PAGE-matrix or non-cross-linked entangled polymer networks, the SDS-protein-complexes are separated by their Stokes’ radius and thus by their MWs. Thus, the sieving effect has a greater impact and overcomes the charge effect [3]. The MW is determined by plotting the logarithm of the MW of MW-markers against the relative migration distance (RF) in SDS-PAGE.

The shortcomings of conventional SDS gel electrophoresis are its limited quantifiability, the use of toxic reagents, and its labor-intensive nature, mainly caused by gel staining and destaining steps [10]. Therefore, the development of a CE-based SDS separation technique improved upon this conventional method to an automated platform [11]. By virtue of superiorities like on-column UV or fluorescence detection, automation, enhanced resolution, and facilitating accurate quantitation of proteins and determination of their MW with software, CE-SDS became an essential tool for biopharmaceutical applications [12]. CE-SDS is well established for the characterization and quality control of therapeutic proteins, especially monoclonal antibodies [4]. The work of Kahle et al. provides an impression about the state-of-the-art performance of some recently developed CE-SDS instruments [13].

In 1994, Guttmann and Nolan compared the results of the MW determination of 65 different proteins by CE-SDS and SDS-PAGE. This study already illustrates that a few proteins show a different behavior in the SDS-PAGE and the CE-SDS system [14]. Reasons for the deviation from literature data are specific side groups like carbohydrates (glycoproteins), lipids (lipoproteins), or other prosthetic groups that may bind SDS differently [15–19]. The irregular binding of SDS causes a different mass to charge ratio for these molecules resulting in inaccurate estimates of the apparent MW [15,20]. Moreover, Engel et al. investigated the challenges of protein analysis of five proteins varying in their glycan moieties by microchip gel electrophoresis and showed that there are deviations in sizing proteins between microchip gel electrophoresis and SDS-PAGE [21]. A second source of error in MW evaluations is that protein mobility in gel electrophoresis is more a function of molecular size than of MW. Generally, it is supposed that SDS-bound-protein exists in a random coiled form, so the relationship between length and mass is constant. However, it is also possible that the actual shape may deviate from this ideally assumed one. Reasons for this are, for example, an incomplete unfolding of the protein due to an insufficient reduction and thus remaining disulfide bonds. Glycan structures can also change the protein form. All these reasons can lead to changes in the migration behavior. For gels with a constant degree of cross-linking or rather constant polyacrylamide concentration, the linearity extends over a limited range, which is determined by the size ratio of MW to pore diameter. If proteins no longer match to the linear range due to their size, deviations in size calculation occur. Remedy is found in the Ferguson method, determining the protein’s MW by separating the same sample in gels of various concentrations [22–24].

In order to figure out the disparity in performance of MW determination of proteins, SDS-PAGE was compared to CE-SDS.

For this purpose, the CE-SDS system Maurice (ProteinSimple, a Bio-Techne Brand) and furthermore Simple Western system Wes (ProteinSimple, a Bio-Techne Brand) were used. In Simple Western, proteins are also separated by CE-SDS followed by a total protein detection assay inside the capillary. The protein is covalently bound to the inside of the capillary after separation, followed by a biotin-streptavidin-horseradish peroxidase (HRP) detection and chemiluminescence or fluorescence readout (see further description about the system in [25]). Rustandi et al. evaluated the automated and quantitative CE-based Size Western Blot for therapeutic proteins and vaccines [26].

In order to closely compare the systems, specific proteins with an MW range from 14 to 179 kDa were chosen, which were traditionally used as MW markers. The proteins lysozyme, myoglobin, carbonic anhydrase, BSA, phosphorylase B, and β-galactosidase (Escherichia coli) are not glycosylated, whereas ovalbumin and α-2-macroglobulin are glycosylated. The advantages of these proteins are that they are relatively stable and very well characterized. Moreover, the MW of each protein is well known since these proteins have been used for a long time as objects of investigation in biochemistry [27–37]. Additionally, Matuzumab, a therapeutic IgG mAb, was selected as a representative of IgG antibodies, which are conventional test objects in CE-SDS [37].

The reference MWs, which were stated by the manufacturers, are based on the amino acid sequence of the six aglycosylated model proteins, whereas the reference MWs of ovalbumin and α-2-macroglobulin include the carbohydrate structure (see Table S1, Supporting Information). For comparability, the reference MWs were defined as the true value to calculate the trueness. Based on this, the trueness was determined by dividing the measured MW by the reference MW. In this context, trueness is defined according to the ISO 5725-1 standard, where trueness is described as the agreement of an arithmetic mean of a set of measurements with the true or assumed true reference value [38].

To determine factors influencing the trueness of the three different methods and how sensitively these methods react to certain influences, various sample preparation conditions of the model proteins were examined, including sample buffer composition, denaturation conditions, and three different reducing agents (β-mercaptoethanol (β-ME), DTT, and Tris(2-carboxyethyl)phosphine (TCEP)). The prerequisite for comparing the respective results between the methods were nearly identical sample preparation steps.

Moreover, the influence of the applied MW standard for calculating the MW of the sample proteins was analyzed by comparing MW markers from different suppliers.
2 Materials and methods

2.1 Materials

2.1.1 Proteins

Lysozyme from chicken egg white (14.4 kDa) and carbonic anhydrase from bovine erythrocytes (29.0 kDa) were provided by SERVA Electrophoresis GmbH (Heidelberg, Germany), while myoglobin from equine heart (17.8 kDa), albumin from chicken egg white (ovalbumin, lyophilized, 44.3 kDa), bovine serum albumin (BSA, 66.0 kDa), phosphorylase B from rabbit muscle (97.0 kDa), β-galactosidase from E. coli (116.25 kDa), and α-2-macroglobulin from human plasma (179.0 kDa) were provided by Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Matuzumab (light chain of Matuzumab (lc-M) 23.63 kDa and heavy chain of Matuzumab (hc-M) 49.66 kDa), provided by Merck KGaA (Darmstadt, Germany) as 10 mg/ml stock solution, was included to this study as potential therapeutic IgG antibody.

Molecular Weight Markers Maurice CE-SDS (ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA, specified size range: 10–270 kDa) and ProteomeLab™ MW Sizing Standard (AB Sciex Germany GmbH, Darmstadt, Germany, specified size range: 10–200 kDa), Benchmark™ Standard (New England Biolabs GmbH, Frankfurt am Main, Germany, specified size range: 10–200 kDa), and Benchmark™ Unstained Protein Standard (New England Biolabs GmbH, Frankfurt am Main, Germany, specified size range: 10–250 kDa), Unstained Protein Standard (New England Biolabs GmbH, Frankfurt am Main, Germany, specified size range: 10–200 kDa), and Benchmark™ Unstained Protein Ladder (Novex by life technologies, Thermo Fisher Scientific, MA, USA, specified size range: 10–220 kDa) were chosen as typical SDS-PAGE markers. The SDS-PAGE markers are ready-to-use and contain DTT.

As system suitability tests (SST), the Maurice CE-SDS IgG Standard for CE-SDS as well as HeLa lysate controls for Simple Western were provided by ProteinSimple, a Bio-Techne Brand. The Maurice CE-SDS 25 × Internal Standard (10 kDa recombinant protein, ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA) was added to each sample to calculate the MW.

2.1.2 Reagents

Tris (ultrapure grade (>99.9%), Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and SDS (Dodecylsulfate-Na-salt, electrophoresis grade, SERVA Electrophoresis GmbH, Heidelberg, Germany) were used for buffer preparation. As reducing agents 14.2 M β-ME (99%, Carl Roth GmbH, Karlsruhe, Germany), Bond-Breaker™ TCEP Solution (0.5 M TCEP with neutral pH, Thermo Fisher Scientific, MA, USA) and 400 mM DTT out of the Simple Western EZ Standard Pack (ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA) were used.

For the SDS-PAGE, Bio-Safe™ Coomassie G-250 Stain, 10× Tris/glycine/SDS buffer, 2× Laemmli Sample Buffer,Mini-PROTEAN® TGX™ Gels 10% 10-well comb, 30 µL long shelf life precast gels for use with Tris/Glycine buffers (Bio-Rad Laboratories GmbH, Feldkirchen, Germany), Laemmli 2× Concentrate Sample Buffer (Sigma–Aldrich Chemie GmbH, Steinheim, Germany), Bromphenol blue (BPB) sodium salt for electrophoresis (Sigma-Aldrich, Co., St. Louis, MO, USA), Glycerol ≥99.5% (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and Mini-PROTEAN® Tetra System (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) were required.

When performing CE-SDS experiments on the Maurice, the Maurice CE-SDS Size Application Kit Reagents and Consumables (ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA) are required. They contain two CE-SDS cartridges, reagent vials and 96-well plates, as well as general reagents (Maurice 1× Sample Buffer, Separation Matrix, Conditioning Solution 1 and 2, Wash solution, Running buffer top and bottom). For the Simple Western, 8×25 capillary cartridges 12–230 kDa (containing Simple Western plates, sample buffer, wash buffer, and cartridges; ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA), EZ Standard Packs (containing lyophilizedes of DTT, Fluorescent 5× Master Mix and the Biotinylated Ladder, ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA) and the Total Protein Detection Simple Western (containing Wes Antibody Diluent, Total Protein Reconstitution Agent 1 and 2, Total Protein Strep-tavidin HRP, Luminol and Peroxide, ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA) were utilized.

2.2 Methods

2.2.1 General information about sample preparation

Proteins were dissolved in 100 mM Tris and 1% SDS (pH 8.0) to a final concentration of 1 mg/mL. The samples were divided into aliquots and stored at −20°C. All experiments were conducted with ultrapure water produced by an arium pro VF system (Sartorius AG, Göttingen, Germany). On CE-SDS and SDS-PAGE, a final concentration of 710 mM β-ME, 20 mM DTT, and 10 mM TCEP was used. On Simple Western, the following concentrations achieved the best resolution: 40 mM β-ME, 40 mM DTT, and 2 mM TCEP. Note that 50 mM iodoacetamide is commonly added to the samples reduced by DTT in CE-SDS to prevent the occurrence of a DTT peak as mentioned in the application note. To ensure the comparability, iodoacetamide was not added to the reducing agents in this study.

2.2.2 Sample preparation for CE-SDS and SDS-PAGE

The eight model proteins were diluted to a final concentration of 0.5 mg/mL with the Maurice 1× Sample buffer, while Matuzumab (10 mg/mL stock solution) was diluted to a final concentration of 1 mg/mL. Afterward, the reducing agent (2.5 µL 14.2 M β-ME, 2.5 µL 400 mM DTT or 1 µL 0.5 M
TCEP) and 2 μL CE-SDS 25× Internal Standard Maurice (10 kDa recombinant protein) were added to the samples. The samples were vortexed for homogenization, centrifuged, and heated to 70°C or 95°C for 5 or 10 min. After cooling down on ice and repeating the vortex and centrifugation step, 50 μL of each sample was loaded into the 96-well plate, according to the manufacturer’s protocol [39]. Furthermore, the lyophilizates of the Molecular Weight Markers CE-SDS as well as the Maurice CE-SDS IgG Standard for CE-SDS were diluted to 50 μL with the Maurice 1× Sample buffer. They were also treated as the samples. The above-mentioned reducing agents, as well as 2 μL CE-SDS 25× Internal Standard Maurice, were always added to these samples.

For SDS-PAGE, the samples were prepared as above mentioned with the exception of the sample buffer. Instead, SDS-PAGE sample buffers were used. In order to ensure comparability with CE-SDS, the sample preparation for SDS-PAGE was also carried out by adding the CE-SDS 25x Internal Standard Maurice. Concerning the protein samples, 10 μL each were loaded onto the gel, which corresponds to a protein amount of 5 μg when using a sample concentration of 0.5 mg/mL. From the MW marker 7.5 μL were loaded onto the gel. Due to the unknown concentration, it is not possible to provide information about the amount of protein. The MWs were determined by using the Molecular Weight Markers Maurice CE-SDS.

2.2.3 Sample preparation for Simple Western

Sample preparation was performed as described in the operation protocol [40]. For each run, the samples, the MW marker, the HeLa lysate as SST, and a blind sample were prepared. The samples were treated at 95°C for 5 min. 40 mM DTT as reducing agent and three fluorescence markers (in the Fluorescent 5x Master Mix) to recalculate the MW were added to the samples. The final concentration of the eight model proteins was 0.2 mg/mL and was 0.1 mg/mL for Matusumab.

2.2.4 Preparation for sample buffer studies

According to Laemmli [41], the sample buffer, based on the final concentration in the sample, contains 62.5 mmol/L Tris-HCl, 10.0% glycerol, 2.0% SDS, 0.001% BPB, and 5.0% β-ME resulting in a pH of 6.8. An example of a buffer that differs from this composition is the 2× Laemmlli sample buffer by Bio-Rad, which was included in the experiments. With 32.9 mmol/L, it contains significantly less Tris-HCl than the original formulation, again based on the final concentration in the sample. Glyceraldehyde, however, is contained to a slightly higher extent of 13.05%. The SDS concentration is approximately halved to 1.05%. BPB on the other hand is contained in five times the amount (0.005%). The pH of 6.8 and the amount of β-ME, which is added manually later and therefore being variable anyway, are identical. The second buffer examined was the Laemmli sample buffer by Sigma. This buffer differs from the original composition only in the doubled concentration of BPB (0.002%). To ensure comparability with CE-SDS, the aim was also to investigate the Maurice 1× Sample buffer by ProteinSimple, a Bio-Techne Brand, on the SDS-PAGE. A simple replacement of the buffer is not possible, because the CE-SDS sample buffer does not contain any additives like density-increasing glycerol to help the sample sink into the well of the gel or a dye to track the running front. Before being diluted, the Maurice 1x Sample buffer contains 100 mM Tris and 1.0% SDS with a pH of 9.5. In order not to change the concentrations of the other components when adding glycerol and BPB and thus to remain as close as possible to the original product, a part of the water of the buffer was evaporated and then replenished to the previous volume with the above-mentioned additives. Regarding the final concentration in the sample, the following concentrations apply: 50 mM Tris, 0.5% SDS, 10% glycerol, 0.001% BPB, and 5.0% β-ME.

2.2.5 Sample preparation for the ladder comparison study on CE-SDS

25 μL of each SDS-PAGE marker (concentrations of stock solutions are unknown) were diluted with 25 μL of the Maurice 1x Sample buffer before the injection into the CE-SDS instrument. The Molecular Weight Markers Maurice CE-SDS and ProteomeLab™ MW Sizing Standard were prepared as recommended in the application notes, downscaled to 50 μL [42]. By way of derogation from this provision, these markers were heated to 95°C for 5 min and reduced by 710 mM β-ME. For the ProteomeLab™ MW Sizing Standard, Maurice 1× Sample buffer was used.

For SDS-PAGE, the CE-SDS markers were prepared as described above with the exception of the sample buffer. The 2× Laemmlli Sample Buffer was utilized. CE-SDS (25× Internal Standard Maurice, 2 μl) was added to all the markers to evaluate the relative migration times on CE-SDS as well as the Rf on SDS-PAGE.

2.3 Instrumentation

2.3.1 CE-SDS

The CE-SDS instrument Maurice (ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA) was used. The effective length of the capillary in the cartridge is 15 cm with a diameter of 50 μm. The sample load was performed electrokinetically. At the beginning of each run, the Molecular Weight Marker Maurice was injected once for 20 s at 4600 V and separated for 35 min at 5750 V. The other MW markers were loaded into the capillary and separated like mentioned before. The reduced IgG was injected afterward and at the end of each sequence for 20 s at 4600 V and separated for 35 min at 5750 V to guarantee the system suitability. Each sample was injected three times for 20 s at 4600 V and the proteins...
with an MW less than 70 kDa were separated for 25 min at 5750 V, while the larger proteins were separated for 35 min at 5750 V. The default parameters were utilized. The detection was performed at a wavelength of 220 nm. The data were evaluated by Compass for iCE 2.0.11 (ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA). Using the software tool analysis, the position of the MW marker was defined after the run was completed and each peak of the MW marker was assigned to a predefined MW based on the migration time of the peak. The CE-SDS 25× Internal Standard Maurice (10 kDa recombinant protein) was also part of the MW marker. Each migration time of the single protein marker was linked to the known MW, also the 10 kDa CE-SDS 25× Internal Standard Maurice. Therefore, the MW of each sample was recalculated from the RMT in relation to the CE-SDS 25× Internal Standard Maurice, which was added to all samples. The baseline correction was automatically generated. More detailed information is described by Kahle et al. [13].

### 2.3.2 SDS-PAGE

The SDS-PAGE experiments were performed on 10% polyacrylamide gels. The selection of the gel, i.e., the concentration of polyacrylamide, depends on the research question to be addressed and the proteins to be separated. To ensure good reproducibility, precast gels by Bio-Rad were used. The sample preparation as well as the electrophoresis itself was carried out according to the Laemmli procedure [41]. SDS-PAGEs were performed on the Mini-PROTEAN® Tetra System by Bio-Rad in a Tris/glycine/SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) and an applied voltage of 200 V (29.9 V/cm), resulting in a current of about 50 mA. Under these conditions, an electrophoresis run for two gels at a time took about 40 min. After completion of the electrophoresis, the gels were washed three times with deionized water over a period of about 15 minutes to avoid disturbance by SDS from the running buffer during subsequent staining. Afterward, the gels were stained with Bio-Safe™ Coomassie G-250 Stain for at least 2 h. Ordinarily, destaining of the gels with deionized water was done overnight, to achieve the best contrast between the stained bands and the gel’s background. For documentation, transmitted light photos were taken using an imager (Vilber Lourmat Peqlab FUSION SL gel documentation system 2012, Vilber Lourmat Deutschland GmbH, Eberhardzell, Deutschland) in connection with the program Infinity by Vilber Lourmat (version 15.06). The evaluation was carried out using the image processing program ImageJ 1.52a (Wayne Rasband, National Institutes of Health, USA). It uses a grayscale analysis to evaluate images and offers a function to convert defined lanes into electropherogram-like plots with the abscissa representing the running distance and the ordinate representing the intensity of the band or the grey level, respectively. By evaluating the peak tops, x-values were obtained that were used to calculate the RF of the corresponding band in relation to the CE-SDS 25× Internal Standard Maurice being used as a reference value. Apparent MWs were calculated by inserting the RFs of the respective band in the gel into the linear equation obtained by plotting the logarithmic MW of the marker used against its RIs.

### 2.3.3 Simple Western

The Simple Western system Wes (ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA) was implemented to this study as an additional new method to determine the MW of proteins using the Total Protein Assay. Each protein sample was measured in duplicate and was separated for 25 min at 375 V. After immobilization of the proteins on the capillary wall, they were covalently labeled with biotin for 30 min, washed, and then incubated with Streptavidin HRP conjugate for 30 min. The proteins were subsequently detected by chemiluminescence and evaluated by Compass for SW 4.1 (ProteinSimple, a Bio-Techne Brand, San Jose, CA, USA).

All measurements were performed three times on Maurice and Wes and four times on SDS-PAGE.

### 3 Results and discussion

The investigation of the MW determination is based on reference MWs, according to the manufacturer’s specifications of the eight model proteins. To verify the MW and the influence of glycosylation pattern and other posttranslational modifications, the sequences were also compared with data from the bioinformatic databank UniProt (universal protein database, www.uniprot.org). The reference MW and the MW found on UniProt are nearly identical for all proteins, except for ovalbumin and α-2-macroglobulin. Their MWs in UniProt are smaller than the reference MWs due to the carbohydrate groups (see Table S1, Supporting Information).

Furthermore, as basis for good comparability, the same marker (Molecular Weight Markers Maurice CE-SDS) and the CE-SDS 25× Internal Standard Maurice was used for both CE-SDS and SDS-PAGE. By using 10% polyacrylamide gels, proteins can be separated in an MW range of 20–250 kDa with an optimum separation range of 30–150 kDa according to the manufacturer’s instructions [43]. Correspondingly, a similar MW range is obtained with Maurice (10–270 kDa) and Wes (12–230 kDa). Besides the separation module 12–230 kDa, Simple Western also offers two further separation modules, 2–40 kDa and 66–440 kDa, to increase the separation capabilities of the platform that in total can cover 2–440 kDa. A wider MW range in SDS-PAGE could be covered, for example, by using gradient gels. Gradient gels are characterized by an increasing concentration of polyacrylamide in the course of the gel (e.g., 4–20%). These gels are particularly suitable for estimating the approximate MW of an unknown protein. For a more precise observation and calculation of the MW, a
gel with a suitable uniform concentration of polyacrylamide is selected [44].

The usual evaluation method for MW determination by SDS-PAGE is the linear fit approach by plotting the corresponding logarithmic MW values versus the relative migration distances. A closer investigation of the linear range (see Section 3.4) reveals that 10% polyacrylamide gels cover a representative linear range of 20–100 kDa. This investigation led to an exclusion of the SDS-PAGE study for proteins with an MW out of this range, i.e. lysozyme, myoglobin, β-galactosidase, and α-2-macroglobulin. Based on other evaluation methods (Maurice and Wes), a wider MW range can be covered. Therefore, these four proteins could additionally be evaluated for both CE-SDS systems (see Tables S4 and S5, Supporting Information). Considering these effects, the results of carbonic anhydrase, ovalbumin, BSA, and phosphorylase B are chiefly discussed in the following.

3.1 Investigation of sample preparation effects

3.1.1 Effects of buffer compositions on SDS-PAGE

In order to investigate whether the sample buffer has an influence on the calculated MW, three sample buffers, differing in their composition, were examined by comparative SDS-PAGE analysis. Although most of the sample buffers available on the market are called “Laemmli buffers”, it would be more correct to speak of “Laemmli-based systems”, because there are sometimes deviations from the original composition of Laemmli [41]. On the SDS-PAGE, two SDS-PAGE sample buffers of different manufacturers (Bio-Rad and Sigma) and one CE-SDS sample buffer (Maurice 1 x Sample buffer, ProteinSimple, a Bio-Technne Brand) were examined for their influence on the MW determination. It was determined that the use of different Laemmli based SDS-PAGE sample buffers, as well as of the adapted CE-SDS buffer, does not cause remarkable differences in MW determination. Independent of the sample buffer, similar MW results are obtained for the proteins investigated. A maximum percent error of 3% based on the protein’s calculated MWs between samples being prepared with different sample buffers, was shown for repetitive measurements (see Table S2, Supporting Information). It should therefore be noted that the choice of sample buffer does only have a minor influence on these results.

Most commercially available sample buffers do not contain a reducing agent. It is added during sample preparation since they generally have limited stability in solution. Hence, there is a flexibility in the choice of the reducing agent. The tested sample buffer from Sigma, however, already contains β-ME, so that a free choice of the reducing agent or an SDS-PAGE under non-reducing conditions would not be possible. This might be the only factor to be considered in the selection process of a suitable sample buffer.

3.2 Effects of the denaturation steps

3.2.1 Effects of various denaturation temperatures

The influence of the denaturation temperature during sample preparation on the MW determination of the three methods was investigated using heating temperatures of 70 or 95°C at two incubation periods (5 or 10 min). The calculated MWs of the eight model proteins determined by CE-SDS are shown in Table S3, Supporting Information with respect to the individual denaturation step (70 and 95°C for 5 and 10 min). Furthermore, the MWs resulting from each temperature condition were compared to each other and the difference between the smallest and highest value was calculated, e.g. 32 kDa – 31 kDa = 1 kDa (in relation to the higher value: 3%) for carbonic anhydrase. Based on these calculations, the smaller proteins (lysozyme, myoglobin and carbonic anhydrase) with MWs less than 35 kDa show minor differences of less than 1 kDa (3%) between the various denaturation steps, while for the larger proteins (ovalbumin up to α-2-macroglobulin) a maximum difference up to 11 kDa (4%) for α-2-macroglobulin between 70°C for 10 min and 95°C for 5 min was observed. The main differences appear to arise between the temperatures used (95°C versus 70°C) rather than the incubation time. Due to these observations, one could consider that for larger proteins the standard denaturation conditions generally lead to additional measurement uncertainty, due to insufficient denaturation or more general measurement inaccuracies due to size. The higher denaturation temperature could lead to stronger unfolding of the proteins so more SDS binds, thus the proteins become more negatively charged and larger. Looking at the size, one would assume that the protein now migrates more slowly caused by the sieving effect, so that the protein peak shifts to higher migration times resulting in higher MW estimates [3]. However, the increase in negative charge would cause the protein to move faster towards the anode. It is difficult to say which of the two effects is more pronounced. In any case, a changed migration behavior will occur. On the other hand, the denaturation temperature could be insufficient, leading to incomplete denaturation and thus to a lower measured MW [4]. The difference in MW obtained between proteins incubated for 5 min and 10 min per temperature condition is less than 4 kDa, except for β-galactosidase and α-2-macroglobulin. Due to these results, further comparisons between SDS-PAGE and Simple Western were limited to 95°C for 5 min and 70°C for 10 min, which are typical denaturation conditions used for CE-SDS and SDS-PAGE.

After these preliminary experiments, the MWs of carbonic anhydrase, ovalbumin, BSA, and phosphorylase B were determined by CE-SDS, SDS-PAGE, and Simple Western (see Table 1). Furthermore, the trueness of the MW determinations of all three techniques was calculated in relation to the reference MW.

The comparison of the MW determination by CE-SDS and SDS-PAGE (10% gels) reveals, that the trueness, regarding the comparison of the both denaturation conditions,
Table 1. Influence of the denaturation temperature (70°C for 10 min versus 95°C for 5 min) and different reducing agents (reduced by β-ME, DTT, and TCEP at 95°C for 5 min) on the MW determination

| Method      | Carb | Ova | BSA | Phos |
|-------------|------|-----|-----|------|
|             | Mean [kDa] | Trueness | Mean [kDa] | Trueness | Mean [kDa] | Trueness | Mean [kDa] | Trueness |
| CE-SDS      |      |     |     |      |
| β-ME*       | 31   | 1.07 | 45  | 1.02  | 66   | 1.00  | 102   | 1.05  |
| β-ME        | 32   | 1.10 | 46  | 1.04  | 69   | 1.05  | 104   | 1.07  |
| DTT         | 32   | 1.10 | 45  | 1.02  | 68   | 1.03  | 108   | 1.11  |
| TCEP        | 31   | 1.07 | 45  | 1.02  | 66   | 1.00  | 102   | 1.05  |
| SDS-PAGE    |      |     |     |      |
| β-ME*       | 28   | 0.97 | 44  | 0.99  | 67   | 1.02  | 95    | 0.98  |
| β-ME        | 28   | 0.97 | 44  | 0.99  | 68   | 1.03  | 99    | 1.02  |
| DTT         | 28   | 0.97 | 45  | 1.02  | 67   | 1.02  | 100   | 1.03  |
| TCEP        | 27   | 0.93 | 41  | 0.93  | 66   | 1.00  | 99    | 1.02  |
| Simple Western |    |     |     |      |
| DTT*        | 36   | 1.24 | 48  | 1.08  | 63   | 0.95  | 100   | 1.03  |
| DTT         | 36   | 1.24 | 48  | 1.08  | 63   | 0.95  | 102   | 1.05  |
| β-ME        | 37   | 1.28 | 48  | 1.08  | 62   | 0.94  | 99    | 1.02  |
| TCEP        | 36   | 1.24 | 47  | 1.06  | 62   | 0.94  | 101   | 1.04  |
| r-MW [kDa]  | 29.0 | 44.3 | 66.0| 97.0  |

Reference molecular weight (r-MW) according to manufacturer's specification; *denatured at 70°C for 10 min; carbonic anhydrase (Carb), ovalbumin (Ova), bovine serum albumin (BSA), phosphorylase B (Phos)

The samples were comparatively analyzed by all three methods. The results of the MW determination and the trueness in relation to the reference MWs are shown in Table 1. In SDS-PAGE, the trueness is between 0.93 and 1.03. In CE-SDS, the trueness covers a range of 1.00 to 1.11. Thus, this study also confirms the results of Guttman and Nolan (1994) as well as Weber and Osborn (1969) [3, 14]. Moreover, this study shows, by investigating proteins in the range of 20 to 100 kDa, that 10% SDS-PAGE has a slight advantage for MW determination in comparison to CE-SDS. In Simple Western the trueness ranged from 0.94 to 1.08, with the exception of carbonic anhydrase at 1.28.

For investigating the influence of reducing agents on proteins with higher or lower MW, trueness calculation compared to reference MWs as well as MW determination on CE-SDS and Simple Western for myoglobin, lysozyme, β-galactosidase, and α-2-macroglobulin were performed. The results are shown in Table S5 of the Supporting Information. The trueness is in the range of 0.84 to 1.45 for CE-SDS and in the range of 0.98 to 1.11 for Simple Western. The highest trueness value with 1.45 was calculated for α-2-macroglobulin for CE-SDS and could be explained by the glycosylation pattern (see Section 3.3).

In addition, the influence of the reducing agents on the MW of each protein was compared within one method. For CE-SDS a maximum percent error of 6% for MW determination could be detected within a series of measurements of one protein treated with different reducing agents, whereas in SDS-PAGE a maximum percent error of 9% and for Simple Western a maximum percent error of 3% could be determined.

3.2.2 Effect of various reducing agents

The reduction step during sample preparation is necessary to cleave the disulfide bonds in addition to the H-bonds. Besides the conventional reduction agents β-ME and DTT, TCEP was investigated, because it has become increasingly important in the pharmaceutical industry due to safety reasons of the other reducing agents [45]. The four proteins were heated to 95°C for 5 min. The best results for protein concentrations of 0.5 mg/mL measured by CE-SDS and SDS-PAGE were achieved with 710 mM β-ME, 20 mM DTT and 10 mM TCEP. For Simple Western, following concentrations for reducing agents were applied: 40 mM β-ME, 40 mM DTT and 2 mM TCEP.
Western a lower concentration of Matuzumab (0.1 mg/mL) was analyzed and the concentrations of the reducing agents were 40 mM β-ME, 40 mM DTT, and 2 mM TCEP. In order to ensure that the reduction step with the respective reducing agent for Matuzumab has been completed successfully, a control experiment was included in which the separation time was extended to 35 minutes on CE-SDS (see Fig. S1, Supporting Information). No peak of the intact antibody could be measured for all three reducing agents, which means that the reduction step was carried out successfully under the stated conditions.

As shown in Section 3.2.1 and 3.2.2, the denaturation temperature and various reducing agents have minor influence on the MW determination. By comparison within each analytical approach with the various reducing agents, for CE-SDS a maximum percent error of 4%, for Simple Western a maximum percent error of 3% and for SDS-PAGE a maximum percent error of 2% was determined. In contrast, for CE-SDS the temperature condition 70°C for 10 min (710 mM β-ME) causes lower calculated MWs of lc-M and hc-M due to potentially incomplete unfolding of the polypeptide. That illustrates the importance of optimizing the reduction method for each protein.

Furthermore, the trueness of the lc-M and hc-M in relation to the reference MW (calculation based on the amino acid sequence out of the patent) was evaluated. The trueness of the lc-M is in the range between 1.06 and 1.14 for CE-SDS, 1.14 for SDS-PAGE and between 1.31 and 1.35 for Simple Western. Compared to the lc-M, the hc-M shows higher trueness values for CE-SDS between 1.33 and 1.39, while the other techniques produced trueness values for the hc-M: 1.11–1.13 in terms of SDS-PAGE and 1.19–1.21 for Simple Western. These differences in trueness of the three methods can be explained by posttranslational modifications of the IgG antibody [4, 54]. In general, the MW determination of hc-M shows higher values than the reference data because the heavy chain is glycosylated. The MW of the carbohydrate structures is not considered in the reference MW data that is only based on the amino acid sequence of the heavy chain. In addition, the hydrophilic carbohydrates have the effect that they do not bind SDS and therefore the charge-to-mass ratio is lower compared to unglycosylated samples, thus the protein migrates more slowly [19]. Nevertheless, there are clear differences in trueness of hc-M measured by CE-SDS and SDS-PAGE. Wang et al. suspected that the various glycan chains can also interact with the different gel matrices, thus can result in higher MW determination by CE-SDS in contrast to SDS-PAGE [54].

### 3.3 Investigation on Matuzumab (IgG mAb)

The investigation of a therapeutic IgG antibody, namely Matuzumab (lc-M 23.63 kDa, hc-M 49.66 kDa, see Patent WO 2009/043490 A1 [37]), was included for the comparability to previous studies [10, 12, 46–53]. Typically, SDS-PAGE and CE-SDS were compared in terms of purity and size heterogeneity assays, to examine the variety in analyzing antibodies. Therefore, reduced IgG was evaluated.

In this study, Matuzumab was denatured at 70°C for 10 min and 95°C for 5 min and reduced by β-ME, DTT, and TCEP like the model proteins before, to evaluate the impact of various sample preparation conditions (see Table 2). In CE-SDS and SDS-PAGE experiments, 1 mg/mL Matuzumab was tested and the concentrations of the reducing agents were 710 mM β-ME, 20 mM DTT, and 10 mM TCEP. In Simple Western a lower concentration of Matuzumab (0.1 mg/mL) was analyzed and the concentrations of the reducing agents were 40 mM β-ME, 40 mM DTT, and 2 mM TCEP. In order to ensure that the reduction step with the respective reducing agent for Matuzumab has been completed successfully, a control experiment was included in which the separation time was extended to 35 minutes on CE-SDS (see Fig. S1, Supporting Information). No peak of the intact antibody could be measured for all three reducing agents, which means that the reduction step was carried out successfully under the stated conditions.

As shown in Section 3.2.1 and 3.2.2, the denaturation temperature and various reducing agents have minor influence on the MW determination. By comparison within each analytical approach with the various reducing agents, for CE-SDS a maximum percent error of 4%, for Simple Western a maximum percent error of 3% and for SDS-PAGE a maximum percent error of 2% was determined. In contrast, for CE-SDS the temperature condition 70°C for 10 min (710 mM β-ME) causes lower calculated MWs of lc-M and hc-M due to potentially incomplete unfolding of the polypeptide. That illustrates the importance of optimizing the reduction method for each protein.

Furthermore, the trueness of the lc-M and hc-M in relation to the reference MW (calculation based on the amino acid sequence out of the patent) was evaluated. The trueness of the lc-M is in the range between 1.06 and 1.14 for CE-SDS, 1.14 for SDS-PAGE and between 1.31 and 1.35 for Simple Western. Compared to the lc-M, the hc-M shows higher trueness values for CE-SDS between 1.33 and 1.39, while the other techniques produced trueness values for the hc-M: 1.11–1.13 in terms of SDS-PAGE and 1.19–1.21 for Simple Western. These differences in trueness of the three methods can be explained by posttranslational modifications of the IgG antibody [4, 54]. In general, the MW determination of hc-M shows higher values than the reference data because the heavy chain is glycosylated. The MW of the carbohydrate structures is not considered in the reference MW data that is only based on the amino acid sequence of the heavy chain. In addition, the hydrophilic carbohydrates have the effect that they do not bind SDS and therefore the charge-to-mass ratio is lower compared to unglycosylated samples, thus the protein migrates more slowly [19]. Nevertheless, there are clear differences in trueness of hc-M measured by CE-SDS and SDS-PAGE. Wang et al. suspected that the various glycan chains can also interact with the different gel matrices, thus can result in higher MW determination by CE-SDS in contrast to SDS-PAGE [54].

### 3.4 Investigation of the influence of the ladder

As previously described, for MW estimation, standard curves are constructed by plotting the logarithm of the MW as a linear function of the relative migration distance (Rf) in SDS-PAGE measurements respectively the reciprocal migration time in CE-SDS (see Fig. 1).
When the logarithmic MW is plotted against the Rf of all bands of the marker, however, it is noticeable that there is no linearity over the entire examined range (see, e.g., Fig. 1A). If, in case of the Precision Plus Protein Standard, all bands of the marker are included in the evaluation, the linear regression results in the following: Pearson R-square related to the linear equation ($R^2$) = 0.95865 and RMSE = 0.09694 respectively (see Table 3.1). If in turn, excluding the deviating points, namely the ones for 10, 150 and 250 kDa and performing linear regression with the remaining values again, the R-square improves to $R^2$ = 0.99343 and RMSE = 0.02455 respectively (see Table 3.2).

Moreover, the patterns of residuals were compared (Fig. 1C and D) and illustrate how the residuals fit to the linear equation generated from the whole range of data points in contrast to the linear equation generated from 20 to 100 kDa. It can therefore be concluded that there is no linear relationship over the entire range of the gel between the Rf of the band in the gel and the MW of the corresponding protein. Repetitive experiments showed that the used 10% gels show an approximately linear relationship in the range of 20 kDa to about 100 kDa (see Fig. 1B). Therefore, the choice of gels must be made based on the MW range of the proteins studied.

When comparing different markers among themselves by SDS-PAGE, it was also noted that bands, which according to the manufacturer represent proteins of identical MW, show different Rf's, contrary to expectations. This phenomenon can be seen, for example, when comparing the 50 kDa (55 kDa for the Molecular Weight Marker Maurice CE-SDS by ProteinSimple) and 100 kDa (103 kDa for the Molecular Weight Marker Maurice CE-SDS by ProteinSimple) bands of the different markers (see Fig. 2).

This inevitably results in different standard calibration curves for different markers, either shifted in parallel or with different slopes, which in turn means that the calculated MWs for the examined proteins differ depending on the marker used (see Table 3.2). Moreover, this effect can easily be demonstrated with an example. If one wants to determine the MW of a protein that shows an Rf = 0.3, the calculation with the Precision Plus Protein™ Standard results in an MW of 87 kDa. If Rf = 0.3 is again inserted into the equation given by the ProteomeLab™ MW Sizing Standard by Sciex in...
Table 3. Parameters of the linear regression analysis of the tested MW markers while all data points are included (3.1) and statistic parameters of the linear regression analysis with deviating points excluded (3.2) based on the results of the evaluation of MW markers by 10% SDS-PAGE

### 3.1 Statistic parameters of linear regression analysis with all data points included

| Plot                  | Bio-Rad       | ProteinSimple | Sciex       | Biolabs      | Benchmark  |
|-----------------------|---------------|---------------|-------------|--------------|------------|
| Intercept             | 2.46464 ± 0.06728 | 2.5239 ± 0.04812 | 2.40168 ± 0.04696 | 2.4276 ± 0.043 |
| Slope                 | -1.3945 ± 0.10947 | -1.51466 ± 0.07963 | -1.30887 ± 0.07902 | -1.31633 ± 0.0747 |
| Residual sum of Squares | 0.06578 | 0.01924 | 0.05474 | 0.06658 |
| Adj. R-Square         | 0.95865 | 0.98637 | 0.96483 | 0.9628 |
| R-Square              | 0.95865 | 0.98637 | 0.96483 | 0.9628 |
| Root-MSE              | 0.09694 | 0.06203 | 0.07399 | 0.07449 |

### 3.2 Statistic parameters of linear regression analysis with deviating points excluded

| Plot                  | Bio-Rad       | ProteinSimple | Sciex       | Biolabs      | Benchmark  |
|-----------------------|---------------|---------------|-------------|--------------|------------|
| Intercept             | 2.2643 ± 0.02728 | 2.36456 ± 0.01462 | 2.25705 ± 0.02083 | 2.28441 ± 0.0143 |
| Slope                 | -1.07723 ± 0.04379 | -1.27054 ± 0.02408 | -1.06194 ± 0.03511 | -1.06794 ± 0.02417 |
| Residual sum of Squares | 0.00241 | 1.84557E-4 | 0.00358 | 0.00217 |
| Adj. R-Square         | 0.99179 | 0.99892 | 0.99132 | 0.99541 |
| R-Square              | 0.99343 | 0.99995 | 0.99924 | 0.99952 |
| Root-MSE              | 0.02455 | 0.00835 | 0.00961 | 0.02262 |

Bio-Rad = Precision Plus Protein™ Standard unstained by Bio-Rad; ProteinSimple = Molecular Weight Marker Maurice CE-SDS by ProteinSimple; Sciex = ProteomeLab™ MW Sizing Standard by Sciex; Biolabs = Unstained Protein Standard by New England Biolabs; Benchmark = Benchmark™ Unstained Protein Ladder by Novex by life technologies

Table 3.2, MW would be 96 kDa, that causes a percent error of about 9%. With $R_f = 0.8$ the MWs calculated by means of the two ladders would be 25 kDa and 22 kDa, respectively a percent error of even 12%.

For this reason, five different MW markers were examined on SDS-PAGE (10% gels) as well as with CE-SDS (Figs. S4–S8, Supporting Information). The effect of non-linearity, when considering the entire size range, is shown on the SDS-PAGE for all markers, as it is caused by the gel itself. Looking at the residual plots of the respective markers, it becomes apparent that the values do not fluctuate around the predicted ordinate value in a normally distributed way (see Fig. 1C and Figures S4C–S8C, Supporting Information). On the contrary, the trend is approximately similar for all markers. To investigate whether all these mentioned effects can be observed as well when using CE-SDS, the markers were also tested with this method (see Figs. S4C–S8C, Supporting Information). Figure S3 in the Supporting Information shows the electropherograms of the five investigated markers on CE-SDS.

The evaluation of the CE-SDS data confirms the results of the SDS-PAGE. Looking at the same case as described above, the following results are obtained for the Precision Plus Protein Standard when all values are considered for a linear fitting: $R^2 = 0.98762$ and RMSE = 0.0535. When, in turn, excluding the deviating points below 20 kDa as well as above 150 kDa and performing the linear fit again, the fit statistic parameters improve to $R^2 = 0.98867$ and RMSE = 0.03728 respectively. It can therefore be concluded that both SDS-PAGE and CE-SDS have different linear ranges depending on the gel matrix used.

This detailed examination of these five commonly used protein ladders in both SDS-PAGE and CE-SDS showed that the selection of the marker has a substantial influence on the calculated MWs, owing true to the term, apparent MW. Depending on the marker used, the deviations in MW determination can result in more than 10%. Moreover, the comparison of the same marker by CE-SDS and SDS-PAGE shows that both methods have different linear ranges. Thus, the linear range of a marker depends on the separation matrix that is used.

### 4 Concluding remarks

This present study shows the comparability of the protein MW determination by CE-SDS, respectively Maurice and Simple Western, and the method of origin: SDS-PAGE. Moreover, it illustrates which influencing factors for MW determination should be considered if one method is to be replaced by the other.

By comparison of three different sample buffers by SDS-PAGE, it was shown that the use of different Laemmli based SDS-PAGE sample buffers does not cause remarkable differences in MW determination. A percent error of 3% could be detected between samples prepared in different buffers. Furthermore, temperature and time conditions in the protein denaturation step do not have influences on MW determination by CE-SDS, SDS-PAGE, and Simple Western.
For the entire investigated MW range, respectively for all analyzed model proteins, no remarkable percent errors caused by using different reducing agents (β-ME, DTT, or TCEP) for the denaturation step could be determined by CE-SDS, SDS-PAGE, and Simple Western. The same applies to Matuzumab. However, the prerequisite for comparable MW results by using different reducing agents is a successfully reduced protein.

By investigating different MW ladders by CE-SDS (Maurice, ProteinSimple, a Bio-Techne Brand) and SDS-PAGE, it becomes apparent that the linear ranges of both CE-SDS and SDS-PAGE are different from each other, possibly due to different separation matrices. Thus, the linear range of the CE-SDS separation ranges tested lies between 20 and 150 kDa and in 10% SDS-PAGE the linear range is between 20 and 100 kDa. As expected, the choice of the MW marker most strongly influences the MW determination. Different markers produce different standard curves, regardless of the method used. When using different MW markers, the deviation in MW determination can be over 10% for the same protein. This is why a comparison of MWs, obtained by different methods, should therefore be based on the same markers, as done in the presented study.

The direct comparison of the MW determination of the four model proteins by all three methods revealed that trueness to the given reference MW, which refers to the manufacturer’s specifications, is between 1.00 and 1.11 for CE-SDS, 0.94–1.08 for Simple Western similar to CE-SDS and between 0.93 and 1.03 for SDS-PAGE, depending on the experimental conditions used in all cases. In contrast to that, the results of lc-M and hc-M show the following values regarding trueness: 1.06–1.14 for lc-M and 1.33–1.39 for hc-M measured by CE-SDS, 1.14 for lc-M and 1.11–1.13 for hc-M determined by SDS-PAGE, respectively. One explanation for these deviations is probably due to glycosylation. These observations illustrate the reasonable choice of unglycosylated model proteins for a direct comparison of the three methods. The influence of glycosylation will be closer examined in a follow-up project. Additionally, the precision and repeatability of MW determination for several instrument platforms will be compared.
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