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J. Biochem. Biophys. Methods 60 (2004) 281–293
JOURNAL OF biochemical and biophysical methods
www.elsevier.com/locate/jbbm

Comparison of selected analytical techniques for protein sizing, quantitation and molecular weight determination

H. Goetz[a,*], M. Kuschel[a], T. Wulff[a], C. Sauber[a], C. Miller[b], S. Fisher[b], C. Woodward[c]

[a] *Agilent Technologies Deutschland GmbH, Waldbronn, GER-76337, Germany*
[b] *Agilent Technologies Inc., Santa Clara, CA, USA*
[c] *Agilent Technologies Inc., Wilmington, DE, USA*

Abstract

Protein analysis techniques are developing fast due to the growing number of proteins obtained by recombinant DNA techniques. In the present paper we compare selected techniques, which are used for protein sizing, quantitation and molecular weight determination: sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), lab-on-a-chip or microfluidics technology (LoaC), size exclusion chromatography (SEC) and mass spectrometry (MS). We compare advantages and limitations of each technique in respect to different application areas, analysis time, protein sizing and quantitation performance.

Keywords: Protein analysis techniques; Sizing; Performance comparison; Molecular weight determination

1. Introduction

With the Human Genome Project close to completion, attention is now turning to the next step—how to utilize this genetic data to better understand diseases and develop targeted therapeutics more efficiently. Proteins, rather than genes, convey most cellular functions.

Understanding protein expression and protein function is crucial to the identification of new targets for drug development. As we now enter this new, postgenomic era, proteins will move into the focus of attention.

* Corresponding author.

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doi:10.1016/j.jbbm.2004.01.007

A universal task involved in protein characterization is purification, quantitation and identification. Determination of protein size and concentration is a routine procedure in many research laboratories. For example, protein quantitation is required to calculate and monitor the protein yield after various enrichment or purification processes as well as to optimize and standardize downstream experiments such as protein–protein interaction studies. Protein sizing is commonly used to identify proteins, oligomers and monomers.

2. Discussion

2.1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Although protein analysis technologies are developing fast, the current standard method for protein sizing is still denaturing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [1–3].

Sodium dodecyl sulphate (SDS) is an anionic detergent with a net negative charge. It binds to most soluble protein molecules in aqueous solutions over a wide pH range. The amount of bound SDS is proportional to the size of the molecules. The SDS eliminates most of the complex secondary, tertiary or quaternary structure of proteins, which is one requirement for protein sizing by SDS-PAGE. Furthermore, it is usually necessary to reduce protein disulphide bridges before the proteins adopt the random-coil configuration necessary for separation by size. This is achieved with reducing agents such as 2-mercaptoethanol or dithiothreitol. In addition, SDS also confers a negative charge to the polypeptide, which is proportional to its length. This negative charge is utilized to separate the protein in an electrical field within polyacrylamide gels. The polyacrylamide forms porous gels allowing to separate molecules by size. A polyacrylamide gel with a certain acrylamide concentration restrains larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in molecular weight (Mw) of polypeptides. In a gel of uniform pore size the relative migration distance of a protein (Rf) is negatively proportional to the logarithm of its Mw. If proteins of known Mw are run simultaneously with the unknowns, the relationship between Rf and Mw can be plotted, and the Mws of unknown proteins determined.

The sizing accuracy of SDS-PAGE depends on the protein characteristics, such as amino acid sequence, isoelectric point, structure and the presence of certain side chains or prosthetic groups. Sizing accuracy may therefore vary for particular proteins, as some proteins, e.g. glycosylated proteins, are not truly migrating according to their molecular weight. The sizing precision that can be achieved is approximately 2–7% (see Table 1). The size resolution can be improved by changing the gel composition to achieve optimal resolution for the desired experiment.

Protein separations by SDS-PAGE are commonly used to determine the approximate molecular weights of a protein and the relative abundance of major proteins in a sample. Usually the relative abundance of proteins within one gel is compared; gel-to-gel compar-

Table 1
Accuracy and precision of molecular weight by SDS-PAGE (for electropherogram and analytical conditions refer to Fig. 1)

Reference molecular weight (kDa)[a]	SDS-PAGE average (kDa)	Error (%)	RStDev (%)
116	128.1	−10.4	3.9
97	88.0	9.3	1.6
66.2	68.2	−3.0	1.9
45	47.2	−4.8	2.1
31	27.4	11.7	2.0
21.5	18.2	15.3	2.4
14.4	not separated from 21.5 kDa protein		

[a] Theoretical value.

isons are difficult to perform due to the bad reproducibility between gels (see Table 1). Carefully controlling the experimental conditions for SDS-PAGE such as temperature, pH, gel composition and staining times allows to significantly improve reproducibility. However, protein quantitation is therefore preferably performed with colorimetric protein quantitation assays such as Lowry et al. [4] or Bradford [5]. All commonly used protein stains used for protein detection exhibits some degree of varying staining efficiency when assaying different proteins, affecting quantitation accuracy (Fig. 1; Table 1).

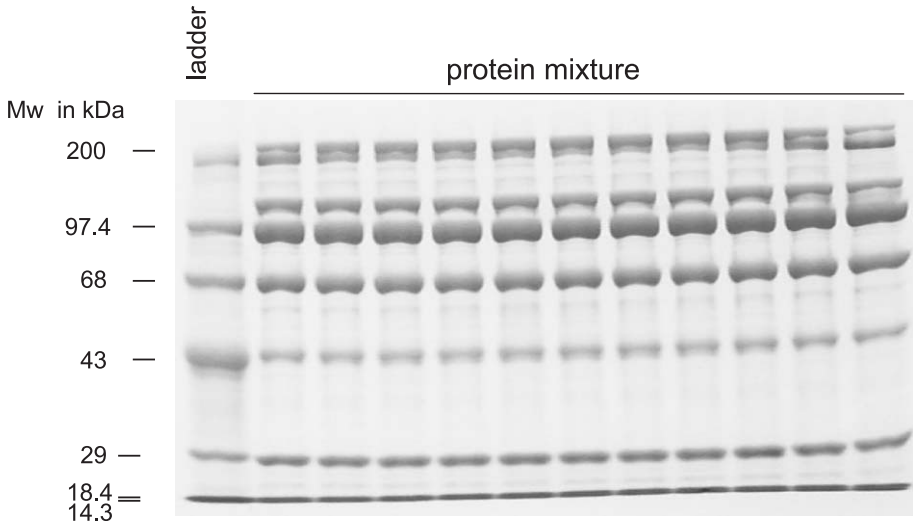


Fig. 1. SDS-PAGE: Gel electrophoresis was performed with 10% pre-cast Tris–glycine Gels (gel size: 8 cm×8 cm×1.5 mm) according to the instructions provided by the manufacturer. An equal volume Tris–glycine SDS sample buffer (2×) was added to the samples, and they were denatured for 5 min at 95 °C before loading onto the gel. The separation was performed for approximately 100 min at constant 125 V. Gels were stained with a non-colloidal Coomassie (R-250) staining kit and destained overnight. A Kodak DC 290 digital camera was used for imaging and analysis was performed with the Kodak 1D Image Analysis Software.

2.2. Lab-on-a-chip technology

The aim of lab-on-a-chip or microfluidics technology is to shrink processes, in this case chemical and analytical, to very small dimensions, thus allowing to handle very little sample volumes. In addition, it has the potential to shorten analysis times significantly and to automate the process of analysis. The technology allows for the active control of fluids in microfabricated channels that are only a few micrometers in dimensions without any moving parts. Typically a number of functional elements are combined on a chip: the emulation of pumps, valves and dispensers for sample handling on the chip, a separation column for electrophoretic separation, a reaction system and finally the detection.

The Agilent 2100 bioanalyzer, which was developed in collaboration with Caliper Technologies (Mountain View, CA, USA), is the first fully commercialized implementa-

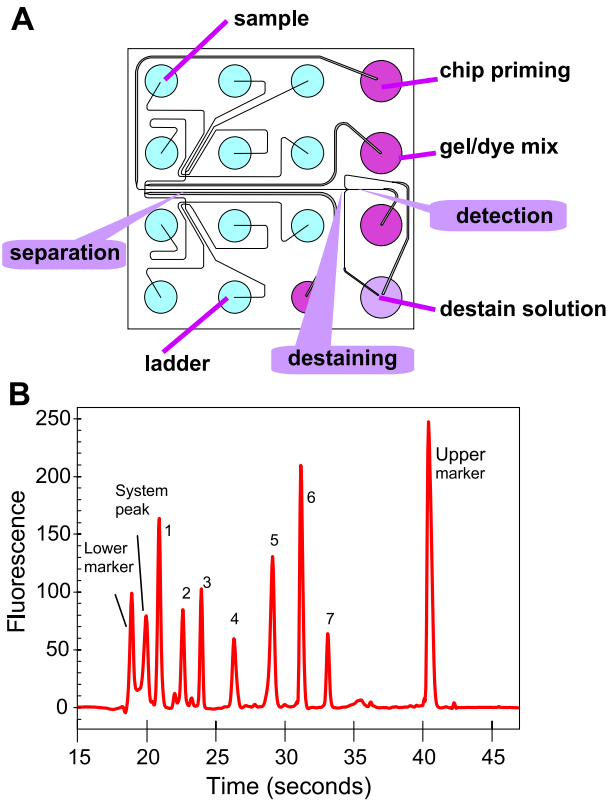


Fig. 2. (A) Channel layout of a microfluidic protein chip. The separation channel, the destaining cross and the detection area are marked. The wells on the chip will be filled with reagents as indicated. (B) An example electropherogram of a protein mixture of seven proteins is shown. The sample was analyzed once on 29 chips to determine sizing accuracy and precision (see Table 2a). The chip-based protein analysis was performed on the Agilent 2100 bioanalyzer using the Protein 200 Plus LabChip kit and dedicated assay software. All chips were prepared according to the protocol provided with the Protein 200 Plus LabChip kit.

tion of microfluidics technology to date [6,7]. Besides the analysis of RNA and DNA as well as simple flow cytometric measurements, the multifunctional instrument is also an alternative tool to characterize various protein samples in respect of size and quantitation determination.

The instrument uses disposable chips which consist of two glass plates bond together, one layer with microfabricated channels etched into it and another layer with 16 through wholes that form the sample and buffer reservoirs and provide access to the channels (Fig. 2a). The principle of the analysis is an electrophoretic process, where denatured proteins negatively charged through the interaction with SDS molecules are moved across the chip. The channels are filled with a sieving polymer in order to separate the proteins according to their size and an intercalating fluorescent dye, which stains the proteins. Before the laser induced fluorescence detection of the different proteins a destaining step is integrated on the chip. Thus 10 protein samples can be analyzed unattended within less than 30 min. The software automatically evaluates the data and displays a detailed result table containing the size and relative concentration of each protein as well as the percentage of the total protein content of the sample. Therefore it is very easy to determine protein production, to monitor and optimize protein purification processes or to determine purity and yield of a purified protein [8–10].

To achieve accurate sizing of unknown protein samples, a sizing ladder is run on each chip. The software automatically generates a standard curve of the ladder protein versus their known molecular weights, which is used to determine the size of the unknown proteins. As in SDS-PAGE sizing accuracy is dependent on the protein characteristics. The sizing precision ranges from 0.5% to 5% (see Table 4). Proteins between 5 and 200 kDa can be analyzed with a resolution of 5–10% (see Tables 2a and 4).

The Agilent 2100 bioanalyzer software provides two functionalities for quantitation. The first possibility is to determine the relative concentration of the individual proteins. This value is determined automatically by the software based on a one-point calibration with the upper marker (myosin), which is used as an internal quantitation standard in every sample (see Fig. 2b). To determine the relative concentration, the peak area of the unknown sample is compared to the peak area of the upper marker with known

Table 2a
Sizing accuracy and precision of the Agilent 2100 bioanalyzer

Peak no.	Protein	Reference Mw (kDa)[a]	Bioanalyzer average Mw (kDa)	Error (%)	RStDev (%)
1	Lysozyme	14.4	12.6	12.5	2.4
2	Trypsin inhibitor	21.5	20.8	3.3	1.3
3	Carbonic anhydrase	29	28.9	0.3	1.3
4	Ovalbumin	45	45.3	0.7	0.8
5	Serum albumin	66	68.4	3.3	1.4
6	Phosphorylase B	97	95.2	1.9	0.9
7	B-galactosidase	116	119.6	3.1	0.6

The protein mixture of Fig. 2 was analyzed once on 29 chips (n=29).

[a] Theoretical value.

concentration. The inclusion of the upper marker in each sample corrects for differences in sample injection into the separation channel and allows for reproducible quantitation. However, due to some staining variability between the upper marker and the protein of interest, this value is not as accurate as absolute quantitation, using a pure sample of the target protein as reference. The Agilent 2100 bioanalyzer software supports absolute quantitation, which can be obtained by a user generated protein quantitation calibration curve.

Table 2b shows that quantitation accuracy was greatly improved with the absolute quantitation feature compared to the relative concentration. The quantitation error for carbonic anhydrase (CA) was reduced from an average error of 42% to 6%, from 28% to 11% for ovalbumin (OV) and for bovine serum albumin (BSA) from 19% to 15%. The quantitation reproducibility was approximately 10% and comparable between both quantitation methods.

2.3. Size exclusion chromatography (SEC)

Aqueous size exclusion chromatography (SEC), often referred to as gel filtration chromatography (GFC), is an entropically controlled separation technique in which molecules are separated based on hydrodynamic volume. SEC is especially well suited for protein analysis applications such as quantification, impurity testing, reaction monitoring, product purification, folding studies, and the desalting and exchange of sample buffer. In all but the last of these applications, the protein's molecular mass may be simultaneously determined by calibrating SEC column retention times or elution volumes

Table 2b
Comparison of relative and absolute quantitation for bovine serum albumin (BSA), ovalbumin (OV) and carbonic anhydrase (CA)—accuracy and precision

Protein	Reference concentration (μg/ml)	Relative concentration (μg/ml)	RStDev (%)	Error (%)	Calibrated concentration (μg/ml)	RStDev (%)	RStDev (%)
BSA	1250	1523.3	9.8	21.9	1430.8	8.1	14.5
	750	911.6	8.9	21.5	856.1	6.9	14.1
	200	245.2	9.5	22.6	230.2	6.7	15.1
	40	36.4	9.5	9.1	34.2	6.1	14.6
OV	1250	985.7	3.4	21.1	1438.2	4.0	15.1
	750	597.6	3.7	20.3	872.7	6.2	16.4
	200	138.5	6.0	30.8	202.4	9.3	1.2
	40	24.3	12.9	39.3	35.6	15.1	11.1
CA	1250	1663.2	11.0	33.1	1142.0	12.0	8.6
	750	1083.8	10.0	44.5	742.7	8.6	1.0
	200	315.3	13.2	57.6	215.4	8.9	7.7
	40	53.7	28.8	34.3	36.8	27.7	8.1

The first six wells of the chip were loaded with standards of the indicated proteins and four "unknown" target concentrations of each protein were loaded into well seven through ten. The "unknown" concentrations were evaluated using either the upper marker (relative concentration) or the calibration curve generated with the standards (absolute concentration; n=6).

with an appropriate series of macromolecular standards or by employing molecular mass sensitive detection methods such as viscosimetry or light scattering.

SEC can handle a very wide range of molecular weights, from several hundred to several million Dalton, and also an extremely wide range of polarities, from easily water-soluble to hydrophobic membrane proteins. A 50–100% difference in molecular weight is typically required. Both, the quantitative data and the molecular weight information are obtained within a single analysis in good precision and accuracy. SEC does not denature the separated protein, which can easily be collected for further investigation. The accuracy of the molecular weight data depends on the calibrant proteins [11], instrument [12] and software performance [12]. Column calibrations should actually be performed with macromolecules of the same conformational class as the unknown [11]. When this prerequisite is fulfilled, the accuracy is typically about 2%. Because proteins of the same conformational class as the unknown are often not available, water-soluble, globular proteins with known molecular weight are often used instead. Le Maire et al. [11] list a series of 15 standard proteins, which were found to be suitable for column calibration. The molecular weights of uncharacterized proteins can differ significantly from the true molecular weight due to different elution behavior between the standard and the unknown proteins. Table 3 summarizes SEC Mw results for proteins of different conformation.

When the scientist is not satisfied with molecular weight results relative to standard proteins viscosimetric or light scattering detection can be used. Because the viscosimetric and light scattering response is directly proportional to the molar mass of the sample, no calibrant proteins are needed [13,14]. Light scattering and viscosimetric detection require in addition a concentration sensitive detector, typically refractive index, because both of these detector responses are proportional to the concentration of the sample.

Table 3
Comparison of molecular weight data obtained by SEC and reference methods

Protein	Reference molecular weight	SEC molecular weight	Difference (%)
Ovalbumin	45,000[a]	42,700	2.8
Human albumin	66,000[a]	65,000	1.5
Antibody	157,000[b]	149,000	5
Myoglobin	17,000[c]	17,100[d]	0.5
Ferritin	460,000[a]	459,000	0.2
Erythropoetin (EPO)	35,000[d]	54,000	55
Granulocyte colony stimulating factor (G-CSF)	18,800[d]	14,200	24
		18,800[e]	3

[a] Theoretical value.
[b] Obtained by Lab-on-a-Chip Technology with the Agilent 2100 Bioanalyzer.
[c] Obtained by SDS-PAGE.
[d] Obtained with MALDI-MS.
[e] The SEC calibration curve was further optimized by the addition of the calibration data of a well characterized G-CSF protein.

The precision of the SEC molecular weight information is about 2%. The precision and accuracy in protein and impurity quantitation by SEC is typically better than 0.5% (see Table 4).

Fig. 3 shows a typical application for protein SEC: determination of impurities and peak identification by molecular weight determination within a single run. The sample is recombinant granulocyte colony stimulating factor (r-G-CSF). G-CSF is a hydrophobic protein which requires for elution the addition of a detergent to the eluent [15]. The chromatogram shows the absence of protein oligomers, but the presence of lower molecular weight impurities in a significant concentration. The molecular weight information agrees well with the MALDI-MS value when the original SEC calibration curve is modified by the addition of the calibration data obtained with a 2nd G-CSF sample (Table 3). The molecular weight of this 2nd g-CSF was known from MALDI-MS. Without the modification the difference compared with the MALDI-MS molecular weight is 24% (see Table 3).

2.4. Mass spectrometry

Matrix-assisted laser desorption ionization (MALDI) [16] and electrospray ionization (ESI) [17] are complementary ionization techniques for mass spectrometry that allow the molecular weight determination of large biomolecules. MALDI in combination with time-of-flight (TOF) mass analyzers is employed for analyzing peptides/proteins up to 300 kDa or more. A limited dynamic range and a negative influence on sensitivity from salts or

Table 4
Comparison of analytical techniques for protein sizing, quantitation and molecular weight determination

	SDS-PAGE	LoaC	SEC	LC/MS (Quad)
Analysis time	>2 h for one gel (10–20 samples)	30 min per chip (10 samples)	30 min per sample	>3 min per sample
Instrument cost	$3000 to 15,000 (incl. imaging)	<$25,000	$25,000–50,000	>$100,000
Sizing				
Accuracy	Depending on protein	Depending on protein	Depending on protein	0.01%
Precision	2–7%	0.5–5%	<2%	na
Resolution	Depending on gel	5–10%	50–100%	na[a]
Size range	5–500 kDa	5–200 kDa	0.1–10,000 kDa	<100 kDa
Absolute quantitation				
Accuracy	10–50%	5–20%	<1%	na
Precision	10–50%	5–20%	<0.5%	na
Limit of detection	Depending on stain (e.g. Coomassie R-250: 50–100 ng)	80 ng BSA in PBS 160 ng BSA in 0.5 M NaCl	20 ng BSA in 50 ul	Low picomol
Linear dynamic range	$<10^2$	10^2 (e.g. 20–2000 ng/μl BSA in PBS)	10^4	10^3

[a] Chromatographic resolution varies with the chosen HPLC method and the separated proteins. Mass spectral resolution is dependent on the mass analyzer and is between 1000 and 1,000,000 ($\Delta M/M$).

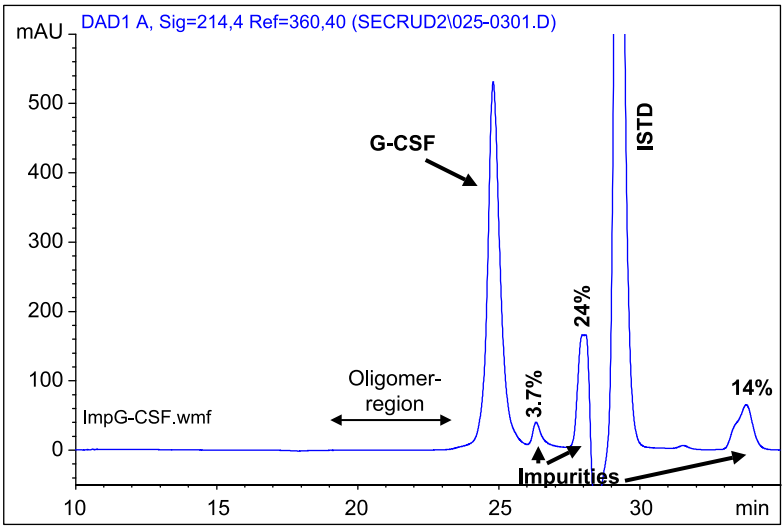
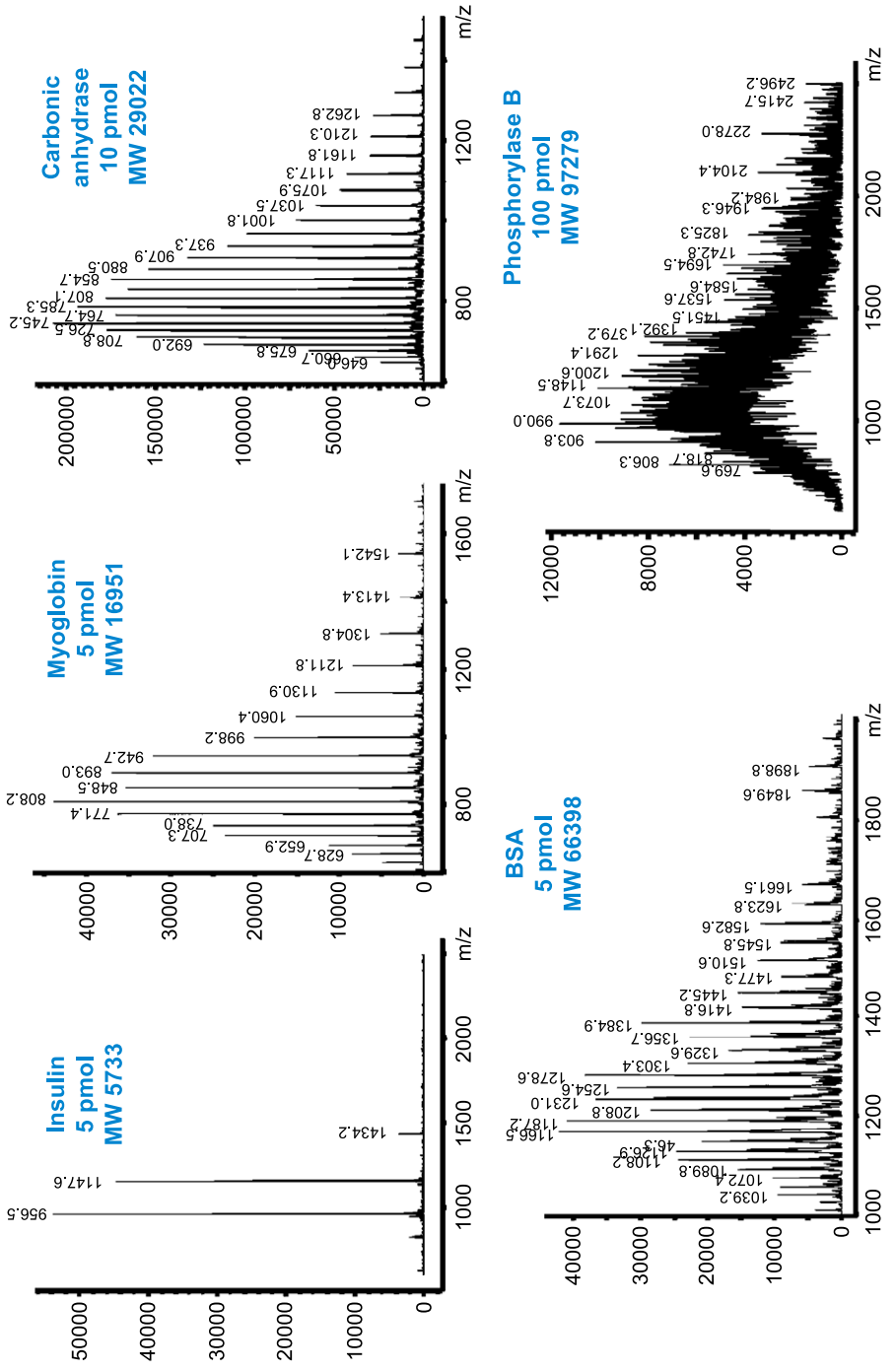


Fig. 3. The SEC analysis was performed with the Agilent 1100 Series GPC-SEC analysis system. The data was transferred to the HPLC 3D ChemStation with GPC-SEC data analysis software. The columns (Agilent TSK SW 2000 [part number 79911S3-597] in series with Agilent TSK SW 3000 [part number 79912S3-597) were purchased from Agilent Technologies, Germany. Mobile phase: 0.15 M NaCl, 20mM Na-phosphate, 0.01% Tween 20, pH 6.7. SEC calibration was performed by injecting globular, water-soluble proteins (supplier: Sigma, Munich, Germany) comprising a range of known molecular weights and then graphing the log of these values against retention time/elution volume. The curve fit was optimized according to international GPC-SEC standards [15] using software calculated %-deviation-value between the actual calibration point and the user-selected curve.

detergents deserve a clean sample for best results. ESI produces multiply charged ions of biomolecules, therefore even simple and cheap quadrupole mass analyzers with a mass range up to *m*/*z* 3000 allow a correct determination of protein molecular weights. However, a mathematical algorithm is needed [18,19] to determine the molecular weight from the mass spectrum, which shows an envelope of multiply charged ions from one protein (Fig. 4). In the on-line combination with liquid chromatography, proteins up to about 100 kDa could be injected, chromatographically separated and detected without tedious sample preparation steps.

In ESI, the number of charge states increases with increasing molecular weight. Secondly, the more charge states for a protein, the poorer the sensitivity because the signal is divided over more ions. This explains the limitation in mass to around 100 kDa.

Due to the complexity of overlapping charge envelopes, it may not be possible to determine the molecular weight for heterogenic proteins without chromatographically separating the isoforms. Therefore high separation capacity is needed in order to chromatographically separate protein mixtures/isoforms and avoid overlapping envelopes. Traditional silica-based reversed phase columns did not allow fast and efficient protein separations, as mass diffusion in and out the pores was a limiting factor. The development of Poroshell columns [20] with superficially porous silica-microsphere packing with a porous outer shell yielded silica-based columns combining the capacity advantage of traditional protein columns and the rapid mass transport of 2-μm non-


Insulin
5 pmol
MW 5733
956.5
1147.6
1434.2
Myoglobin
5 pmol
MW 16951
628.7
652.9
707.3
738.0
771.4
808.2
848.5
893.0
942.7
998.2
1060.4
1130.9
1211.8
1304.8
1413.4
1542.1
Carbonic
anhydrase
10 pmol
MW 29022
646.0
660.7
675.8
692.0
708.8
726.5
745.2
764.7
785.3
807.1
854.7
880.5
907.9
937.3
1001.8
1037.5
1075.9
1117.3
1161.8
1210.3
1262.8
BSA
5 pmol
MW 66398
1039.2
1072.4
1089.8
1108.2
1126.9
1166.5
1187.2
1208.8
1231.0
1254.6
1278.6
1303.4
1329.6
1356.7
1384.9
1416.8
1445.2
1477.3
1510.6
1545.8
1582.6
1623.8
1661.5
1849.6
1898.8
Phosphorylase B
100 pmol
MW 97279
769.6
818.7
806.3
903.8
990.0
1073.7
1148.5
1200.6
1291.4
1392.1
1379.2
1451.5
1537.6
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m/z

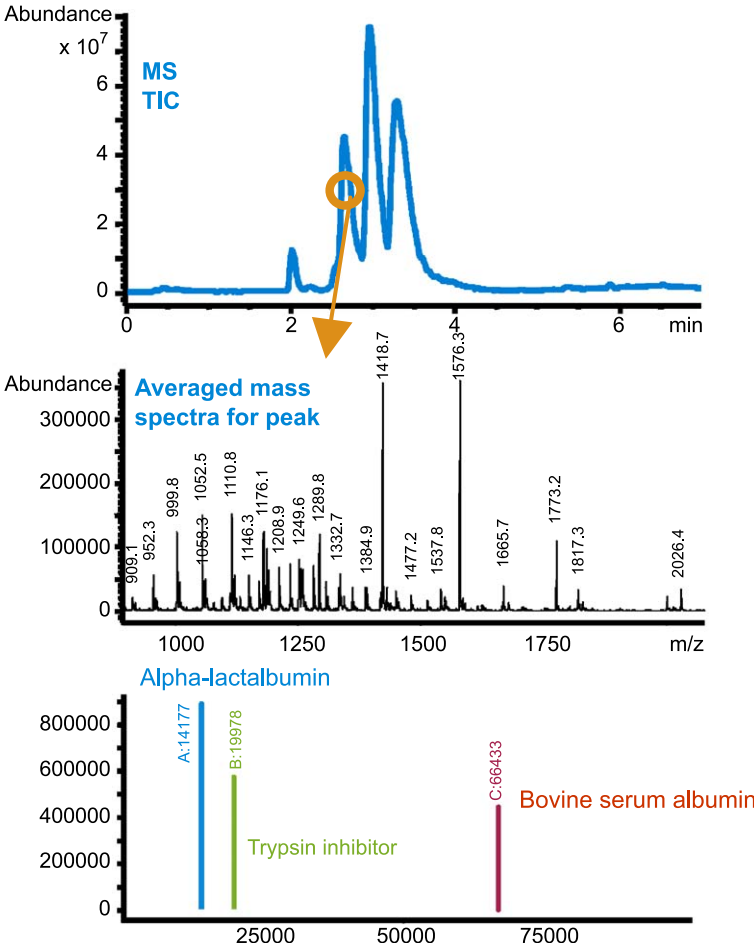

Fig. 5. A six-protein mixture plus sucrose was analyzed by LC/MSD. Although three proteins co-elute resulting in an overlap of their charged envelopes, all three proteins could be still deconvoluted correctly (A: alpha-lactalbumin 14 kDa, soy-bean trypsin inhibitor 20 kDa, bovine serum albumin 66 kDa).

porous columns. Typically a water/acetonitrile gradient with up to 0.1% formic acid is used. The reduced backpressure for superficially porous silica-microsphere packing allows higher flow rates with constant separation efficiency leading to overall runtimes as short as 3–5 min (Fig. 5).

Fig. 4. Mass spectra of several proteins. These mass spectra were acquired with the Agilent 1100 LC/MSD system. Standard proteins from Sigma were dissolved and diluted to the appropriate concentrations. The LC separation was done with a rapid water/acetonitrile/gradient (20–100% acetonitrile in 5.5 min) containing 0.1% formic acid and an Agilent Technologies Poroshell (2.1×50 mm) column at a flow rate of 500 μl/min, 60 °C.

In general, this packing material allows a rapid and very efficient separation of large biomolecules and enables LC/MS with an easy-to-operate and cheap quadrupole bio-analyzer to be used for protein sizing in a rapid, exact, and sensitive way.

3. Conclusion

We compared the main techniques for protein sizing, quantitation and molecular weight determination.

Protein analysis technologies are developing fast, but many still rely on traditional methods such as SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which include a number of laborious, time-consuming manual steps and are difficult to automate.

Although resolution can be optimized with different gels according to the molecular weight of interest, the repeatability in quantitation is limited (Table 1).

A good alternative is lab-on-a-chip or microfluidics technology, which is a miniaturized electrophoresis based technique for rapid and automated analysis of proteins on a chip.

Proteins from 5 to 200 kDa can be analyzed with a resolution of 5–10% and much better sizing precision and quantitation capability than SDS-PAGE.

SEC is a mainstay technique for the analysis of proteins and offers 2% sizing precision and a 0.5% quantitative accuracy together with ease of use and very good automation capabilities.

One mayor advantage of the technique is that it can handle a very wide range of molecular weights, from about 0.1 to about 10,000 kDa. The columns and mobile phases can be adapted to almost any type of protein polarity. However, the proteins need to differ at least 50% in molecular weight to get separated.

Excellent mass accuracy of 0.1% and very high sensitivity is obtained by mass spectrometry, for example by MALDI-MS. Therefore it allows the identification of unknown proteins at very low concentrations, which is not possible with the other three techniques. The molecular weight is limited up to approximately 120 kDa. The dynamic range varies significantly for the discussed techniques from two to four orders of magnitude (Table 4). SDS-PAGE and SEC allow both analytical and preparative analyzes, while the lab-on-chip technology and mass spectrometry are purely analytical techniques.

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