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Quantitation of proteins using HPLC-detector response rather than standard curve comparison ☆

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Abstract

Modern high performance liquid chromatography (HPLC)-diode array detectors with features such as multiple wavelength monitoring are capable of maintaining a high degree of response reproducibility over extended periods of time. This reproducibility suggests that detector response factors, rather than dilution based standard curves, might be used to measure concentrations of proteins and pharmaceuticals. Four different HPLC methods were used to analyze a single protein and to test the accuracy and precision of measurements using response factors. These results were compared to the accuracy and precision obtained using fitting to a standard curve. Protein solutions were analyzed by HPLC after the concentration was determined by quantitative amino acid analysis. The extinction coefficient at 277 nm of these protein solutions was determined by UV spectroscopy as well as calculated based on the known amino acid composition. The theoretical extinction coefficient calculated by summing the extinction coefficient of the individual amino acids was within 2% of the experimental value.

Response factors at 215 and 277 nm were calculated using the peak area produced by the injection of a known amount of protein. When the experimental extinction coefficient was used to calculate the expected HPLC-signal response (peak area = absorbance × duration), the recovery of the protein (accuracy) was 100% if measured at 215 nm and between 90 and 94% when measured at 277 nm. The ruggedness of the recovery was between 2.6 and 4% relative standard deviation, depending on the HPLC-method. It was found that the quantitation was at least as accurate when calculated from the peak area using the response factor as when a standard curve was used.

Keywords: HPLC-response factor; Quantitation of proteins; Extinction coefficient of proteins; Fibroblast growth factor

1. Introduction

A general procedure to quantify proteins in complex mixtures does not exist. A variety of methods are currently in use [1], each with their advantages and shortcomings. The total protein content in protein solutions is generally determined by Lowry, Bicinchoninic acid (BCA), or Bradford assays, which relate the reactivity or binding capacity of an unknown protein solution to the same characteristics of a known protein standard solution.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [1] separates the components of complex mixtures by size, and, when followed by scanning of Coomassie stained gels, has also been used to estimate protein content. Amino acid analysis (AAA) is laborious, and is quantitative only if the

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protein is in a pure state. Elemental analysis (CHN assay) is effective only if the protein is pure and no interfering substances are present. Ellman's titration [2] is very reproducible but can only be applied to a pure protein which contains cysteines where all the cysteines are in a controlled reduced state. All of the above assays require standards for each application.

The only method known that assays the total amount of protein present without having to use a standard for every assay is UV spectroscopy in solution. Once the extinction coefficient is determined with an appropriate standard, all future measurements can be related to this extinction coefficient. However, UV spectroscopy can only be applied to a small range of protein concentrations and if no other absorbing components are present in solution.

SDS-PAGE and isoelectric focusing (IEF) are capable of separating the components of protein mixtures by size or isoelectric point. The amount of a given protein in the mixture can be estimated by scanning stained gels containing known amounts of standard proteins. Unfortunately, variations in the staining of individual proteins make this method prone to significant error.

An ideal combination of instrumentation to isolate the protein of interest and perform UV analysis is high performance liquid chromatography (HPLC) with a diode array UV detector. In the last 20 years, HPLC has become the method of choice for the separation of peptides and proteins. Microprocessor controlled high pressure pumps, mixing, and injection devices allow accurate and reproducible buffer mixing, flow control, and reproducible sample injection. Diode array technology has combined sophisticated UV spectrophotometry with mechanically well-designed HPLC-instrumentation. New software and data processing technology allow complicated integration techniques to consider baseline and noise disturbance. Thus, HPLC-instruments are now high precision tools, not only for concentrating and separating proteins of interest, but also for quantifying complicated dilute mixtures in one step. This highly refined technology makes sample handling by the operator the limiting step for reproducibility and accuracy.

Traditionally, quantitative measurements on HPLC instruments were performed by comparing the duration of the output signal of the HPLC-separation with the duration of the output signal of a known internal or external standard or standard curve [3]. Rossi et al. [4] compared static UV spectra taken in the HPLC-detector cell with the added spectra of the separated components and found a very high precision of the detector signals of a dynamic measurement. We present in this article the reproducibility of protein quantitation using the response factors of four different HPLC-methods and compare the results with values calculated using traditional standard curves. The application of this procedure can give preliminary estimates of the quantity of a protein of known amino acid composition, without knowledge of the static extinction coefficient. It allows accurate quantitation of protein if the static extinction coefficient is known. It eliminates the need for standard curves in the quantitation of proteins of known extinction coefficient. This method led to improved accuracy in mass determination by eliminating random and systematic errors introduced by the preparation and use of standard curves.

2. Materials and methods

Recombinant basic Fibroblast Growth Factor (bFGF) was produced at Scios Nova Inc. by the recombinant bacterial expression method in E. coli described by Thompson et al. [5].

Five different protein lots were assayed on three different occasions within 6 months. All formulations were prepared on ice. Placebos, samples and references were filtered through a 0.22 μm filter (Gelman Acrodisc 13) before analysis. Filters were pre-saturated with protein and leachables were washed off (the first three drops of filtrate were discarded). On the day of analysis, three lots were diluted to approximately 1 mg ml^{-1}. The concentrations of the samples were determined in quadruplicate by AAA. Quantitative AAA was performed in quadruplicate on each protein lot by the Protein Structure Lab. at the University of California at Davis on a Beckman 6300 amino acid analyzer, utilizing a sodium citrate buffer system optimized for hydrolyzed proteins and peptides.

The samples were simultaneously analyzed in heptaplets by UV spectroscopy and by one or more of the following methods: reverse phase (RP)-HPLC, ion exchange (IE)-HPLC, heparin affinity-HPLC and size exclusion (SE)-HPLC.

HPLC analysis was carried out on three different HP 1090L instruments (Hewlett Packard) equipped with a diode array detector, a refrigerated auto sampler, and a PV5-ternary solvent delivery system (low pressure mixing system with metering pump). The flow rate accuracy was ±1 µl and the flow rate precision was 0.3%.

2.1. Accuracy

AAA was used as a measure of accuracy of the results from the use of a standard curve and response factor.

2.2. Precision

The precision of a standard curve obtained by a sequential dilution was compared with the precision of the quantitation obtained by the use of response factors. Precision is comprised of repeatability (short term precision), reproducibility (long term precision), and ruggedness. In order to obtain ruggedness data, the response factor was obtained from eight different lots of FGF, injected by four different operators, analyzed over three different columns, on three different instruments and four different analytical methods over a period of 6 months.

The precision of the dilution operation was determined by diluting six batches at three different time points over a period of 6 months. The batches were diluted to 1 mg ml^{-1}. The concentration was verified by AAA and the precision was determined from the UV spectroscopic data.

2.3. HPLC conditions used

(A) Reverse phase HPLC

Sample loads were 50.0 µl (35 µg). The flow rate was 1.0 ml min^{-1}. Column: protein C-4; 150 mm × 4.6 mm, 5 µm, 300 Å, Cat. #214TP5415, Vydac. Buffer: A, 0.1% TFA in water; B, 0.08% TFA in acetonitrile. Gradient: 0.5% acetonitrile min^{-1}.

(B) Ion exchange chromatography

Sample loads were 50.0 µl or 100 µl (35/70 µg). The flow rate was 1.0 ml min^{-1}. Column: polyLC "PolyCAT A"; 200 mm × 4.6 mm; 5 µm, 1000 Å, Cat. #204CT0510, PolyLC, Inc. Columbia, MD. Buffer: A, 20 mM Na phosphate (pH 6.0), 1 mM Na EDTA, 100 mM $(NH_4)_2SO_4$; B, 20 mM Na phosphate buffer (pH 6.0), 1 mM Na_2EDTA, 500 mM $(NH_4)_2SO_4$. Gradient: 8 mM $(NH_4)_2SO_4$ min^{-1}.

(C) Heparin affinity chromatography

Sample loads were 50.0 µl (35 µg). The flow rate was 1.0 ml min^{-1}. Column: TOSOHaas heparin-5PW, 75 mm × 7.5 mm, 1000 Å; Cat. #13064. Buffer: A, 100 mM K phosphate buffer (pH 6.5), 1 mM Na_2EDTA; B, 100 mM K phosphate buffer (pH 6.5), 1 mM Na_2EDTA, 3 M NaCl. Gradient: 180 mM NaCl min^{-1}.

(D) Size exclusion chromatography

Sample loads were 10.0 µl (7 µg). The flow rate was 0.75 ml min^{-1}. Column: TOSOHaas TSK-Gel, G2000 SWXL, 5 µm, 300 mm × 7.8 mm; Cat. #08540. Buffer: 100 mM K phosphate buffer (pH 6.5), 1 mM Na_2EDTA, 1 M NaCl. Gradient: isocratic.

Detection wavelength

This was 277 or 215 nm with a band width of 4 nm. The reference wave length was 550 nm with a band width of 100 nm. The detector path length was 6 ± 0.07 mm. The instrument was calibrated to an output of 500 mV for 1 absorbance unit (AU) and was offset to +50 mV before each run. The noise level was 0.000 009 V.

2.4. Data analysis

Data were processed by the Nelson Analytical Integration System supported by Nelson Analytical Access*Chrom software (Nelson Analytical Inc., Cupertino, CA) on a VAX (3600 Digital Equipment) computer system.

2.5. Calculation of the response factor

To obtain the response factor of the HPLC-data, all peak areas (in µV s) of each sample HPLC-chromatogram were added. Peak areas that appeared on a placebo run were not considered. The total peak area was divided by the total amount of injected protein. The response factor, peak area per µg protein, was further divided by the applied flow rate and divided by the detector cell path length in order to obtain the final flow rate and detector path length independent response factor in

$mV\ s^2\ mg^{-1}\ cm^{-4}$. All outliers were removed according to the DIXON-test [6].

2.6. Sample preparation

UV spectroscopy

All UV spectrophotometric data were collected on a Cary 3 (Varian) spectrophotometer. For the determination of the extinction coefficient, each lot and the placebo were aliquoted into seven pairs of cuvettes and scanned at 1800 nm min^{-1} from 500 to 200 nm with the appropriate buffer in the reference cuvette. The composition of the HPLC-buffers was corrected to match the actual buffer composition at the apex of the peaks.

Standard curve preparation

The standard was stored at -80 ± 10 °C. After thawing, three appropriate aliquots of the standard solution were diluted in 0.5–1 ml placebo buffer to final concentrations of 0.5, 1.0 and 1.3 mg ml^{-1}.

3. Results

3.1. Calculations of the theoretical response factor

The test protein, bFGF, contained one tryptophan and seven tyrosine residues. The theoretical extinction coefficient at 277 nm was calculated to be 0.931 ± 0.0067 AU ml mg^{-1} cm^{-1} (Trp, 5500 AU M^{-1} cm^{-1}; Tyr, 1493 [7]; Crys, not considered; FW, 17 124). The extinction coefficient at 215 nm was determined by UV spectroscopy in the same buffer composition as occurs at the apex of the main peak (corrected for dwell volume). From the calculated extinction coefficient, a detector response factor at 277 nm was calculated in two steps as follows.

(A) Peak height response factor from extinction coefficient

Beer's Law defines the relationship between the absorbance of a solution at a given wavelength and the concentration of the absorbing component in solution:

$$A_\lambda = E^{0.1\%} C d$$

where A_λ is the absorbance at wavelength λ, $E^{0.1\%}$ is the extinction coefficient of $0.1\% = 1$ mg ml^{-1} solution, C is the concentration in mg ml^{-1}, and d is the path length in cm.

For FGF, the calculated theoretical extinction coefficient was

$$E^{0.1\%} = 0.931 \pm 0.0067 \text{ (AU ml mg}^{-1}\text{ cm}^{-1}\text{)}$$

To calculate the height response factor or the concentration in the flow cell at any instant, the extinction coefficient was converted to detector signal output units (in μV).

The detector output was fixed at 1 AU = 500 mV, and the extinction coefficient was referenced to a detector path length $d = 1$ cm. The response factor for the peak height (Rf_h) was found by converting the absorbance into the output signal unit (mV):

$$500 \text{ (mV AU}^{-1}\text{)} \times A_\lambda = 500 \text{ (mV AU}^{-1}\text{)} \times E^{0.1\%} C d$$

$$Rf_h = 500 E^{0.1\%} \text{ (mV ml mg}^{-1}\text{ cm}^{-1}\text{)}$$

$$Rf_h = 466 \pm 3.4 \text{ (mV ml mg}^{-1}\text{ cm}^{-1}\text{)}$$

Rf_h = response factor for peak height

The concentration (mg ml^{-1}) at any point of the peak is calculated by dividing the height above the baseline (in mV) by Rf_h and by the detector cell path length 'd' (cm).

(B) Peak area response factor from extinction coefficient

In order to obtain the peak area response factor from the extinction coefficient the peak height has to be integrated over its duration (i.e. peak area). The duration is determined by the flow rate 'f' (ml/60 s):

$$A_\lambda t = E^{0.1\%} t C d$$

$$500 \text{ (mV AU}^{-1}\text{)} \times A_\lambda s = 500 \text{ (mV AU}^{-1}\text{)} \times E^{0.1\%} t C d$$

Since

$$tC = q f^{-1}$$

where q = eluted quantity (mg) and

$$500 \text{ (mV AU}^{-1}\text{)} \times E^{0.1\%} = Rf_h$$

$$500 \text{ (mV AU}^{-1}\text{)} \times A_\lambda s = \text{peak area}$$

$$\text{Peak area} = Rf_h f^{-1} q d \text{ (mV ml mg}^{-1}\text{ cm}^{-1}\text{) (s ml}^{-1}\text{)}$$

$$\text{Peak area} = Rf_A q d \text{ (mV s mg}^{-1}\text{ cm}^{-1}\text{)}$$

since

$$Rf_h f^{-1} = Rf_A = 466 \times 60 \text{ (mV mg}^{-1}\text{ cm}^{-1}\text{)(s ml}^{-1}\text{)} = 27930 \pm 22 \text{ (mV s mg}^{-1}\text{ cm}^{-1}\text{)}$$

Table 1
Response factors at 277 nm (Table 1A) and 215 nm (Table 1B). The repeatability, reproducibility and ruggedness are listed as the relative standard deviations (RSDs), which are not significantly different between 277 and 215 nm. The amount of protein injected was determined by amino acid analysis
(A)

	Response factor ($V\ s\ mg^{-1}\ cm^{-1}$)	Repeatability (% RSD)	Reproducibility (% RSD)	Ruggedness (% RSD)	n
Heparin-affinity-HPLC	2.64	0.71	0.60	n.d.	19
Reverse-phase-HPLC	2.36	1.50	1.30	n.d.	20
Ion exchange-HPLC	2.52	0.35	0.69	n.d.	20
Size exclusion-HPLC	2.36	0.43	0.24	n.d.	19
Calculated from AA composition	2.80	n.d.	n.d.	n.d.	30
Calculated from AA analysis	2.81	n.d.	n.d.	n.d.	27

(B)

	Response factor ($V\ s\ mg^{-1}\ cm^{-1}$)	Repeatibility (% RSD)	Reproducibility (% RSD)	Ruggedness (% RSD)	n
Heparin-affinity-HPLC	44.4	0.39	0.46	1.3	40
Reverse-phase-HPLC	44.3	0.78	1.9	2.1	68
Ion exchange-HPLC	41.3	0.92	2.3	2.8	68
Size exclusion-HPLC	39.3	0.95	2.0	6.4	55
Calculated from AA composition	42.5	n.d.	n.d.	0.0	6
Calculated from AA analysis	42.7	n.d.	n.d.	0.0	6
1:10 dilution of FGF to $0.7\ mg\ ml^{-1}$	n.a.	n.d.	n.d.	3.2	7

If instead we used the experimentally determined extinction coefficient (by AAA), the calculated peak area response factor was $28\,100 \pm 100$. These numbers are not significantly different.

The injected quantity is calculated:

$$\text{Peak area} = Rf_A q d\ (mV\ s)$$

where q = peak area/($Rf_A d$) (mg), Rf_A = area response factor for peak area at $f = 1\ ml\ min^{-1}$, t = time (s) and q = eluted quantity (mg)

3.2. Calculations of the experimental area response factor determined by HPLC

To obtain the experimental peak area response factor the detector output signal (mV s) was divided by the injected quantity (mg) determined by AAA, divided by the detector path length (cm):

$$\text{Peak area} = Rf_A q d\ (mV\ s)$$

$$Rf_A = \text{peak area} \times q^{-1} d^{-1}\ (mV\ s\ mg^{-1}\ cm^{-1})$$

The measured response factors at 277 nm and at 215 nm are listed in Tables 1A and 1B. The area response factors at 215 nm were about 15 times larger than those at 277 nm. Although the area response factor at 215 nm provides a higher detector sensitivity, it is less specific for proteins. Fig. 1 shows typical chromatograms for all four HPLC-methods used.

The response factors experimentally determined by HPLC-detector response at 277 nm were between 6 and 10% lower than the calculated area response factor (Table 1A, Fig. 2(A)). Although the source of the lower apparent recovery is not known, the measured area response factor was highly reproducible over a period of 6 months, independent of instrument, operator or column.

Accurate determination of the extinction coefficient from AAA at 215 nm was more difficult than at 277 nm, because buffer and excipients interfered and because changes in pH or salt concentration affected the protein UV absorption at 215 nm more than the absorption at 277 nm (see Fig. 3). The extinction coefficient was determined by visually reading the absorbance off the Y-axis. In spite of the variability of the extinction coefficient at 215 nm, the measured area response factor at 215 nm when compared to the calculated area response factor was nearly 100% (Table 1B, Fig. 2(B)). The precision (average repeatability and reproducibility, all four HPLC-methods)

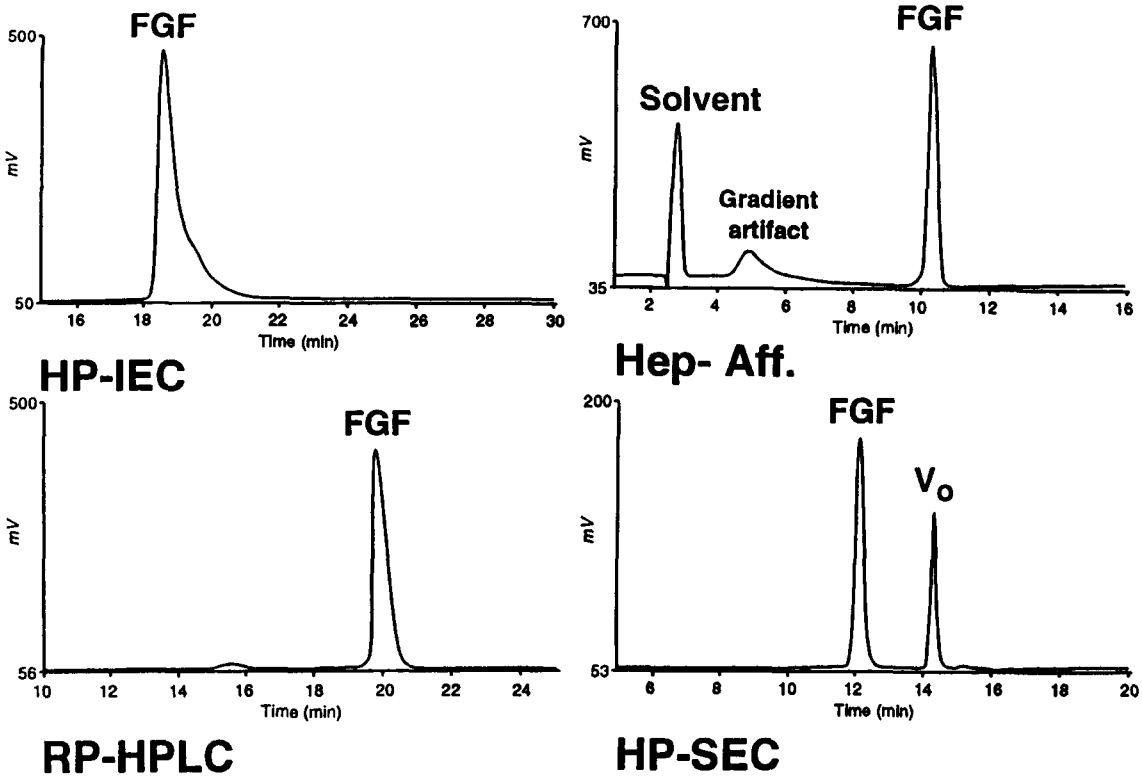

Fig. 1. HPLC-chromatograms of one bFGF sample analyzed by ion exchange chromatography (HP-IEC), heparin-affinity chromatography (Hep-Aff), reverse-phase chromatography (RP-HPLC) and size exclusion chromatography (HP-SEC). All chromatograms show a 15 min time window. The peak area of interest for the determination of the response factor was determined by subtraction of placebo signals. The injected mass was 100 μg for HP-IEC, 50 μg for Hep-Aff and RP-HPLC, and 10 μg for HP-SEC.

of the area response factor at 277 nm was 0.73% RSD compared to 1.22% RSD at 215 nm.

3.3. Comparison of the precision of the mass determined by standard curves or by the use of response factor

When only experimentally determined response factors are used, the RSD of the response of nine independent dilutions at three different times over a period of 6 months using different instruments, columns and operators was less than 2.8% for all HPLC-methods (Fig. 4). In contrast, when a 'traditional' standard curve (Fig. 5) approach was used for conversion of peak area to protein mass, the RSD of seven independent dilutions at three different times over a period of 6 months was 3.2%, demonstrating that the determination of the injected mass from the peak area is at least as accurate as comparison with a standard curve obtained by direct dilution (Fig. 4). It is essential to note that the error associated with the standard curve is determined from the 95% confidence interval around the curve and not by determining the RSD of independent measurements read from the curve. A typical four point standard curve with two independent measurements at the concentration of interest is shown in Fig. 5. The standard curve looks linear, but a 2.6% RSD is attached to all data derived from this standard curve. The concentration obtained using the standard curve was 1.08 ± 0.021 and by use of the area response factor was 1.04 ± 0.016 mg ml^{-1}. When data collected over a period of 6 months and for two different analytical methods (RP- and IE-HPLC) were compared after analysis by response factor or by traditional standard curve comparison, no significant difference in the results could be detected, but the data obtained using area response factors have 30% tighter confidence limits (Table 2).

4. Conclusions

This report introduces the idea of using HPLC-response factors to quantify proteins for

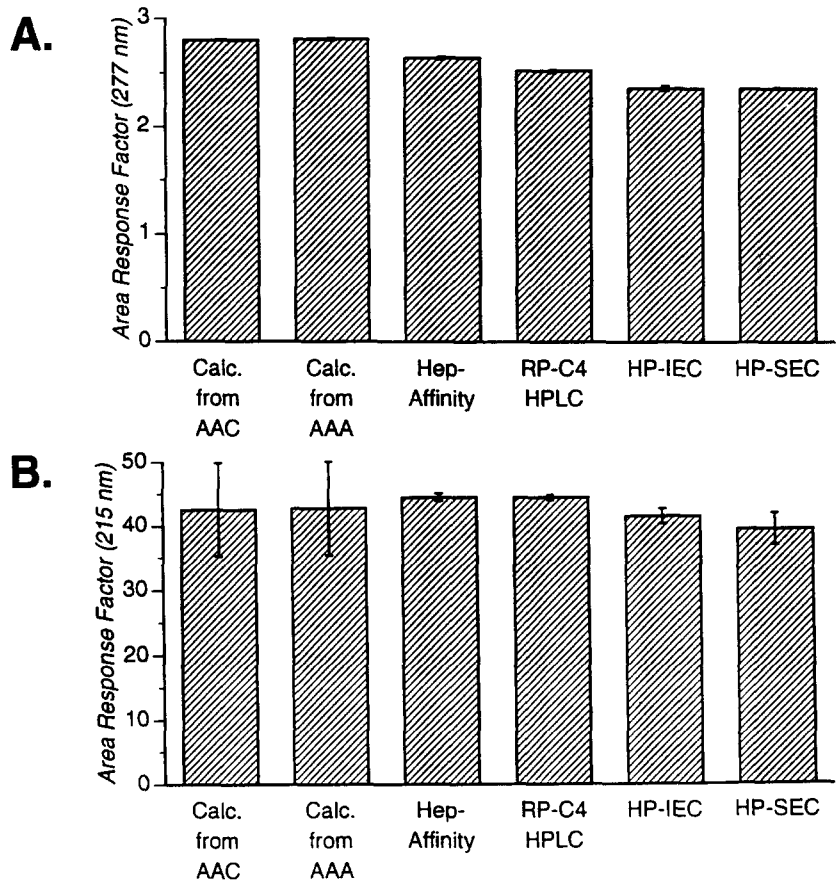

Fig. 2. Experimentally obtained area response factors at 277 (A) and at 215 nm (B) compared with the area response factor that was calculated from the theoretical amino acid composition (AAC) and from amino acid analysis (AAA). The area response factors are expressed in V s mg^{-1} cm^{-1}. The relatively low confidence of the calculated data at 215 nm results from the difficulties in determining accurately the UV absorbance at 215 nm. The recovery at 277 nm relative to the calculated response was between 90 and 94%. The recovery at 215 nm was 100% of the calculated value.

stability studies, release testing and quality control. In the case of bFGF, the variation in response factor of four different HPLC-methods was <4%. Although the measured response factor at 277 nm was 90–94% of the theoretically calculated value, the measured response factor was highly reproducible, repeatable and rugged.

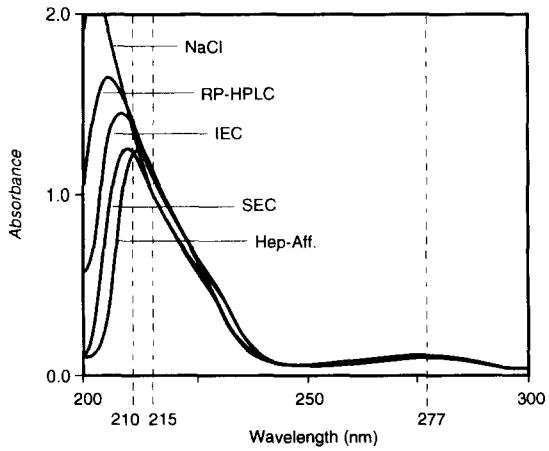

Fig. 3. UV spectra of bFGF in various buffer systems. The extinction coefficient at 215 nm was determined by UV spectroscopy for the same HPLC-buffer composition as occurs at the maximum of the main HPLC-peak (corrected for dwell volume).

The described procedure can give estimates of the quantity of a protein of known amino acid composition, but unknown extinction coefficient. The static extinction coefficient can be estimated.

Finally, and most importantly, protein content determinations by HPLC-response factors were at least equal to, if not more precise than, the values determined using a standard curve. It may be preferable to invest extra effort to determine the response factor accurately and use it for further determinations of concentrations. By doing so, all problems associated with the use of standard curves in quantitation can be avoided. This surprisingly high reproducibility of detector response makes the absolute

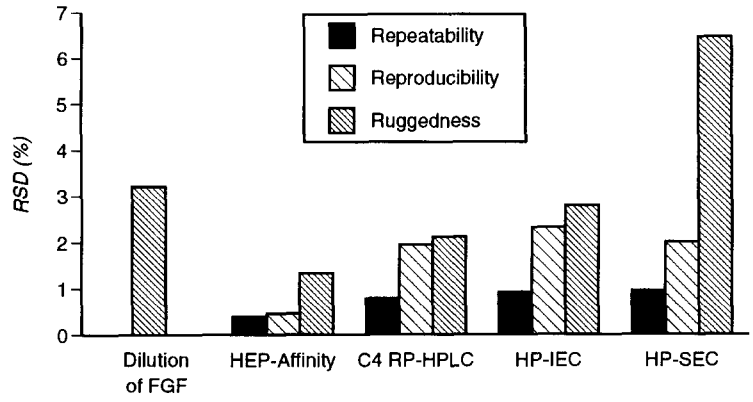

Fig. 4. Repeatability (short time standard deviation), reproducibility (long term (6 month) standard deviation), and ruggedness (different instruments, columns, times of analysis, operators and manufacturing batches) compared with the ruggedness of the dilution ($n = 7$). The precision is expressed as the relative standard deviation.

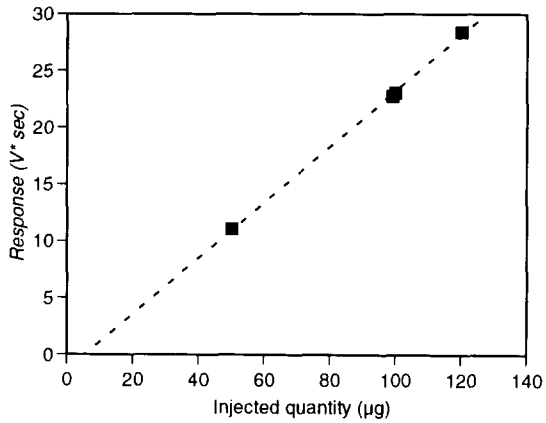

Fig. 5. Typical standard curve for ion exchange-HPLC used to obtain concentrations from the HPLC-response reading.

quantitation of proteins in mixtures possible (AAA and UV absorbance can provide absolute quantitation of only pure proteins or proteins without any other UV-absorbing components). This is a development in technology that would not have been attainable with the fixed wave length detectors, pump and mixing systems available 20 years ago.

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Table 2
Data obtained from a stability study after 0, 1, 3, and 6 months. Analysis was performed by RP and IEC-HPLC. A standard curve was prepared at each time point and was used for both analytical methods (left two columns). Time points and repetitive measurements were pooled. The data in the right-hand columns were obtained by use of the response factor from Table 1B

	Concentration of FGF			
	from standard curve		from response factor	
#	IEC	RP	IEC	RP
1	1.09	1.07	1.01	1.02
2	1.10	1.07	1.02	1.02
3	1.08	1.07	1.02	1.04
4	1.07	1.07	1.01	1.03
5	1.11	1.12	1.03	1.06
6	1.10	1.11	1.02	1.05
7	1.03	1.03	1.08	1.06
8	1.03	1.03	1.08	1.07
9	1.11	1.04	1.03	1.07
10	1.11	1.03	1.03	1.07
11	1.02		1.07	
n	11	10	11	10
Average	1.08	1.06	1.04	1.05
RSD	3.2	3.0	2.6	1.9
	IEC + RP		IEC + RP	
n	21		21	
Average	1.07		1.04	
RSD	3.3		2.3	

References

[1] Robert A. Copeland, Methods for Protein Analysis, Chapman & Hall, New York, 1994.
[2] P.W. Riddles, R.L. Blakeley and B. Zerner, Reassessment of Ellman's Reagent, in C.H.W. Hirs and S.N. Timasheff (Eds.), Methods of Enzymology, Vol. 91, Academic Press, New York, 1983, 49 pp.

[3] High Performance Liquid Chromatography, H. Knox (Ed.), Edinburgh University Press, 1986, 170 pp.
[4] D.T. Rossi, F. Pacholec and L.M. Dawkins, Integral method for evaluating component contribution to total solution absorbance from chromatographic data, Anal. Chem., 58 (1986) 1410–1414.
[5] S.A. Thompson, A.A. Protter, L. Bitting, J.C. Fiddes and J.A. Abraham, Cloning, Recombinant Expression, and Characterization of Basic Fibroblast Growth Factor, in Methods of Enzymology, Peptide Growth Factors, Pat C, Vol. 198, Academic Press, New York, 1991, pp. 96–116.
[6] J.K. Taylor, in Quality Assurance of Chemical Measurements, Lewis, Chelsea, 1987, 35 pp.
[7] H. Mach, C.R. Middaugh and R.V. Lewis Statistical determination of the average values of the extinction coefficients of tryptophan and tyrosine on native proteins, Anal. Biochem., 200 (1992) 74–80.