Journal of Chromatography B, 921–922 (2013) 75–80

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A universal, high recovery assay for protein quantitation through temperature programmed liquid chromatography (TPLC)

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ARTICLE INFO

Article history:
Received 4 October 2012
Accepted 22 January 2013
Available online 29 January 2013

Keywords:
Protein quantitation
UV absorbance
Intact protein
Reversed phase
Temperature programmed liquid chromatography

ABSTRACT

As an alternative to direct UV absorbance measurements, estimation of total protein concentration is typically conducted through colorimetric reagent assays. However, for protein-limited applications, the proportion of the sample sacrificed to the assay becomes increasingly significant. This work demonstrates a method for quantitation of protein samples with high recovery. Temperature programmed liquid chromatography (TPLC) with absorbance detection at 214 nm permits accurate estimation of total protein concentration from samples containing as little as 0.75 μg. The method incorporates a temperature gradient from 25 to 80 °C to facilitate elution of total protein into a single fraction. Analyte recovery, as measured from 1 and 10 μg protein extracts of *Escherichia coli*, is shown to exceed 93%. Extinction coefficients at 214 nm were calculated across the human proteome, providing a relative standard deviation of 21% (versus 42% at 280 nm), suggesting absorbance values at 214 nm provide a more consistent measure of protein concentration. These results translate to a universal protein detection strategy exhibiting a coefficient of variation below 10%. Together with the sensitivity and tolerance to contaminants, TPLC with UV detection is a favorable alternative to colorimetric assay for total protein quantitation, particularly in sample-limited applications.

1. Introduction

Accurate estimation of total protein concentration is integral to the proteome analysis workflow. Direct UV absorbance measurement at 280 nm (A_{280}) is the classic approach to quantify single proteins in a sample [1]. The protein's extinction coefficient at 280 nm is primarily dependent on the tyrosine and tryptophan content and can be determined computationally [2,3] or empirically [4]. Given the extinction coefficient, A_{280} measurements afford high accuracy and sensitivity for determination of protein concentration, while circumventing the need for standard calibration. However, when the protein sequence is unknown, or when estimating total protein in a mixture, A_{280} measurements will only approximate the protein concentration (absorbance of 1.0 = 1.0 mg/mL) [1]. The direct absorbance assay is also highly susceptible to interference by non-protein substances, making it unavailable for numerous proteome applications [5]. Consequently, a direct UV absorbance assay is not the favored tool for total protein quantitation.

Colorimetric reagent assays (e.g. bicinchoninic acid (BCA) [6], Bradford [7], or D_C (Lowry) [8]) provide an alternative to direct absorbance measurements. Such assays provide improved selectivity toward proteins which minimizes the concerns of interfering substances. Nonetheless, with a differing response toward various protein types, colorimetric assays require calibration with an appropriate standard. Of particular concern, however, is the use of a colorimetric assay in protein limited applications. Given the sensitivity of colorimetric assays (micrograms per milliliter), and depending on the volume requirements of the spectrometer, these assays may consume a significant portion of the available sample. Therefore, the development of a sensitive and selective quantitative assay with high protein recovery would provide a desirable alternative for sample limited proteome applications.

Coupling UV absorbance with reversed-phase liquid chromatography (RPLC) is a ubiquitous tool for quantitative analysis. Chromatographic separation affords improved detection selectivity as analytes are separated from interfering compounds. Although most interferences are removed, protein quantitation by LC–UV at A_{280} continues to impart high response variability between proteins. For this reason, LC–UV at 280 nm is most commonly used to quantify single proteins relative to a calibrated response curve of

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http://dx.doi.org/10.1016/j.jchromb.2013.01.021

the purified standard. Alternatively, the concentration can be determined relative to a calculated response factor based on the amino acid sequence [9], or can be referenced to an internal standard [10]. With emphasis on proteins lacking absorbance at 280 nm (i.e. without aromatic residues), Kuipers and Gruppen estimated the molar extinction coefficient of proteins and peptides at 214 nm based on the amino acid composition [11]. They demonstrate that protein absorbance measurements at 214 nm provide increased sensitivity, and in incorporating the peptide bond into the response, allow a more universal protein detection strategy. Following a similar strategy, LC–UV at 214 nm has been used to quantify the total peptide concentration in a complex mixture [12]. This method used a standard curve constructed from a set of four digested proteins to calibration the response of a complex peptide sample and conveniently provides a form of automated sample cleanup [12]. To date, the strategy has not been applied to quantify intact proteins from a proteome mixture.

One of the limiting factors of an LC–UV approach to quantify total protein in a mixture is the variable recovery of intact proteins from reversed phase separation. Choosing the correct solvent system and stationary phase are of utmost importance for improving quantitative results [13]. Along with optimization of column and solvent conditions, the use of elevated temperatures has been shown to improve separation efficiency and recovery of proteins, approaching 100% recovery [14–17]. The objective of this research is to incorporate a reversed phase approach involving temperature-programmed LC (TPLC) to recover intact proteins in high yield and allow protein quantitation. Eluting proteins are recovered over a narrow time window, and are observed as a sharp quantifiable peak. Through calibration with a single protein standard, TPLC demonstrates high recovery and accurate quantitation of complex protein mixtures in sample limited proteome applications (<1 μg).

2. Materials and methods

2.1. Reagents and protein extraction

All standard proteins as well as formic acid were obtained from Sigma (Oakville, Canada). Blocking grade skim milk power was purchased from Bio-Rad (Hercules, CA). Materials for casting and running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as well as Quick Start™ Bradford and DC™ protein assay reagents were also from Bio-Rad. The bicinchoninic acid (BCA) protein assay was obtained from Pierce (Rockford, IL). All solvents were of HPLC grade and, along with trifluoroacetic acid (TFA), were obtained from Fisher Scientific (Ottawa, Canada).

Escherichia coli was grown and harvested according to established protocols (Qiagen Manual for Good Microbiological Practices). *E. coli* proteins were extracted by suspending the cell pellet in 1% SDS, with heating to 95 °C for 5 min. The rat proximal tubule cell line NRK-52E was a gift from Dr. Dawn MacLellan (IWK Health Center, Halifax, Canada) and was grown according to manufacturer's instructions (American Type Culture Collection, Burlington, Canada). Protein was isolated from NRK-52E cells by suspending the cells in water and homogenizing for 30 s using a Pelletpestle™ (Fisher).

A known mass of blocking grade milk powder (Bio-Rad) was suspended in pure water (18 MΩ cm) at an estimated concentration of 10 mg protein/mL (assuming a protein content of 30% by weight). The sample was diluted to 0.1 mg/mL protein and subject to precipitation as described previously [18]. The resulting protein pellet was resolubilized to its original volume using 1% SDS (for BCA) or 70% formic acid (for LC–UV) in preparation for quantitative analysis. A 15%T SDS PAGE gel was loaded with approximately 10 μg protein per lane (based on 30% protein content), noting that equal volumes of the sample were compared before versus after precipitation. Proteins in the gel were visualized through silver staining and photographed with a digital camera.

2.2. Calibration curves

Standard curves for the protein assays were generated using bovine serum albumin (BSA) as per the manufacturer's recommendations. The colorimetric assays were recorded on an Agilent (8353) spectrometer (Mississauga, Canada). For LC–UV, BSA and lysozyme were used to generate standard curves through triplicate injection of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 20, 40, 60, and 80 μg of protein, as determined by weighing of the standard protein on an analytical balance. Column blank runs were included following injections exceeding 5 μg of protein to assess carry-over and insure accurate elution areas. For LC–UV testing, the proteome mixtures were injected at approximately 10 μg, as determined through the BCA assay.

2.3. Calculation of protein extinction coefficients

The extinction coefficients were calculated for all proteins in the human Uniprot database (downloaded August, 2012) containing 70,101 entries (http://www.uniprot.org/taxonomy/9606) following the methods of Pace et al. [2] at 280 nm and Kuipers and Gruppen [11] at 214 nm. Individual extinction coefficients were normalized to the mean and displayed on a $\log_2$ scale (see Section 3).

2.4. Coefficient of variance for protein assays

Stock solutions of the protein standards were prepared by mass at an approximate concentration of 1.0 mg/mL and the 'true' concentration was determined using the extinction coefficient of the pure proteins at 280 nm (or 410 nm for cytochrome c), calculated using the ExPASy web-based tool for calculating the theoretical extinction coefficient of proteins (ProtParam) based on methods by Pace et al. [2]. The concentration of the complex proteome extracts was determined through UV absorbance measurements as described previously [1]. For each assay tested, the protein stock was diluted to the center of the linear portion of the curve, taking five measurements of independent solutions, as well as reagent blanks. The coefficient of variation was determined from the average response factor of each protein standard, relative to the response of BSA.

2.5. SDS removal and proteome resolubilization

The SDS-containing *E. coli* samples were subject to detergent removal through protein precipitation in acetone (4:1, acetone:sample), with inclusion of a single acetone wash as described previously [19]. Prior to HPLC injection, the protein pellet was redissolved in a small volume (10–20 μL) of 70% formic acid and diluted with water such that the protein concentration was within the linear range of the calibration curve. All remaining samples were directly acidified with 0.1% TFA and diluted to an appropriate concentration prior to HPLC analysis.

2.6. Chromatographic instrumentation and data analysis

Chromatographic experiments were conducted on an Agilent 1200 HPLC system (Mississauga, Canada) constituting an autosampler equipped with a 100 μL sample injection loop, column thermostat, diode array detector recording at 214 nm or 280 nm and equipped with a 50 nL flow cell and fraction collector. Separations were on a 1 mm × 50 mm self-packed column containing

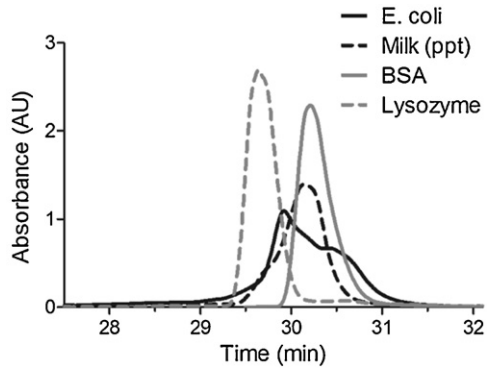


Fig. 1. The elution of individual proteins and of proteome mixtures from a reversed phase column (1 mm × 50 mm) by employing an 8 min solvent gradient (5–95% ACN) and following application of a temperature program from 25 °C to 80 °C (load–elute). The absorbance was recorded at 214 nm, with a single fraction collected over the time interval 26–41 min.

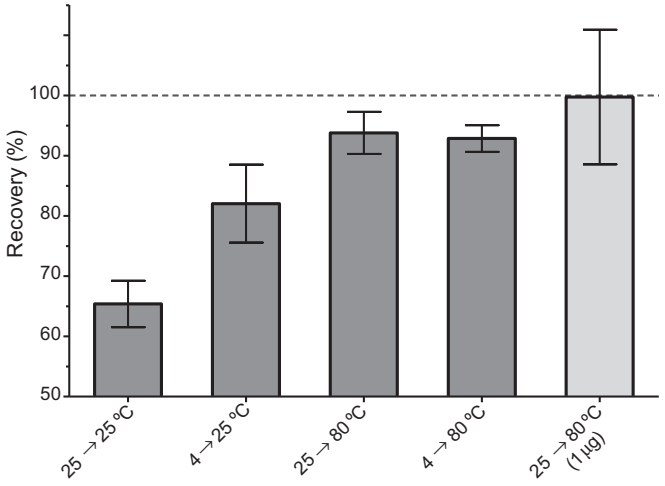


Fig. 2. Assessment of protein recovery following injection of 10 μg (gray bars) or 1 μg (white bar) *E. coli* total proteome extract. The temperature of the column is listed below the bars as (load temperature) → (elution temperature). Error bars represent the standard deviation from 5 replicate injections.

POROS® R2 beads (Applied Biosystems, Carlsbad, CA). The flow was set to 100 μL min^{-1} and fractions were collected in 1.5 mL vials. Peak areas were obtained by exporting the data from ChemStation software to Microsoft Excel for integration, obtained by summing all intensities measured in 0.4 s intervals over the fraction collection timeframe (see Section 3). Peak areas are reported following subtraction of the average blank peak area (triplicate injection) of a solvent blank collected immediately prior to the sample injection.

2.7. Solvent gradient conditions

The solvent gradient was varied through injection of a constant mass (~10 μg) of SDS-extracted *E. coli* protein. Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The final gradient includes an initial hold at 5% B for 16 min, followed by a linear increase to 95% B over 8 min.

2.8. Temperature programming liquid chromatography (TPLC)

The temperature of the column was adjusted using the column heating compartment of the Agilent HPLC system. Samples were loaded at 4 °C, 25 °C, or 80 °C. Eluting temperatures of 25 °C and 80 °C were explored. In each case, the temperature of the column was allowed to equilibrate to a constant value for approximately 2 min prior to applying the solvent gradient.

2.9. Sample recovery determination

Protein recoveries were obtained through injection of an equal volume of the corresponding protein sample through the complete HPLC system, but with omission of the column [14]. Fractions were collected over a 15 min time interval (from 26 to 41 min) to insure complete recovery of the eluting fraction. Samples were fully dried in a SpeedVac and re-suspended in water with 1% SDS and diluted appropriately for recovery determination by the BCA assay.

3. Results and discussion

Quantitation of total protein by reversed-phase liquid chromatography (RPLC) is herein accomplished by eluting all components over a narrow window. This facilitates integration of the sample peak, further enabling high protein recovery within a single collection vial. Fig. 1 displays the resulting chromatograms of ~10 μg injected protein using a rapid solvent gradient from 5 to 95% over 8 min. The combination of temperature and solvent gradients consistently elutes protein as a single peak over an approximate 2 min window. Shifting retention times of the eluting proteins do not affect the quantitative assay, as the chromatogram is integrated over a broader interval (15 min). An instantaneous solvent ramp from 5 to 95% B also yielded a single peak. However, higher background variability generated by the solvent front increased the limit of quantitation. Therefore the 8 min gradient was selected to assess total protein concentration.

3.1. Effect of temperature on protein recovery

Conducting reversed phase separations at constant elevated temperature has previously been shown to provide high protein recovery, approaching 100% [14,15]. However, as applied here, separations conducted at a constant 80 °C led to a gradual degradation of column performance. Specifically, protein recovery dropped from a high of 90 to 95% and ended below 60% after 10 replicate injections. Although the polymeric stationary phase (POROS R2 beads) employed in this study was selected for its heat stability and favorability for intact protein separation, the performance of the column could not be restored through simple washes. Given the reduced recovery, an alternative heating strategy was investigated.

Previous studies have shown that varying the column temperature can have a profound effect on analyte retention. In fact, a 4–5 °C change in temperature is shown to correspond to an approximate 1% change in solvent composition [20]. Fig. 2 shows that at a constant 25 °C, the solvent gradient alone yielded approximately 65% recovery. The resulting protein loss corresponds to components which retain on the column, as evident by the absence of protein in the non-retained fraction (injection peak). Application of a temperature change from 4 °C during loading to 25 °C for elution provides an increase in protein recovery to 82%, clearly demonstrating the influence of temperature on protein retention. Extending the temperature program from 4 to 80 °C (load–elute) further improved recovery to 93%. However, given the added time requirements to cool the column below room temperature (~100 min total run time), a temperature program from 25 to 80 °C was selected, and found to provide similar protein recovery (94%). It is further noted that no degradation of column performance was observed when employing the temperature program. The reversed phase column remained stable over the entire course of the experiments (>100 sample injections). Thus, the 25–80 °C temperature program is the preferred method for maintaining high protein recovery across a complex proteome mixture.

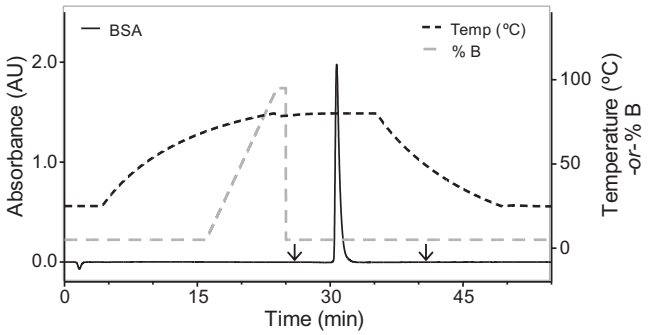


Fig. 3. The TPLC method employs a solvent gradient from 5 to 95% acetonitrile, over an 8 min period, following elevation of the column temperature to 80 °C. The protein fraction elutes as a sharp peak and is collected in a single vial (arrows indicate time over which fraction is collected).

The efficacy of the 25–80 °C method was tested at lower protein loading by injecting a 1 μg sample of *E. coli* total proteome extract. As shown in Fig. 2, recovery remains high (99%), illustrating the potential for temperature programmed liquid chromatography (TPLC) to permit quantitative analysis of proteins without sacrificing the sample. A sample chromatogram displaying the final TPLC method is provided in Fig. 3. Column temperature is allowed to equilibrate at maximum temperature for a minimum of 2 min prior to analyte elution. The collection window is also noted in Fig. 3 where, at a flow rate of 100 μL min^{-1}, a 15 min fraction can be collected in a single 1.5 mL vial.

3.2. Wavelength selection

Accurate assessment of total protein concentration by response curve generation assumes the sensitivity of the calibrant is similar to that of the unknown. A_{280} measurements are known to display high signal variability, given that the extinction coefficient depends primarily on the aromatic amino acid content of the protein [2,3]. At 214 nm, the sensitivity is reported as approximately 15–20 fold higher than at 280 nm [11]. Furthermore, in probing the peptide bond, all proteins will show appreciable absorbance at this wavelength. Fig. 4 summarizes the protein-to-protein variation of computationally derived molar extinction coefficients at 280 and 214 nm, as calculated across the entire human proteome. The average extinction at 214 nm (calculated at 673,043 M^{-1} cm^{-1}) was approximately 16.5 fold higher than at 280 nm (40,771 M^{-1} cm^{-1}). For ease of comparison, the individual extinction coefficients were therefore normalized with respect to their average values at each wavelength.

As seen in Fig. 4A, on a molar basis, significant variation in signal response exists for individual proteins across the human proteome at both wavelengths. This is to be expected, as low molecular weight

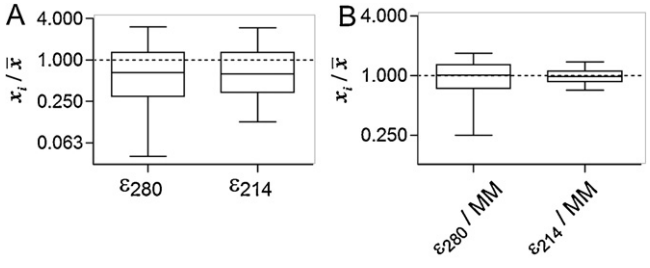


Fig. 4. Box and whisker plots display the variation in calculated protein extinction coefficient across the human proteome at 214 and 280 nm. (A) Plots the 1st and 3rd quartiles (box) together with median (solid line in box) and the 5th and 95th percentile (bars) for the molar extinction coefficient (cm^{-1} M^{-1}), following normalization to the average at each wavelength. (B) Shows an equivalent plot of the extinction coefficient accounting for the molecular mass of the protein (cm^{-1} g^{-1} L).

proteins will generally exhibit a lower molar extinction coefficient. The coefficient of variance was similar at these wavelengths, equating to 133% at 214 nm versus 126% at 280 nm. The box and whisker plots demonstrate the skew from a normal distribution, as the median (line at center of box) is significantly lower than 1 (the normalized average). Translating these values for measuring protein concentration, it can be concluded that absorbance measurements at 214 and at 280 nm would provide significant differences in sensitivity on a per mole basis (e.g. mol/L). However, total protein quantitation is more typically assessed on a mass basis (e.g. g/L). Thus, comparison of extinction coefficients requires consideration of the protein molecular mass.

Fig. 4B demonstrates the spread between extinction coefficients normalized according to the molecular mass (MM) of the protein. The units of these extinction coefficients are cm^{-1} g^{-1} L, meaning that the absorbance readings provide a measure of the protein concentration in g/L. Following normalization, the spread in the extinction coefficient is significantly reduced at both wavelengths. However, the distribution at 214 nm shows lower inter-protein variability, as measured by a coefficient of variation of 21% (versus 42% at 280 nm). At 214 nm, peptide bonds were calculated to account for approximately 51% of the total protein absorbance across the proteome. Thus, accounting for the molecular mass, absorbance measures at this wavelength show tight agreement across the proteome. As seen in Fig. 4B, while both distributions (214 and 280 nm) are approximately normal, the spread at is significantly reduced at 214 nm. These calculations justify the use of 214 nm to probe total protein concentration with minimal variation in signal response across individual proteins. Thus, a representative protein standard can be selected to calibrate the response of all proteins in a LC–UV quantitation assay.

3.3. Protein response curves

Fig. 5 illustrates the response curves of BSA and lysozyme over the range 0.1–80 μg at 214 nm (Fig. 5A) and 280 nm (Fig. 5B). At 280 nm, the sensitivity of lysozyme is significantly greater than that of BSA, being a consequence of the variable extinction coefficients at this wavelength. However, as seen in Fig. 5B, at 214 nm, these two proteins show nearly overlapping signal response across the range 0.1–80 μg. Thus, if BSA is used as a calibrating standard, estimation of the lysozyme concentration would be more accurate at 214 nm than at 280 nm.

The inset in Fig. 5A displays the non-linear signal response of BSA and lysozyme at 214 nm beyond 10 μg injections. This is a consequence of the higher sensitivity at 214 nm which leads to absorbance signal saturation in the sharply eluting peak (peak maximum exceeds an absorbance of 2). The linear range at 214 nm extends to 5 μg, but can be fit to a second order polynomial up to 10 μg injected. Subsequent blank run peak areas showed undetectable protein carry-over with injections over the linear range, extending up to 20 μg injected. Based on the linear regression, a lower limit of quantification (LOQ) of 0.74 μg and 0.71 μg are calculated for BSA and lysozyme, respectively. It is further noted that the response curves at 280 nm are linear up to 80 μg, suggesting that protein recovery remains consistent up to this higher loading amount. Thus, the capacity of the column is not exceeded up to 80 μg. However, higher mass (40–80 μg) injections display carry-over nearing 2.5–5% of total mass injected (by peak area), and therefore require a blank run prior to subsequent sample analysis (data not shown).

3.4. Assay comparison

The accuracy of a protein assay will be influenced by the variation in response between the unknown and the calibrant. The

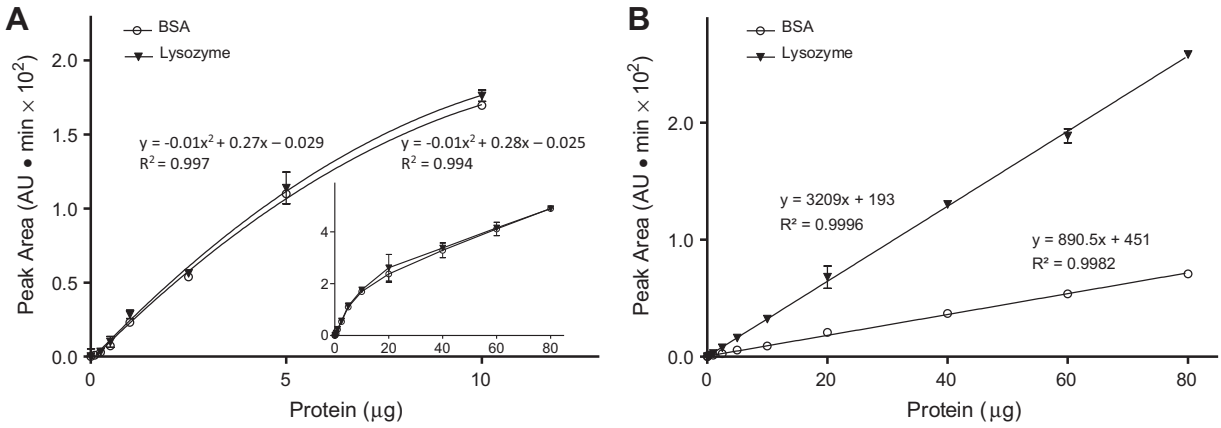


Fig. 5. TPLC response curves of BSA and of lysozyme at (A) 214 nm and (B) 280 nm. The inset of (A) plots the response of BSA and lysozyme over a broader range of 0.1–80 µg. The error bars represent the standard deviation in peak area from triplicate injections (some being too small to observe on the scale). Formula for the regression curves are listed, together with the R^2 values.

Table 1
Coefficient of variation determined from a common set of proteins for various quantitative assays.

Sample	LC–UV (214 nm)	Bradford	DC	BCA
BSA	1.00 ± 0.04	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.03
Lysozyme	1.00 ± 0.10	0.83 ± 0.01	1.36 ± 0.03	1.18 ± 0.02
Cytochrome C	1.13 ± 0.02	1.25 ± 0.02	1.27 ± 0.02	1.08 ± 0.04
Ovalbumin	1.04 ± 0.03	0.77 ± 0.06	1.20 ± 0.04	0.98 ± 0.04
α-Casein	1.19 ± 0.01	0.64 ± 0.02	1.34 ± 0.48	0.86 ± 0.10
Dephosphorylated α-casein	1.03 ± 0.02	0.68 ± 0.01	0.74 ± 0.02	0.58 ± 0.03
E. coli	1.01 ± 0.04	0.83 ± 0.01	1.46 ± 0.04	1.20 ± 0.07
NRK-52E	0.86 ± n/a[a]	0.73 ± 0.04	1.13 ± 0.08	0.99 ± 0.10
Coefficient of variation	9.6	23.6	19.3	20.1

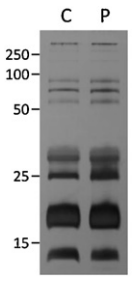
[a] Single injection.

choice of calibrant is therefore an important variable when calculating concentration based on response curves. Here BSA was chosen as the calibrant and used to assess the variation in response for a standard set of five proteins and two proteome mixtures. Direct comparison between the TPLC–UV assay and three commonly employed colorimetric assays (BCA, Bradford, and DC) is provided in Table 1.

The observed coefficient of variation of the conventional colorimetric assays ranged from 19% (BCA) to 23% (Bradford), which is in agreement with the advertised values as reported by the manufacturer. By comparison, the TPLC method displays a CV of 9.6%, with the most variation found in the protein α-casein. This protein is highly phosphorylated, contributing to an increase in detector response relative to a non-phosphorylated standard. As shown in Table 1, dephosphorylated α-casein shows greater agreement to the response of BSA by TPLC, suggesting highly phosphorylated proteins provide higher response TPLC. Application of TPLC for total protein quantitation reveals close agreement to the 'true' protein concentration (determined by $A_{260/280}$), with an error below 14%. These response values are in closer agreement with the response of BSA (as calibrant), compared to the reagent assays. It is further noted that the *E. coli* proteome extract was obtained in solution containing 1% SDS. However, detergent removal and subsequent protein resolubilization in 70% formic acid enables accurate assessment of protein concentration of a sample originally prepared in SDS. When employing a single calibration standard TPLC is shown to be a reliable assay for total protein quantitation.

3.5. Application of TPLC for quantitative analysis

The TPLC method was applied to quantify a sample of milk. The total protein content of this sample is reported to be approximately 30% (Bio-Rad). However, as shown in Fig. 6 the protein content as measured by BCA was 55%. The presence of lactose in the milk sample overestimates the protein concentration through the BCA assay. Protein precipitation removes this interference, and allows a more accurate estimation of protein concentration (25%). The gel image in Fig. 6 shows the high recovery of the precipitation step, suggesting the difference in protein concentration before and after precipitation is a primary consequence of the interference being removed, as opposed to limited protein recovery. TPLC removes lactose and other non-protein contaminants, including buffer additives such as salts, reducing agents and urea prior to quantitation, allowing a more accurate assessment of protein concentration. The protein content in the milk sample as determined by TPLC (before and after precipitation) was similar to the BCA assay following removal of the interference. The TPLC method is compatible with many buffer additives and interfering compounds, however



	Protein content (% w/w)	
Assay	Control (C)	Precipitated (P)
BCA	55.0 ± 0.79	25.1 ±1.92
LC-UV	24.4 ± 1.73	21.6 ± 0.51

Fig. 6. Assessment of protein content in a sample of milk before or after protein precipitation through chloroform/methanol/water. The SDS PAGE image demonstrates the high protein recovery between the unprecipitated control (C) and the pellet (P). The true protein content in the milk sample, as estimated by Kjeldahl nitrogen content, is reported at approximately 30%. Error bars represent the standard deviation of triplicate injections.

detergents must be removed prior to quantitative analysis, as they retain on reversed-phase columns and interfere with quantitation. Here, acetone precipitation is used to remove SDS, and concentrated formic acid is used for RPLC-friendly protein resolubilization and yields good quantitative results.

4. Concluding remarks

A method for total protein quantitation with high analyte recovery has been developed using a single standard protein to calibrate the LC/UV response at 214 nm. Incorporation of temperature programming increases recovery to greater than 90%. This method was validated over a period of several months, with little to no change in sensitivity. This implies that construction of the calibration curve need only be conducted once over the lifetime of the UV lamp. The method is applicable to quantify low quantities of protein, and as a non-destructive technique, is particularly valuable in sample limited applications.

Acknowledgements

The authors thank the National Sciences and Engineering Research Council of Canada for financial support. The authors also thank Courtney Stairs (Department of Biochemistry and Molecular Biology, Dalhousie University) who assisted with the bioinformatics analysis of protein extinction coefficients from the human proteome.

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