Two dimensional liquid chromatography–ultraviolet/mass spectrometric (2DLC–UV/MS) analyses for quantitation of intact proteins in complex biological matrices

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ABSTRACT

A conventional scale online two dimensional liquid chromatography-ultraviolet/mass spectrometric (2DLC–UV/MS) method was developed for simultaneous quantitation of intact proteins. A series of valve switches were utilized between the two LC dimensions and the mass spectrometer to resolve and confirm the proteins of interest from a complex biological matrix. Two model proteins, myoglobin and serum albumin were simultaneously resolved and quantitated from *Escherichia coli* lysate using a strong anion-exchange chromatography and reversed-phase chromatography as the first and second dimension respectively. The method validation consisted of evaluating linearity, precision, and accuracy. A linear relationship ($R^2 > 0.99$) between the concentrations of the two proteins and peak areas was observed over the concentration range; 12.0–120.4 μg/mL and 8.5–85.4 μg/mL for serum albumin and myoglobin, respectively. The average RSD of peak areas for intra-day and inter-day analyses were 5.9% and 9.4% for myoglobin and 6.2% and 10.1% for serum albumin respectively. Over the linear range, the recoveries ranged from −15.4 to 9.0% for serum albumin and −2.5 to 9.4% for myoglobin. The system presented in this work is amenable to a quality control environment for evaluation and quantitation of expression levels of multiple target proteins. To our knowledge, this represents the first 2DLC–UV/MS method depicting the viability of simultaneous quantitation of more than one intact protein from complex biological mixtures in a single run.

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1. Introduction

The quantitation of intact proteins in complex biological matrixes is important in biopharmaceuticals, agriculture, food industry, and biomarker research. Quantitative analysis of proteins is commonly addressed using reversed phase chromatography coupled with mass spectrometric based approaches [1–3]. The majority of these approaches utilize bottom-up strategies, where the protein of interest is first digested and indirectly quantified by using selected internal peptides. These approaches have had some success due to amenability of robust and sensitive quantitation of peptides by mass spectrometric (MS and MS/MS) detection with pre-fractionation using an initial chromatographic step. Although sensitive and robust, these approaches (i) ignore the contribution of selected peptides from isoforms and other proteins with substantial homology and (ii) include potentially problematic digestion steps

[4], which increases the sample complexity (50–100 times), is time intensive and needs to be quantitative and complete [5].

In contrast, if the intact protein/s of interest can be quantitated directly, one can circumvent the above mentioned limitations. Intact proteins of interest have been chromatographically separated, and quantitated previously either by LC–UV [2,6–8] or LC–MS [9,10] approaches. The primary challenge with quantitating at the chromatographic step is the need to resolve the protein of interest from other co-eluting components. In most biological samples, there is substantial sample complexity that makes it difficult to resolve and accurately quantify proteins of interest using only a single dimension. In the last two decades, multidimensional chromatographic approaches have gained popularity for resolving complex protein mixtures [11–13]. The enhanced resolving power delivered by multidimensional approaches over single chromatographic phases has to some extent helped mitigate the complexity of these samples [14]. However, in majority of the multidimensional approaches, the proteins are proteolyzed to shorter peptides either before the first dimension [15] or after the first dimension [16]. In parallel, although to a significantly lesser extent, multi-

dimensional approaches have also been applied to characterize intact proteins [17–19]. The primary objective of these studies was towards identification of large sets of proteins.

To the best of our knowledge, there has been no comprehensive study to evaluate the feasibility of quantitation of intact proteins using online multidimensional chromatographic approaches. In this study, a two dimensional liquid chromatography-ultraviolet/mass spectrometric (2DLC–UV/MS) method was developed and validated for simultaneously quantitating two proteins of interest, myoglobin and serum albumin from *E. coli* lysate, a model complex protein mixture. The proteins of interest were resolved using two orthogonal dimensions of separation while the mass spectrometer provided intact molecular mass confirmation and a handle on selectivity of the method. The intent of this study was to understand the feasibility of the method using robust analytical scale chromatography. A validation study was carried out to evaluate the linearity, accuracy, and precision of the developed method in targeting the proteins of interest in a complex lysate.

2. Experimental

2.1. Materials

Tris–HCl, myoglobin (equine), serum albumin (bovine), phenylmethanesulfonyl fluoride (PMSF); pepstatin A, dithiothreitol (DTT) and E-64 protease inhibitor were purchased from Sigma–Aldrich (St. Louis, MO). Sodium chloride, disodium ethylene diaminetetraacetic acid (EDTA), glycerol, LC/MS grade trifluoroacetic acid (TFA), were purchased from Thermo Fisher Scientific (Pittsburgh, PA). HPLC-grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ). Polypropylene auto sampler vial inserts were purchased from Waters (Milford, MA). Nutrient broth liquid media was purchased from Invitrogen (Carlsbad, CA). For all analyses, Milli-Q (Millipore, Billerica, MA) deionized water was used.

2.2. Sample preparation

E. coli B strain (ATCC 11303) was grown for 16 h in nutrient broth liquid media (containing 3 g of beef extract and 5 g of peptone per liter) at 37 °C. For preparation of *E. coli* total protein lysate, an overnight culture (1L) was harvested by centrifugation at 4 °C for 30 min at 5000 × g. The pellet was re-suspended in lysis buffer (50 mM Tris/HCl, 50 mM EDTA, 1 mM DTT, 5%glycerol, 1 mM PMSF). This was followed by lysis using sonication in the lysis buffer with 20 short bursts of 10 s on ice. To the cell suspension, 15 μL MgCl₂ and 3 μL of DNAase (1 mg/mL) was added and incubated at 4 °C for 30 min. The cell debris was removed by ultracentrifugation at 4 °C for 30 min at 100,000 × g. An aliquot of cocktail of protease inhibitor PMSF, pepstatin A, E-64, EDTA was added to the resulting lysate (12 mL) to prevent protein degradation. The final concentration of proteins in *E. coli* culture as estimated by Bradford's assay was calculated to be 7 mg/mL. The *E. coli* lysate was stored at −80 °C prior to analysis.

2.3. 2DLC–MS conditions

Components from two Agilent 1100 systems (Santa Clara, CA) were combined to create the two-dimensional liquid chromatography system. The system consisted of one autosampler, two degassers, two binary pumps, a thermostated column compartment with integral column switching valve, two external 10-port valves, a multi-wavelength detector and a variable wavelength detector. All the modules were controlled by ChemStation software. Strong anion exchange chromatography was used in the

Table 1
Gradient elution conditions for first and second dimension for separation of myoglobin and serum albumin from *E. coli* lysate.

First dimension gradient			Second dimension gradient		
Time	%B1	Flow (mL/min)	Time	%B2	Flow (mL/min)
2.99	0	1	5	28	0.5
3	17	1	15	55	0.5
8	17	1	17	60	0.5
8.1	21	1	18	32	0.5
13	21	1	20	32	0.5
23	100	1	25	32	0.5
25	100	1	35	40	0.5
25.1	0	1	37	60	0.5
36	0	0	38	28	0.5
36.1	0	0	46	28	0.5
43	0	1			0.5
43.1	0	1			0.5

first dimension. A ProPac strong anion exchange analytical column (4 mm × 250 mm) was purchased from Dionex (Sunnyvale, CA). Reversed phase chromatography was used in the second dimension. A Symmetry C4 column (4.6 mm × 50 mm, 3.5 μm) and two guard columns (3.9 mm × 20 mm, 5 μm) were purchased from Waters (Milford, MA). Two external 10-port valves were purchased from Valco (Houston, TX) for coupling the two dimensions and mass spectrometer. A disposable pre-column filter purchased from Phenomenex (Torrance, CA) preceded the columns in the two dimensions. The separation was performed in the first dimension by mobile phase A1 (10 mM Tris Buffer; pH 8.0) and mobile phase B1 (1 M NaCl; 10 mM Tris Buffer; pH 8.0). The mobile phases in the second dimension were; A2:0.1 v/v%TFA in water and B2:0.085 v/v% TFA in acetonitrile. The details for the gradients in the two dimensions are presented in Table 1. An injection volume of 20 μL was used for all analyses. The column temperature for the first dimension was maintained at 30 °C. The temperature for first reversed phase gradient was 50 °C and second reversed phase gradient was 45 °C. All mass spectra were acquired on Waters Q-ToF Micro mass spectrometer (Milford, MA). Prior to MS inlet, a solution of 7 v/v% glycerol/68 v/v% water/25 v/v% acetonitrile was teed-in to the second dimension eluent from the multi-wavelength detector. Instrumental parameters for mass spectral acquisition were as follows: capillary was set at 2850 V, sample cone at 30 V, extraction cone at 1.5 V, desolvation temperature at 450 °C, source temperature at 100 °C, low and high mass resolution at 5, desolvation gas at 600 L/h, cone gas at 50 L/h, MCP detector at 2350 V, scan time 0.9 s and interscan delay at 0.1 s.

2.4. Assay validation

The proteins of interest, myoglobin and serum albumin were isolated from strong anion exchange chromatography in an online setup followed by their subsequent separation, detection, and quantitation using reversed phase chromatography–UV/mass spectrometry. Absolute concentration of myoglobin and serum albumin was determined by quantitative amino acid analysis. The intra-day and inter-day precision of the assay was evaluated by analyzing duplicates at three different concentrations for one and three days respectively. Assay intra-day and inter-day precision (percent coefficient of variation, % CV) were calculated. In addition, analyte carryover was evaluated by analyzing a solvent blank immediately following the highest standard sample. The percent absolute carryover was calculated as a percent relative to the limit of quantitation levels for the two proteins. A single point calibration was performed for recovery [(expected area counts (based on single point calibration)/observed area counts) × 100] using reference concentrations for myoglobin and serum albumin concentrations at 48.2 μg/mL
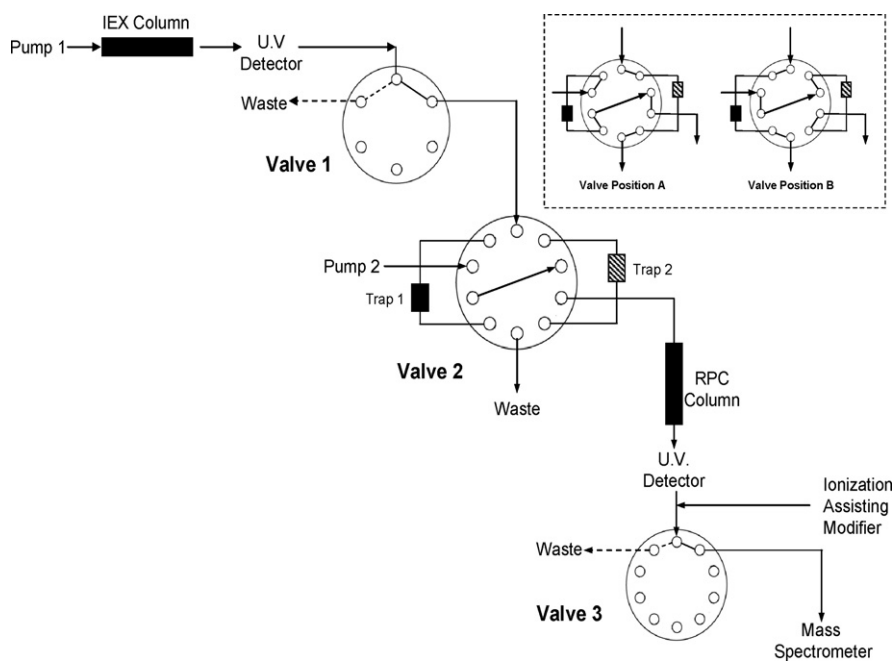
Fig. 1. Schematic of the online two dimension liquid chromatographic-ultraviolet/mass spectrometric (2DLC–UV/MS) configuration for quantitation of intact proteins. Valve positions A and B correspond to marked positions (—) and (- - -) with-in Valves 1 and 3. The inset describes the valve positions A and B and corresponding flow path in Valve 2.

and 34.2 μg/mL, respectively. Linearity of the method was calculated for both myoglobin and serum albumin by analyzing duplicate injections with the two proteins spiked at eight different concentration levels in *E. coli* lysate. A linearity curve was obtained by plotting the respective peak areas for myoglobin and serum albumin with their respective concentrations. A linear regression was used to obtain a linear equation. For the linearity curve calculations, the equation was not forced through the origin. Because of the sample complexity, the 2DLC–UV was coupled with ESI-MS analysis to confirm the eluting proteins of interest. The mass spectrometric characterization of intact proteins was found to be critical during method development for detecting any co-elution from other components in the complex matrix. The assay selectivity was measured by analyzing the unspiked *E. coli* lysate.

3. Results and discussion

3.1. The approach

The 2DLC–UV/MS configuration consisted of three different valves and was adapted from Cohen et al. [17] with several modifications. Valve 1 was a two position 6 port valve, Valves 2 and 3 were two position 10-port valves. Valve 2 consisted of two alternating reversed phase traps while Valve 3 was used to divert salt and glycerol solution to waste when the mass spectrometer was not acquiring the eluent from the second dimension. Fig. 1 and Table 2 depict the schematic for the overall 2DLC–UV/MS configuration and the different switching events used to control the coupling of the three valves and the mass spectrometer. As the protein of interest was detected by the UV detector linked to the first dimension column, Valve 1 was switched to direct the eluent to the first C4 trap of Valve 2. Once the protein of interest was completely eluted from the first dimension, Valve 1 was diverted to waste. It was critical to allow the entire peak to get transferred onto the C4 trap before switching Valve 1. Once the first dimension cut was completely transferred, Valve 2 was switched in order to elute the trapped protein from Trap 1. At this stage, Trap 2 was ready to retain the next protein of interest from the first dimension. Valve 2 was coupled to

an analytical column on which the trapped proteins were further resolved. When the next protein of interest was observed in the first dimension, the same series of events was then repeated. Based on the run time of the second dimension, it is possible to quantitate several proteins in a single 2D-LC analysis. The protein quantitation was carried out using UV detection (214 nm). The glycerol solution was introduced to the eluent from the second dimension before the third valve and was diverted to the mass spectrometer only when the trapped components were eluting. The third valve was found to be critical to prevent salt adduction of proteins with non-volatile salts during ESI-MS analysis. The valve was switched to mass spectrometer after the elution of the salt peak in the two reversed phase analysis.

3.2. Method development

E. coli is known to have more than 4000 estimated translated proteins [20]. Owing to the sample preparation conditions utilized, and snapshot of the *E. coli* proteome at any given time-point, there could be on an average 1000 proteins in the lysate. The *E. coli* lysate was used as a model to evaluate the feasibility of quantitating proteins from a complex matrix sample. For intact proteins,

Table 2
Timing program for controlling the three switching valves in the 2DLC–UV/MS system. Valve positions A and B correspond to marked positions (—) and (- - -) with-in Valves 1 and 3 and for the respective flow path in Valve 2 as shown in Fig. 1.

Time (min)	Valve 1	Valve 2	Valve 3
0	A	A	A
1.62	B	A	A
1.9	A	A	A
2	A	B	A
8	A	B	B
10.6	B	B	B
11.8	A	B	B
20	A	A	B
21	A	A	A
26	A	A	B
44	A	A	A
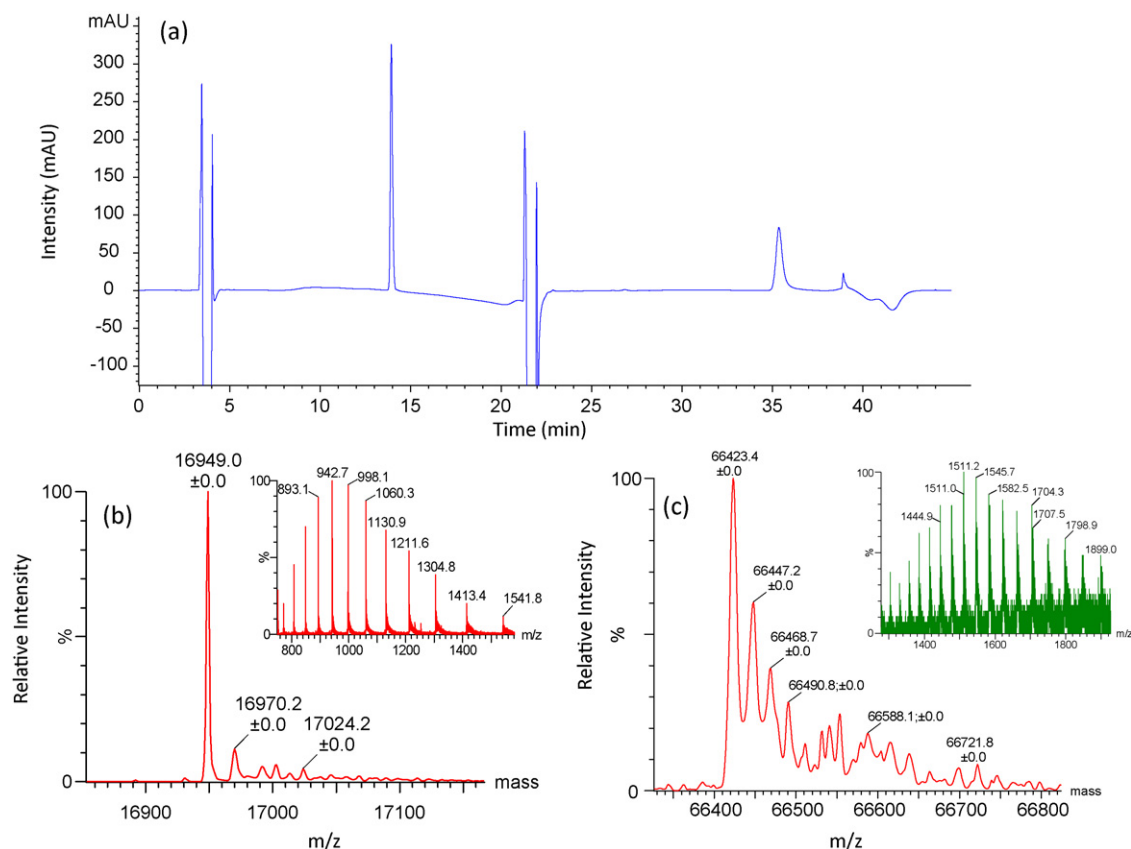
Fig. 2. Characterization of reference proteins standards; (a) representative UV (214 nm) second dimension reversed phase chromatogram for myoglobin and serum albumin, (b) deconvoluted mass spectrum of myoglobin (*inset*: multiple charge envelope mass spectrum) and (c) deconvoluted mass spectrum of serum albumin (*inset*: multiple charge envelope mass spectrum).

ion-exchange chromatography (IEX) together with reversed phase liquid chromatography (RPLC), decoupled using trapping columns has been reported [21–23]. Since more than 70% of cellular proteins have a pI of 7 or below; a strong anion exchange chromatography was used as the first orthogonal dimension of separation [17]. Reversed phase chromatography was used as the second dimension due to its robustness and ease of coupling with mass spectrometric detection. The use of traps in decoupling the two dimensions has multiple advantages as it allows (i) to focus the components eluting from the first dimension and (ii) to run at higher operating pressures in the second dimension thereby circumventing the practical operating pressure limit of ion exchange columns (∼200 bar). [17]

In order to scope the separation conditions for myoglobin and serum albumin, a mixture of the two proteins in the absence of *E. coli* lysate was analyzed. The concentrations of myoglobin and serum albumin utilized were 85.4 μg/mL and 120.4 μg/mL respectively. During the first dimension analysis, myoglobin elutes as a well defined peak before the start of the salt gradient while serum albumin elutes in a step gradient at 21% acetonitrile. Since myoglobin is a relatively hydrophobic protein, as high as 28% acetonitrile was used to trap myoglobin in the first trap column. Similarly, serum albumin was trapped in the second trap with high organic concentration at 25% acetonitrile. Fig. 2a depicts the respective first and second UV chromatograms from the reversed phase analysis of standard solution of serum albumin and myoglobin. For the entire second dimension analysis, the first reversed phase analysis was carried out at 50 °C while the second analysis was carried out 45 °C. It was observed that with C4 Symmetry® column allowed for

a better peak shape at 45 °C for serum albumin than at 50 °C (data not shown). The column re-equilibrated in 1.6 min before serum albumin was introduced onto the analytical column via the 10-port valve. To improve the mass spectrometer response in the presence of TFA, a pre-MS modifier of a solution of glycerol in a water/acetonitrile mixture was utilized as reported previously [8].

Based on the mass spectrum depicted in Fig. 2b, the observed intact molecular mass for myoglobin was found to be *m/z* 16,949.0. This corresponds to a mass difference of 0.011% of the theoretical mass of myoglobin (16,951.4 amu) [24]. Similarly, as depicted in Fig. 2c, the intact average mass for serum albumin was observed to be 66,423.2 amu. This corresponds to 0.010% of the theoretical value for average mass of serum albumin (66,430 amu). The insets in Fig. 2b and c show the respective multiply charged spectra for the two proteins.

Fig. 3a depicts the strong anion exchange chromatography of *E. coli* lysate with spiked proteins of interest. As shown, myoglobin elutes early in the first dimension analysis in a short 0.28 min window cut while serum albumin elutes in a step gradient along with other components from *E. coli* lysate in a 0.8 min cut. Eluting serum albumin in a linear gradient resulted in substantial complexity in second dimension and prevented resolution of serum albumin from other lysate components. In contrast, when a step gradient was utilized, serum albumin was successfully resolved from other components during the second dimension. Fig. 3b depicts the UV chromatograms for separation of the two proteins in the second dimension. The average chromatographic resolution for myoglobin and serum albumin in the second dimension was observed to be 0.7 and 2.0, respectively.
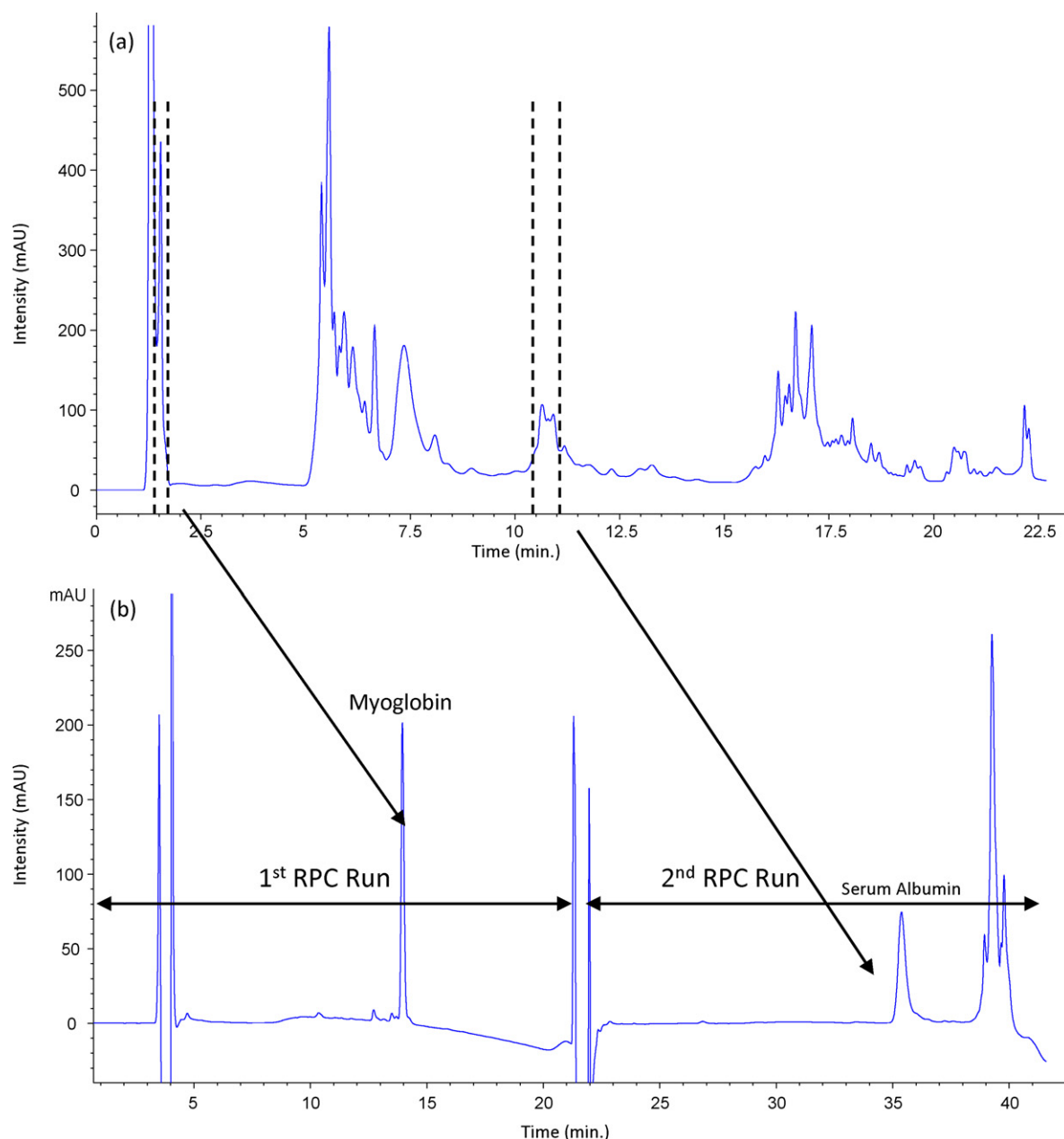
Fig. 3. (a) Representative strong anion exchange chromatogram (220 nm) of *E. coli* lysate spiked with myoglobin and serum albumin. (b) Reversed phase chromatogram depicting the separation of myoglobin and serum albumin from other lysate components.

Table 3
Intra- and inter-day precision analysis for myoglobin and serum albumin in *E. coli* lysate.

	Myoglobin (μg/mL)				Serum albumin (μg/mL)			
Conc.	17.1	42.7	85.4	All	24.1	60.2	120.4	All
Inter-day precision (% CV)	12.1	7.5	8.5	9.4	14.6	8.3	7.4	10.1
n	3	3	3	9	3	3	3	9
Intra-day precision (% CV)	11.5	1.2	5	5.9	12.7	3.2	2.7	6.2
n	3	3	3	9	3	3	3	9

3.3. Assay validation
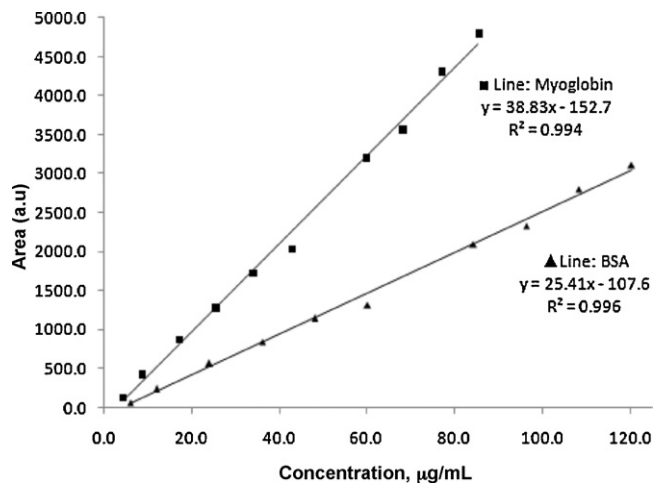
The assay for quantitation of the two proteins of interest in *E. coli* lysate was observed to be linear over the range of 12.0–120.4 μg/mL for serum albumin and 8.5–85.4 μg/mL for myoglobin as depicted in Fig. 4 with an R^2 value >0.99. As a function of total protein in the lysate, this corresponded to 1.7–17.2 μg/mg for serum albumin and 1.2–12.2 μg/mg for myoglobin respectively in this method. The results for inter- and intra-day precision are reported in Table 3. The intra-day precision was evaluated for three sample concentrations with duplicate injections for each of the samples. The overall intra-day precision (% CV) was found to be 5.9% for myoglobin and 6.2% for serum albumin. The inter-day precision was studied on three sets of samples for three consecutive days. The inter-day precision

Table 4
Multipoint recovery analysis for myoglobin and serum albumin spiked into *E. coli* lysate.

Serum albumin				Myoglobin			
Spiked/total protein (μg/mg)	Expected (μg/mL)	Observed (μg/mL)	Recovery (% RE)	Spiked/total protein (μg/mg)	Expected (μg/mL)	Observed (μg/mL)	Recovery (% RE)
1.7	12	10.1	−15.4	1.2	8.5	8.3	−2.5
3.4	24.1	23.8	−1.1	2.4	17.1	17.4	1.8
5.2	36.1	35.6	−1.5	3.7	25.6	25.1	−1.8
6.9	48.2	48.2	0.1	4.9	34.2	34.2	0.1
8.6	60.2	55.6	−7.4	6.1	42.7	40.5	−4.5
12.0	84.3	88.3	4.6	8.5	59.8	63.2	4.9
13.8	96.3	98.4	2.1	9.8	68.3	70.4	2.6
15.5	108.3	118.4	9	11.0	76.9	85.1	9.1
17.2	120.4	131.3	8.8	12.2	85.4	94.8	9.4

Fig. 4. Linearity curves for myoglobin (■) over the range 8.5–85.4 μg/mL and serum albumin (▲) over the range 12.5–125.0 μg/mL, respectively.

was found to be 8.5% for myoglobin and 10.1% for serum albumin. The assay recovery was between −15.4% and 9.0% for myoglobin and −2.5% to 9.4% for serum albumin. The limit of quantitation for myoglobin and serum albumin were found to be 8.3 μg/mL and 12.0 μg/mL, respectively. It was observed that there was a rapid decrease in the accuracy below the limits of quantitation for each of the proteins. The selectivity was measured by analyzing unspiked lysate and checking for any co-elution with the proteins of interest. Fig. 5a and b depicts the separation of myoglobin and serum albumin at the limit of quantitation, respectively. The bottom trace depicts the UV chromatograms of unspiked *E. coli* lysate depicting absence of any co-elution at the retention time of the two proteins of interest. Due to the sample complexity, the use of mass spectrometry greatly assisted method development to detect the presence of any co-eluting proteins. The background signal intensity in the blank run at the retention times for myoglobin and serum albumin was found to be 7.3% and 8.4%, respectively, relative to the limits of quantitation for the respective proteins. The carry-over in the blank run was evaluated after analysis of the highest standard sample. There was no detectable carry-over in the case of myoglobin and 10.1% relative to limit of quantitation for serum albumin. The analyte stability was measured for the two proteins of interest at 4 °C at 1 h and 14 h. After 1 h, the variation was found to be 4.9% for myoglobin and 6.2% for serum albumin. Both the changes are
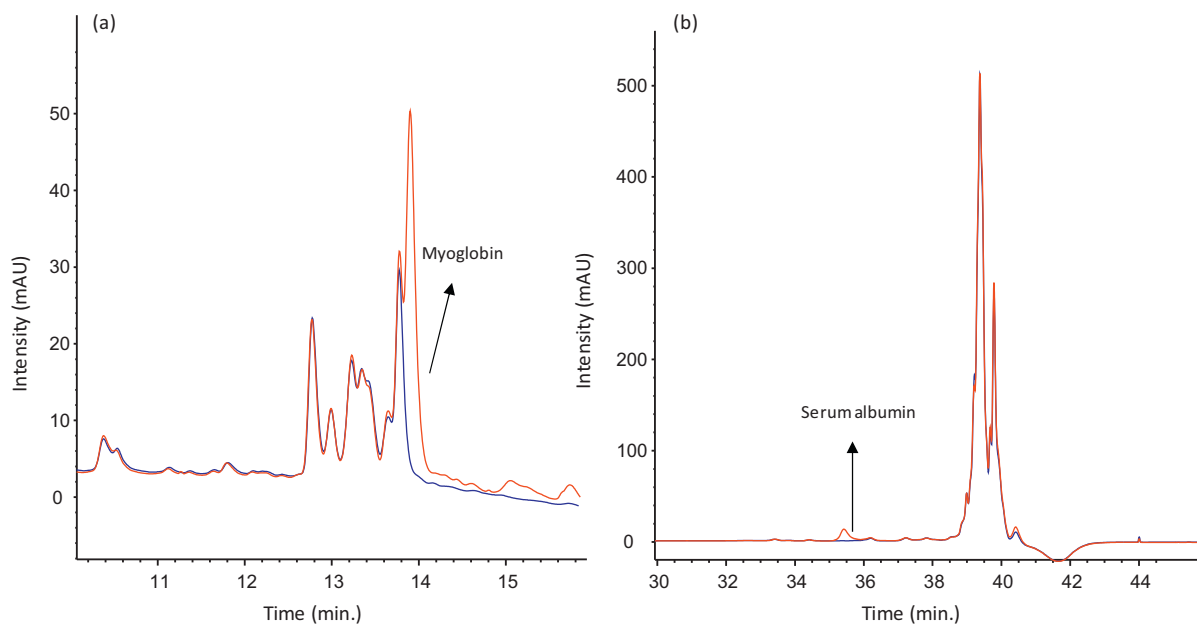
Fig. 5. a) Reversed phase chromatograms for (- - -) lysate spiked with myoglobin and (—) unspiked lysate at limit of quantitation for myoglobin; and b) reversed phase chromatograms for (- - -) lysate spiked with serum albumin and (—) unspiked lysate at limit of quantitation for serum albumin.

within the intra-day precision for the two proteins. The variation for serum albumin and myoglobin after 14 h at 4 °C analysis was found to be 9.4% and 14.2%, respectively. Based on the 2DLC–UV/MS data, it was concluded that the assay was able to differentiate and quantitate the proteins of interest from other putative lysate components (Table 4).

Use of co-extracted proteins expressed from house-keeping proteins [7] could be used as markers for relative protein quantitation. This would further improve the % CV of the method. The speed of the overall analysis could be improved by utilizing UPLC as a second dimension [25]. The absolute UV detector response at conventional flow rate was the limiting factor in sensitivity observed using this method. The limit of quantitation could be further improved by increasing the overall injection volume. Since the method is based on analytical scale flow rates, it is intuitive that the sensitivity constraints associated with lower abundant proteins will not be met. Adding an immuno-precipitation step in conjunction with utilizing micro/nano-scale chromatography in the second dimension will improve the limit of detection to possibly quantitate lower abundant proteins of interest.

4. Conclusions

The study demonstrates the capability of conventional scale 2DLC–UV/MS analysis to simultaneously quantify more than one target protein in a complex biological matrix. The linearity, precision, and recovery are comparable to those of traditional and conventional scale single dimension analysis. When proteins of interest are present at levels addressable by conventional scale LC analysis, quantitation at intact protein level is recommended over other approaches. This approach could be used for isoform or variant analysis, precludes problematic digestion steps, and is relatively straightforward.

Acknowledgements

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