Journal of Chromatography B, 908 (2012) 59–66

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Chromatographic behaviour of peptides following dimethylation with H_2/D_2-formaldehyde: Implications for comparative proteomics

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ARTICLE INFO

Article history:
Received 20 April 2012
Accepted 24 September 2012
Available online 29 September 2012

Keywords:
Dimethyl labelling
Isotopic effects
Deuterium isotope effect
Comparative proteomics
Relative quantitation
Formaldehyde

ABSTRACT

The differential separation of deuterated and non-deuterated forms of isotopically substituted compounds in chromatography is a well-known but not well-understood phenomenon. This separation is relevant in comparative proteomics, where stable isotopes are used for differential labelling and the effect of isotope resolution on quantitation has been used to disqualify some deuterium labelling methods in favour of heavier isotopes. In this work, a detailed evaluation of the extent of isotopic separation and its impact on quantitation was performed for peptides labelled through dimethylation with H_2/D_2 formaldehyde. The chromatographic behaviour of 71 labelled peptide pairs from quadruplicate tryptic digests of bovine serum albumin were analysed, focusing on differences in median retention times, resolution, and relative quantitation for each peptide. For 94% of peptides, the retention time difference (heavy-light) was less than 12 s with a median value 3.4 s. With the exception of a single anomalous pair, isotope resolution was below 0.6 with a median value 0.11. Quantitative assessment indicates that the bias in ratio calculation introduced by retention time shifts is only about 3%, substantially smaller than the variation in ratio measurements themselves. Computational studies on the dipole moments of deuterated labels indicate that these results are consistent with literature suggestions that retention time shifts are inversely related to the polarity of the label. This study suggests that the incorporation of deuterium isotopes through peptide dimethylation at amine residues is a viable route to proteome quantitation.

1. Introduction

In mass spectrometry (MS), the use of stable isotopes is a preferred approach to quantitative proteomics. Various protein labelling strategies incorporate isotopes through metabolic processes [1,2], enzymatic digestion [3–5], or reaction with chemical tags containing ^{2}H, ^{13}C, ^{15}N, or ^{18}O [6–9]. In an LC–MS experiment, the chromatographic profiles of the isotope analogues should ideally exhibit perfect overlap so that the relative MS ion abundance of the eluting peptide pair indicates the concentration ratio of peptides. Unfortunately, the chromatographic separation of isotopically labelled peptide pairs is commonly observed, especially for deuterated compounds. In this case, the heavy isotope generally elutes in advance of its lighter counterpart [10]. This phenomenon has influenced current practices of quantitative proteomics.

Numerous studies have investigated the extent of isotopic separation in proteomics. As seen with the original isotope coded affinity tags (ICAT), Gygi et al. acknowledged a 1–2 s separation between D_0 and D_8-labelled ICAT peptides [6], which could have implications for quantitation. In contrast, it has been observed that labels incorporating isotopes other than deuterium generally lead to a much smaller separation of isotopic pairs. This is not surprising given that there is a doubling of mass for D vs H, while the change for ^{13}C over ^{12}C, for example, is only about 8% [10]. A general consequence of this observation is that labels employing isotopes other than deuterium, though more costly, have become the preferred choice in proteomics studies [11]. To illustrate, while the original SILAC (stable isotope labelling by amino acids in cell culture) reagent employed D_3-leucine [1], chromatographic separation of the resulting peptides (on the order of half a peak width) prompted development of a $^{13}C_6$-arginine SILAC reagent [2]. Likewise, an updated cleavable ICAT reagent eliminated deuterium in favour of ^{13}C [7]. The iTRAQ (isobaric tags for relative and absolute quantitation) reagent also avoids deuterium in favour of ^{13}C, ^{15}N, and ^{18}O [8]. With all such commercial reagents, the isotopic pairs have been shown to co-elute during RPLC, ensuring correlation of the MS intensity of respective isotopic pairs and providing more reliable quantitation based on point measurements.

To address quantitative issues arising from isotopic separation of deuterated peptide pairs in the original ICAT method, Gygi et al.

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http://dx.doi.org/10.1016/j.jchromb.2012.09.035

integrated the peptide peak areas over their respective elution profiles [6]. Given that peak integration can potentially overcome the issue of chromatographic separation of isotopes, numerous researchers have weighed the benefits of deuterated labelling reagents over more costly ^{13}C isotopically labelled compounds. Although peak integration is a potential solution to the problem of isotopic separation, it increases the complexity of automated data analysis. Moreover, comparison of peak areas for chromatographic signals separated in time implies that the sensitivity of the MS detector remains constant. Given the stochastic nature of the electrospray process, such an assumption may not be valid. Thus, a preferred solution to balance cost and quantitative reliability would be to identify deuterated labels that minimize isotopic separation. Such labels have been identified in the literature [12], though they have not come into widespread use, perhaps owing to a lack of commercial availability, or a perception that all deuterated labels lead to isotopic separation.

Considering alternatives to commercial isotopic reagents, one of the simplest and increasingly popular methods is the use of D_0 or D_2 formaldehyde [13–15], which dimethylates primary amines (lysine residues and N-termini of peptides). Through peptide dimethylation, the resulting 4 Da mass difference per label is preferred to the 2 Da difference obtained with ^{13}C formaldehyde. Expanding on previous observations for deuterated analogues, one would assume a chromatographic time shift following reaction with D_0/D_2 formaldehyde. Perhaps surprisingly, in an early study proposing this reagent for quantitative proteomics, Hsu et al. noted a negligible chromatographic time shift [12]. It should be noted however that such an observation was made from a single BSA peptide, and may not be representative of a complex series of labelled peptides.

While deuterium isotopic effects are more pronounced than those of ^{13}C, the incorporation of deuterium alone does not dictate chromatographic separation. Zhang et al. [16] observed that the chromatographic time shift increased as a function of the number of deuterium atoms in the labelled peptide (for structurally similar labels). This gave rise to a concept of "specific resolution", referring essentially to the time shift afforded per deuterium isotope. These authors also noted that the relative time shift was larger for smaller peptides with the same label. Boersema et al. [17] speculated that the partial separation of dimethyl-labelled peptides was due to the higher hydrophilicity of the C—D bond over that of the C—H bond. Such an explanation would imply a differential separation dependent on the relative contribution of the C—D bond to the retention of a given peptide. Furthermore, the location of deuterium in a given isotopic compound would also be important in influencing the relative retention of isotopic pairs on a reversed phase chromatographic support. This argument was presented by Zhang et al. [16], who noted that the incorporation of a deuterium in a more polar (charged) region of the molecule led to smaller observed differences in the chromatographic retention of H/D labelled peptides.

In this work, a comprehensive study was carried out to investigate the effect of dimethyl labelling with H_2/D_2 formaldehyde on the retention characteristics of differentially labelled peptides. The objectives of the work reported here were to (1) determine whether there is a significant difference in retention (measured in terms of time as well as peak resolution) across a complex mixture of peptides, and (2) assess the consequences of any observed separation on proteome quantitation. Consistent with previous observations using deuterium, a statistically significant separation of dimethylated peptides (D ahead of H) was observed. However, this separation is inconsequential in terms of peptide quantitation based on point measurements (i.e. without peak integration). These results are explained with consideration of the relative high polarity of the dimethylated peptides during chromatographic separation.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA), bovine trypsin (catalogue T8802), trifluoroacetic acid (TFA), triethylammonium bicarbonate (TEAB), Tris, formaldehyde, D_2-formaldehyde, sodium cyanoborohydride and formic acid were obtained from Sigma (Oakville, Canada). Dithiothreitol (DTT) and iodoacetamide (IAA) were from Bio-Rad (Hercules, CA). Milli-Q grade water was purified to 18.2 MΩ cm. Solvents were of HPLC grade and obtained from Fisher Scientific (Ottawa, Canada).

2.2. Tryptic digestion

BSA, prepared in 250 mM Tris–HCl (pH 8) was reduced through addition of DTT (in 250 mM Tris) to a final concentration of 5 mM, with incubation at 55 °C for 20 min. Then, IAA (250 mM Tris) was added to a final concentration of 12.5 mM, with incubation at room temperature in the dark for 20 min. The final concentration of reduced BSA was 0.5 g/L. The sample was divided into 5× 100 μL aliquots (50 μg protein) to which 390 μL water was added, along with10 μL trypsin (0.1 g/L in water) per vial. Digestion proceeded overnight (16 h) at 37 °C. The digests were terminated through addition of 50 μL of 10% TFA per vial.

2.3. Isotopic labelling

Digested BSA peptides were subject to sample cleanup via reversed phase HPLC as described by Wall et al. [18]. The strategy employs a C_{18} column with a 0.1% TFA, water/acetonitrile gradient to separate non-protein components (Tris, DTT, IAA) while capturing peptides as a single fraction. The peptide fractions (50 μg per vial) were evaporated to dryness in a SpeedVac ahead of isotopic labelling.

Peptide dimethylation with D_0 (CH_2O) and D_2 (CD_2O) formaldehyde was performed as previously described [12]. Briefly, dried peptide samples (50 μg) were reconstituted in 100 μL of 100 mM TEAB (pH 8.5) to which 3.6 μL of 20% D_0 or D_2 formaldehyde was added. Three of the replicate BSA digests (fractions 1, 3, and 5) were labelled with D_2 formaldehyde ('heavy') while the remaining two fractions (2 and 4) were labelled with D_0 formaldehyde ('light'). The sample was incubated at room temperature for 5 minutes, followed by addition of 4.2 μL of 6 M sodium cyanoborohydride, with incubation for 2 hours at room temperature. Each sample was then subjected to RP-HPLC sample cleanup as previously described [18]. Cleaned fractions were dried, then frozen (−20 °C) until LC–MS/MS analysis.

2.4. LC–MS/MS

Prior to LC–MS/MS, the heavy and light labelled peptide fractions were reconstituted (0.1% TFA, water with 5% acetonitrile), and combined in a 1:1 ratio. Four replicates were prepared, combining the heavy/light labelled fractions (1 + 2, 2 + 3, 3 + 4, and 4 + 5). A combined total of 1 pmol of digested and labelled BSA was injected per analysis. A ThermoFisher LTQ linear ion trap mass spectrometer (Waltham, MA) equipped with a nanospray ionization source coupled to an Agilent 1200 nanoflow HPLC system (Palo Alto, CA) was used to analyse the protein digests. Separation was on a self-packed C_{12} reversed phase column (30 cm × 75 μm i.d., 3 μm Jupiter beads from Phenomenex, Torrance, CA) flowing at 0.25 μL min^{-1}. The gradient consisted of a linear increase from 5% to 30% acetonitrile with 0.1% formic acid, over 100 min, followed by an instantaneous increase to 80% acetonitrile to regenerate the column. The nanospray ionization voltage was set at 2.5 kV and

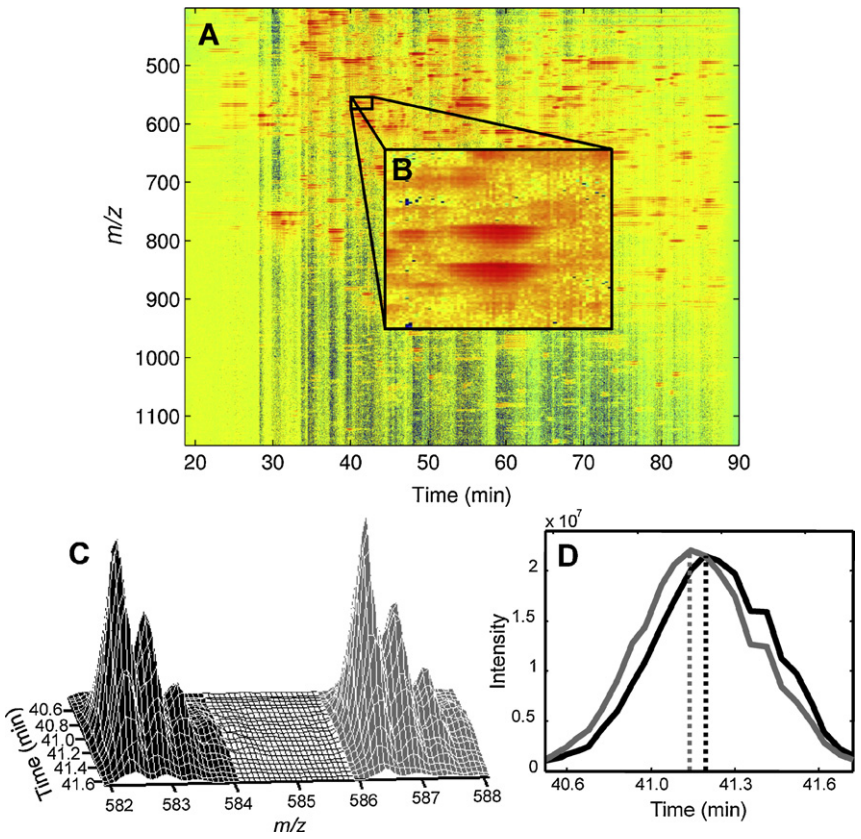

Fig. 1. (A) Two-dimensional representation of a mass chromatogram for one replicate of the BSA digest, with inset (B) showing an example of a peptide pair. (C) shows a three dimensional surface plot of the peptide pair shown in (B), while (D) shows the calculated XIC's for the heavy and light labelled peptides. The dashed lines in (D) indicate the calculated median retention times for each peptide.

the transfer capillary temperature was set to 225 °C. The MS scan range was 400–1700 *m/z*. The ion trap had the maximum fill time set at 100 ms, and the automatic gain control was set to allow up to 1×10^5 ions to enter the trap for MS and 1×10^4 ions for MS/MS. Data acquisition used dynamic exclusion, collecting one MS scan followed by tandem MS scans of the top five ions, with an exclusion duration time of 30 s.

2.5. SEQUEST parameters

Peptide identification was performed using the SEQUEST algorithm within the Thermo Xcalibur Bioworks (v. 3.3) software package. Peptide filters were set to achieve a false positive rate of 1% or less when the reversed BSA database was included in the search. X_{corr} versus charge state was set to 1.50 (+1), 2.00 (+2) and 3.25 (+3) for searches. Peptide probability was set to 0.01 with $\Delta CN \geq 0.1$ and $R_{Sp} \leq 4$. Two searches were completed: the first employed fully tryptic peptides with up to 2 missed cleavages; the second employed fully non-tryptic peptides (i.e. non-specific cleavage at any amino acid), again permitting up to 2 missed cleavages (at K or R). The data set was searched for both fully tryptic as well as non-tryptic peptides against BSA, sequence-reversed BSA, trypsin and a collection of 10 commonly observed protein contaminants.

2.6. Data selection and preprocessing

A multiconcensus SEQUEST search of the database resulted in a total of 155 unique BSA peptides identified as either the heavy or the light tag in one or more of the four replicate runs. The raw data files were obtained from the mass spectrometer and converted into mzXML files using ReAdW software from the Seattle Proteome Center (SPC) [19]. The files were then imported into MatLab® (R2009b, MathWorks, Natick, MA) to carry out the data analysis.

For each of the 155 identified peptides, extracted ion chromatograms (XICs) of the respective isotopic profiles for heavy and light pairs were obtained for all of the replicates in which the peptide was identified. For example, given a mass chromatogram (Fig. 1A) for one replicate, the XICs (Fig. 1D) for peptide pairs are created by integrating the light (black shading) and heavy (gray shading) component regions as indicated in Fig. 1C. The regions correspond to the mass and retention time ranges required to construct the XIC for the particular peptide pair. These regions are different for each peptide pair combination (charge and number of labels). For peptides that were not initially identified in all four replicates, a manual search was carried out, resulting in additional positive hits when the presence of a peptide overlooked by SEQUEST could be verified. The number of peptides used for this study was subsequently reduced to 71 by applying the following criteria: (1) both peptide peaks (heavy and light) needed to be confirmed in all four replicates, and (2) the isotopic patterns needed to be clearly distinguishable from baseline noise and free from obvious mass interferences. From the initial set of 155 peptides pairs, a total of 66 identified peptides were rejected by the first criterion and 18 by the second criterion. The resulting 71 reliable unique BSA peptides were used in this work.

2.7. Signal processing

After preprocessing, statistical parameters including retention time difference, chromatographic resolution and quantitative ratio were calculated using the median retention time and peak widths at half height for each XIC for every replicate of each peptide pair. The median of the elution profiles, representing the time encompassing

half of the peak area, was chosen as the most objective representation of retention time, especially when peak maxima were not clearly defined. A summary table that includes the sequence, masses of the light and heavy molecular ions, charge, number of labels, time difference and resolution for all 71 unique peptide pairs is available in the supplementary material accompanying this paper.

2.8. Computational calculations of compound polarity

All calculations were performed with Spartan (Wavefunction Inc., Irvine, CA)'08 computational software. The DFT functional B3LYP with 6-31G* basis set was used for the dipole moment calculations for the labelled species presented in Fig. 7.

3. Results

A total of 71 unique labelled BSA peptide pairs were identified in all four replicate samples of labelled BSA using the criteria previously noted. In cases of multiple charge states, only the most abundant was included in this list. The 71 peptides included 54 doubly charged ions (14 with one label, 37 with two labels and 3 with three labels), 15 triply charged ions (2 with one label, 9 with two labels and 4 with three labels) and 2 quadruply charged ions (two labels). Out of the 71 peptide pairs, 38 were fully tryptic peptides (28 fully cleaved and 10 with one missed cleavage), 31 were semi-tryptic peptides (29 fully cleaved and 2 with one missed cleavage) and 2 were non-tryptic peptides. The peptides account for a BSA sequence coverage of 67% with masses ranging from 813 to 2303 Da with a median mass of 1375 Da. Retention times varied between 20.9 and 83.5 min with a median value of 49.1 min.

3.1. Retention time differences

Fig. 2 summarizes the time differences calculated from each of the 71 heavy/light peptide pairs, plotted as a function of the apparent mass of the light peptide in the pair. The time difference is calculated as the average retention time difference (light, t_r^L, minus heavy, t_r^H) of each peptide pair across four replicate runs. Here, the retention time is defined as the median time calculated for each XIC, as shown in Fig. 1D. Because the sampling interval was not uniform on the time axis, the median time was determined by trapezoidal integration of the XIC, followed by interpolation to determine the time corresponding to 50% of the total area. The limits for the median calculation were 10% of the maximum peak intensity. In Figs. 2, 4 and 5C and D, the error bars represent one standard deviation of the mean ($\pm s/\sqrt{4}$).

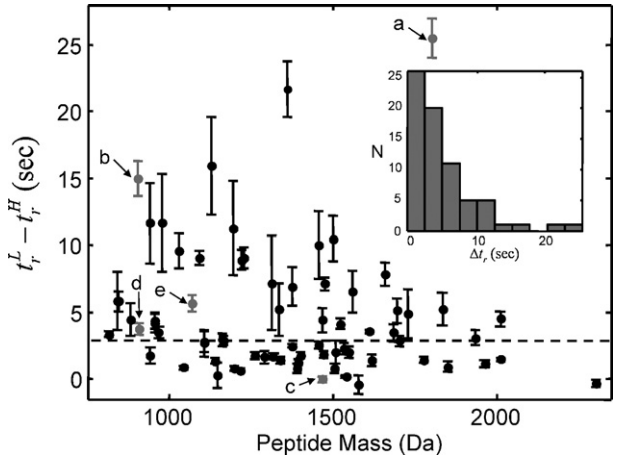

Fig. 2. Calculated mean retention time differences as a function of apparent peptide mass for the 71 unique BSA peptide pairs. A histogram of the time differences is shown in the inset. Error bars indicate one standard deviation of the mean. Representative peptide pairs, shown in Figs. 3 and 4, are labelled 'a' through 'e'. The dashed line corresponds to the median retention time difference (3.36 s) for all peptide pairs.

Fig. 2 shows that the retention time differences of the various peptides are highly variable, ranging from −0.40 to 25.45 s. The majority of the retention time differences (69 out of 71) are in the positive region and correspond to elution of the heavy-labelled peptide ahead of the light. This positive bias is expected based on previous studies [16,20,21]. No meaningful correlation was observed ($r^2 = 0.154$) between retention time difference and the apparent peptide mass. Similarly, the time difference also did not correlate with the retention time of the peptide pairs, their observed mass, charge state, number of labels (related to the number of missed cleavages) or cleavage type (fully or non-specific).

As seen in the inset histogram of Fig. 2, the retention time differences have a tailed distribution with most of the differences between 0 and 8 s and a median value of 3.36 s. Four peptides had an average time difference greater than 15 s. The selected XICs for two of these peptide pairs (indicated by 'a' and 'b' in Fig. 2) are provided in the subplots of Fig. 3 (A and B). As seen from these XIC profiles, these peptides display a significant degree of chromatographic separation, with the heavy component (gray) eluting ahead of the light component (black). The other two peptide pairs with a time difference greater than 15 s were characterized by features similar to the ones shown in Fig. 3A and B. Also shown in Fig. 3 are the selected XICs from a peptide pair with a time difference near zero (Fig. 3C, indicated by 'c' in Fig. 2) and a pair with a time difference near the median value (Fig. 3D, indicated by 'd' in Fig. 2).

3.2. Resolution

The absolute time differences between heavy/light labelled peptide pairs highlight the retention differences experienced by hydrogen vs deuterium labelled peptides. Chromatographic resolution provides a quantitative measure of the degree of separation resulting from this time difference, and is presented in Fig. 4. Again, peak resolution is plotted as a function of the apparent peptide mass of the light peptide in the pair. The resolution for one peptide pair is defined as the average retention time difference ($t_r^L - t_r^H$) divided by the average peak width at half maximum for the light and heavy components. Thus resolution can be negative if the light component elutes before the heavy component.

A peak width was calculated from the maximum of the XIC for both components to the points of earliest intersection at half height as shown in Fig. 3. Despite potential variations in the peak widths of the eluting peptides, the resolution correlates strongly with the time difference ($r^2 = 0.98$). The resolution between peaks ranges from −0.022 to 1.39. The peptides pairs labelled 'a' through 'e' in Fig. 2 are similarly labelled in Fig. 4. The peptide pair with the largest time difference also has the largest resolution. With exception to the peptide pair labelled 'a' in the figure, the remaining peptides have resolution below 0.6, with a median value of 0.114. The inset in Fig. 4 shows the distribution of resolution values (three small negative values are excluded).

3.3. Quantitative ratios

For isotope labelled peptides that do not co-elute, it has been recommended to integrate each peptide signal over their respective elution profiles. The effects of chromatographic separation on the quantitation of peptides following formaldehyde dimethylation are investigated here. Based on experimental design, the theoretical ratio of the heavy to light tagged peptides would be unity. Two approaches were used to calculate the relative ratio of heavy/light peptides, as illustrated in Figs. 5A and B. The first approach (shown

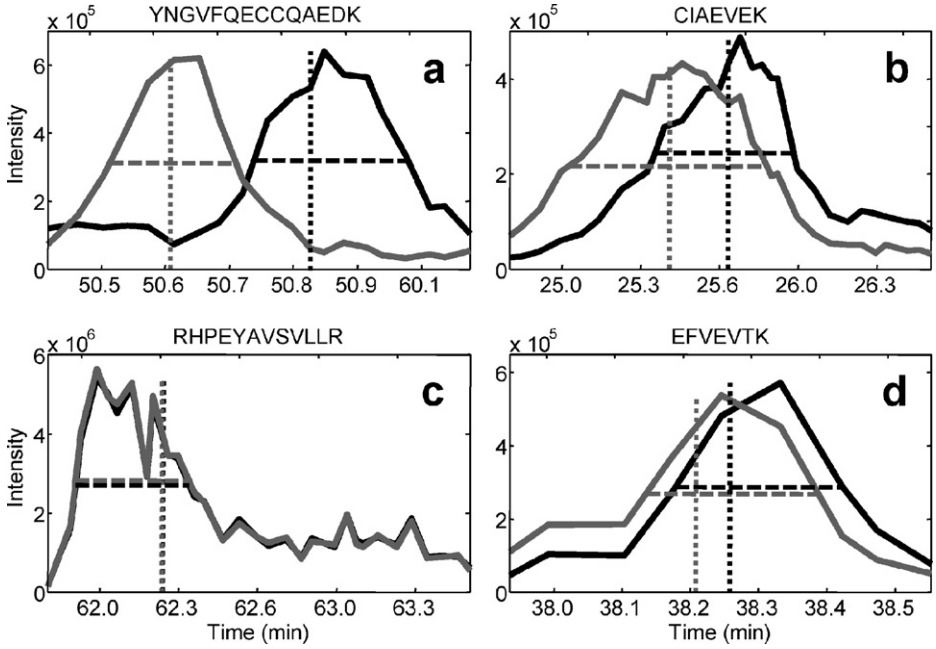

Fig. 3. Selected XIC replicates for the peptide pairs, as indicated 'a' through 'd' in Fig. 2. The vertical lines correspond to the median retention times and the horizontal lines represent the peak widths at half height for the light (black) and heavy (gray) components. The confirmed amino acid sequence of each peptide pair is provided in the figure.

in Fig. 5A) calculates the intensity ratio (R_1) of the two peaks by summing the intensities of the five time channels of the XICs centered on the time channel corresponding to the maximum observed signal for the heavy component. The second approach (Fig. 5B) calculates the intensity ratio (R_2) in the same manner, but centers the intensity measurements at the respective peak maxima for the heavy and light components (multiple time channels were included to improve signal averaging, but the window, ca. 15 s, was sufficiently small to approximate point measurements). Given the observed separation of isotopes, one would assume that the second ratio calculation would lead to a more accurate determination of the relative peptide quantity. Each ratio corresponds to the area over the indicated region associated with the heavy component (gray shading) divided by the area associated with the light component (horizontal lines). The averages for the four replicate ratios for each peptide pair are shown in Fig. 5C (R_1) and D (R_2) as a function of apparent peptide mass. The median values for R_1 and R_2 were 0.95 and 0.92 (the extreme was excluded in this calculation). To examine the change in the ratios, a plot of the difference between R_1 and R_2 for every peptide pair as function of resolution is also shown in Fig. 5E. The ratio for the extreme case is not shown in the main plot for Fig. 5C and E but is indicated by the asterisk in the inset histograms. In Fig. 5E, the error bars represent the 95% confidence interval and the dashed line corresponds to the zero ratio difference.

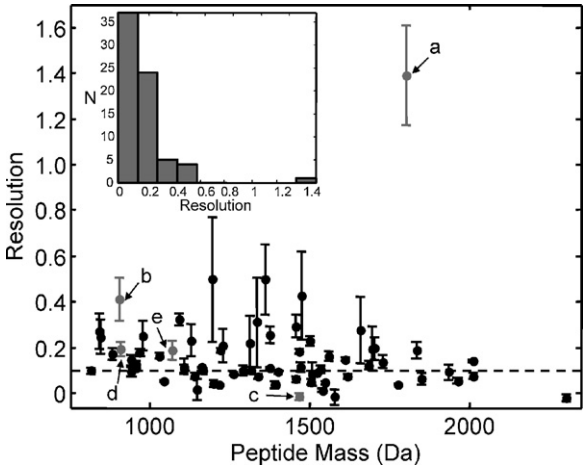

Fig. 4. Calculated mean resolution as a function of apparent mass for the 71 unique BSA peptide pairs. A histogram of the resolutions is provided in the inset. Error bars indicate one standard deviation of the mean. The dashed line corresponds to the median resolution (0.114). The points labelled 'a' through 'e' refer to the same peptide pairs indicated in previous figures.

4. Discussion

As previously stated, Hsu et al. [12], using similar although not identical separation conditions to those employed here, indicated that the inaccuracy in quantification due to an isotopic effect was "negligible" when labelling BSA with the dimethyl method. Although consistent with the results reported here, this conclusion was supported by direct evidence from only one particular BSA peptide, QTALVELLK, which may not be representative of the entire sample. This particular peptide was also observed in the current work (labelled 'e' in Figs. 2 and 4); the XICs for all replicates of this peptide are shown in Fig. 6. The figure shows very similar elution patterns for the differentially labelled peptides across all replicates, consistent with the observations of Hsu et al. for this peptide pair. However, the ratio of signal intensities (heavy to light) is consistently higher on the leading edge of the profile, resulting in a relatively small but reproducible difference in the median retention time. While a small retention time difference is observed for this particular peptide, the magnitude of the isotopic effect varies substantially depending on the peptide pair, as shown in Figs. 2 and 4. Thus, one cannot generalize co-elution of deuterated peptide pairs from this one peptide.

In the context of studies of other isotopic labels, Zhang and Regnier [20] reported that when BSA was labelled with D_0/D_8 ICAT reagents, 4 of 19 peptides (20%) had a resolution greater than 0.5, and no peptides had a resolution below 0.1. It was also reported that, for peptides labelled with ^{12}C and ^{13}C succinate reagents, no peptides exceeded a resolution of 0.01 (i.e. they effectively co-elute). In the present work, only 3% (2 out of 71) of peptides had a

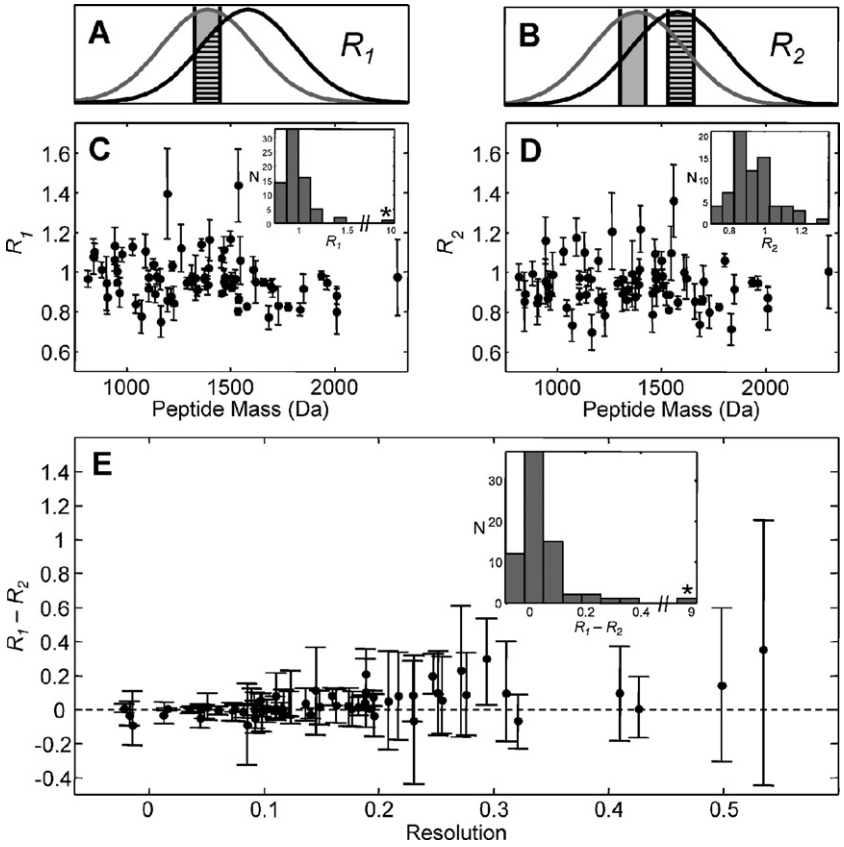

Fig. 5. (A) Strategy for calculation of ratios based on a single time region (R_1) and (B) based on two time regions (R_2); (C) R_1 ratios calculated for 71 peptides with inset histogram; (D) R_2 ratios with inset histogram; (E) ratio differences plotted against resolution.

resolution greater than 0.5, and 42% (30 out of 71) of peptides had a resolution below 0.1. No peptides had a resolution below 0.01, although the error bars suggest that such precise measurements are not meaningful. These results suggest that dimethyl labelling consistently produces resolution values smaller than those for deuterated ICAT reagents, but larger than those for the ^{13}C succinate reagents. Zhang et al. [16,20] reported that there was a negative correlation between the resolution and the peptide mass, wherein a greater isotopic separation was observed for smaller peptides. This trend was not observed in the current study, perhaps owing to the narrower range of peptide masses observed. Low mass peptides (<800 Da) were not observed given the selected mass range of the

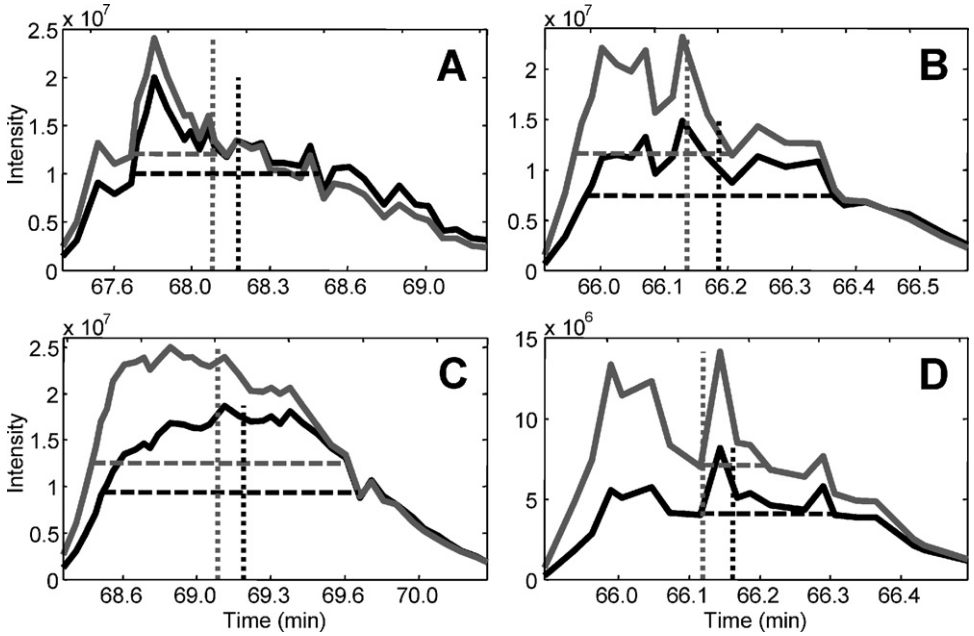

Fig. 6. Shown are the XIC's, extracted through four replicate LC/MS runs, for the peptide pair identified as QTALVELLK. The vertical lines correspond to the median retention times and horizontal lines represent the peak widths at half height for the light (black) and heavy (gray) components.

detector (400–1700 m/z). Such a mass range is consistent with most proteomic investigations.

Zhang et al. [16] have also provided a qualitative rationale for the relative isotopic effects of deuterated labels based on the polarity of the label. Deuterated compounds, in general, are expected to elute earlier because their interactions with the non-polar stationary phase are not as strong as the protonated compounds [10]. It has been shown that chromatographic separation of isotopes is related to the number of deuterium atoms for structurally similar molecules [16]. However, this effect will only be observed if the deuterium atoms are able to interact with the stationary phase. Thus, Zhang et al. argued that the polarity of the label will influence the chromatographic separation, with less polar compounds showing greater separation of isotope pairs. This argument was consistent with a group of deuterated compounds used to for protein labelling, though a quantitative investigation has not been attempted.

To place dimethyl labelling in the context of a quantitative assessment of polarity, computations were carried out in this work to obtain estimates of the polarity of the labels. To achieve this, model compounds were assessed as shown in Fig. 7, representing the product after reaction of the label with 1-butylamine or, in the case of ICAT, methylthiol. Thus, these compounds mimic the environment of the tag following reaction with a lysine or cysteine group. Fig. 7 lists the computed dipole moment (described in Section 2.8). As suggested by Zhang et al., the fragments with the highest polarity correlate with those that have the smallest isotopic effect. In this group, the dimethyl label (7E) falls between the ICAT (7D) and succinic anhydride (7F) labels, which is consistent with the experimental results observed for the resolutions obtained. Thus our observations confirm the arguments previously put forward [16].

The primary purpose of this work was to examine the extent of retention time shifts in dimethyl labelling and their impact on quantitation. While time shifts were observed between heavy and light labelled peptides, these were very small, except for one anomalous peptide pair. To evaluate the effect on quantitation, intensity ratios were calculated based on time synchronous measurements (R_1) and measurements made at chromatographic maxima (R_2), with the latter expected to be more reliable. The majority of the differences of the ratios ($R_1 - R_2$) are in the positive region (Fig. 5E), indicating that R_1 is larger than R_2 for most of the peptide pairs, as expected. This positive bias is also indicated by the median ratios reported. Excluding the anomalous peptide pair (Fig. 3A) and considering the remaining 70 peptide pairs, the mean difference in ratios (R_1 vs R_2) is statistically significant ($P = 2 \times 10^{-4}$ by a paired t-test). However, individually, none of these peptide pairs showed a significant difference in the ratio (i.e. the time shift was unimportant). This is illustrated in Fig. 5E, where the confidence interval of the difference in peptide pair ratios ($R_1 - R_2$) encompasses zero. This was true for all peptide pairs except for a single anomaly. Because proteomics studies (e.g. biomarker studies) often focus on extreme values, the presence of such anomalies should be a consideration, though retention time shifts are expected to be inconsequential for the majority of dimethylated peptides.

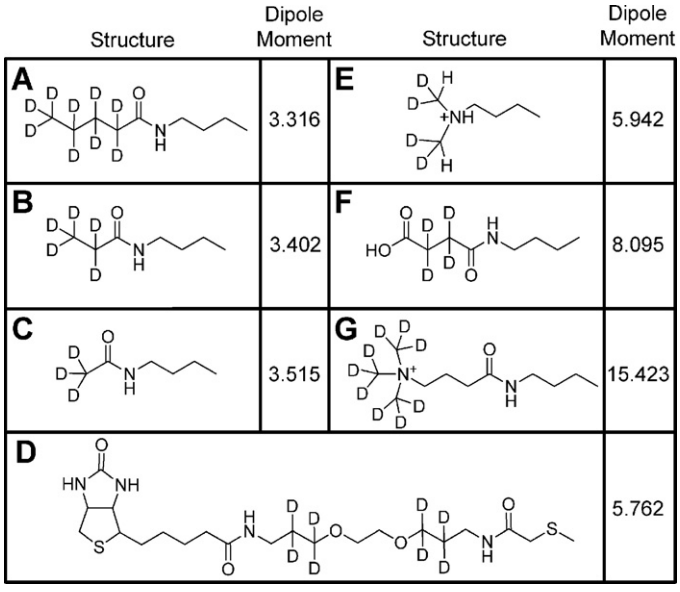

Fig. 7. Calculated dipole moments (Debye) of labelled surrogates (deuterated version) for: (A) pentanoic acid 2,5-dioxopyrrolidin-1-yl ester, (B) propionic acid 2,5-dioxopyrrolidin-1-y ester, (C) acetic acid 2,5-dioxopyrrolidin-1-y ester, (D) ICAT, (E) dimethyl labelling, (F) succinic anhydride, (G) [3-(2,5-dioxopyrrolidin-1-yloxycarbonyl)-propyl]-trimethylammoniumchloride.

It has been suggested in literature that $^{12}C/^{13}C$ based methods generate more reliable quantitative results due to smaller isotopic separation over $^{1}H/^{2}H$ based methods [7]. Although deuterium labelled compounds produce some separation of isotopic peaks, in many cases, as exhibited here, this shift is inconsequential for purposes of quantitation. The majority of $^{12}C/^{13}C$ labelling methods are more expensive and the labelling reagent is commonly larger. It has also been reported that larger labelling reagents may interfere with MS/MS sequencing [22]. Therefore, it is felt on the basis of the present study that deuterium based dimethylation is a viable peptide labelling strategy for relative protein quantitation.

5. Concluding remarks

While the deuterium isotope effect was observed when stable dimethyl labelling was employed for comparative proteomic experiments, this effect is small, resulting in minimal chromatographic resolution for most peptide pairs observed in the normal operational range. This effect is peptide dependent and was found to be more substantial in a few cases. No reproducible trends were observed when retention time difference or resolution was plotted as a function of apparent mass, retention time, or other relevant variables. Based on literature comparison of resolution values, it was determined that the isotopic effect through dimethylation is smaller than that observed through labelling with deuterated ICAT reagents, but larger than when labelling with $^{12}C/^{13}C$ succinate reagents. Through computational studies, it was demonstrated that these results are consistent with arguments that the magnitude isotopic effect is inversely related to the polarity of the label. Except for one anomalous peptide, the effect of time shifts resulting from dimethyl labelling on single point quantitation were found to negligible when compared to measurement uncertainty. It is therefore concluded that it is possible to employ dimethyl labelling to quantitative proteomics without the need for peak integration.

Acknowledgements

The authors gratefully acknowledge Stephen Christensen, who performed the dipole moment calculations in Spartan, and Mark Wall, who provided the BSA data set. Financial support for this work was provided by the Natural Sciences and Engineering Research Council of Canada.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.09.035.

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