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Quantification of Protein Phosphorylation by Liquid Chromatography−Mass Spectrometry

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The identification and quantification of specific phosphorylation sites within a protein by mass spectrometry has proved challenging when measured from peptides after protein digestion because each peptide has a unique ionization efficiency that alters with modification, such as phosphorylation, and because phosphorylation can alter cleavage by trypsin, shifting peptide distribution. In addition, some phosphorylated peptides generated by tryptic digest are small and hydrophilic and, thus, are not retained well on commonly used C_{18} columns. We have developed a novel C-terminal peptide ^2H-labeling derivatization strategy and a mass balance approach to quantify phosphorylation. We illustrate the application of our method using electrospray ionization liquid chromatography−mass spectrometry by quantifying phosphorylation of troponin I with protein kinase A and protein kinase C. The method also improves the retention and elution of hydrophilic peptides. The method defines phosphorylation without having to measure the phosphorylated peptides directly or being affected by variable miscleavage. Measurement of phosphorylation is shown to be linear (relative standard error <5%) with a detection limit of <10%.

Protein phosphorylation is a key regulatory mechanism of numerous biological processes.[1,2] The rapidly reversible phosphorylation-dephosphorylation of specific serine, threonine, and tyrosine residues promotes conformational changes in protein structure that affect protein−protein interactions. The ability to link protein phosphorylation and the regulation of cellular process requires both the identification of specific phosphorylation sites and the quantification of the degree of phosphorylation. Although a variety of methods are available to identify protein phosphorylation, including the use of ^{32}P labeling, phosphospecific antibodies, and phosphospecific gel staining,[3] new mass spectrometry based methods hold significant promise over conventional techniques for the identification of phosphorylation sites in single proteins and complex mixtures of proteins. Nonetheless, the identification of unknown phosphopeptides by mass spectrometry is difficult[4,5] and the quantification of their degree of phosphorylation is even more challenging.

There are several factors that limit mass spectrometry for quantification of protein phosphorylation. For example, peptides generated from the digestion of proteins have unique ionization efficiencies, and therefore, their integrated ion currents cannot be quantified directly. The same problem is also true of phosphorylated and unphosphorylated peptides, each having different ionization efficiencies and, therefore, restricting direct comparison of measured ion currents for quantification.

Zhang et al.[6] defined a method for the quantification of protein phosphorylation using matrix-assisted laser desorption time-of-flight mass spectrometry that circumvented ionization efficiency differences by using a "sample-splitting approach". Zhang et al. digested the protein sample to peptides, split the digest into two equal fractions, dephosphorylated the peptides in one fraction with alkaline phosphatase, and derivatized that fraction with a ^2H-labeled reagent. They derivatized the other fraction with an unlabeled reagent, recombined the fractions, and measured the ^2H-labeled and unlabeled peptides. The degree of phosphorylation was determined from the measured ratio of ^2H-labeled to unlabeled nonphosphorylated peptides. The measurement of the phosphorylated peptide was not needed to determine the degree of phosphorylation using their mass balance relationship. Hegeman et al. applied this method using electrospray ionization (ESI) liquid chromatography−mass spectrometry (LC−MS) and a deuterated methanol label also to determine the degree of phosphorylation.[7]

The approach by Zhang et al. is elegantly simple, but it has limitations. First, the scheme assumes that the peptides are split into equal halves. Second, the scheme assumes that both the

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modified and unmodified forms of the protein behave identically during enzymatic cleavage, i.e., phosphorylation does not affect cleavage. More recently, Steen et al.[8] showed that the degree of cleavage during tryptic digestion of phosphorylated proteins is altered when a phosphorylated amino acid is located near the cleavage site. Steen et al. developed an isotope-free method for the quantification of phosphorylation by ESI-LC−MS that addresses the problem of variable miscleavage of proteins. A key element to the Steen et al. method is the calculation of "flyability constants" that define the degree of peptide cleavage at a particular site and the ionization efficiency of each peptide in a mixture. By defining such constants, this method allows for the direct quantification of peptides and phosphopeptides in a complex mixture. This novel approach accounts for the differential cleavage due to phosphorylation, but the method has drawbacks in practical application. In particular, the determination of the "flyability constant" is not simple and requires repeated measurements of samples in varied degrees of dephosphorylation.

We have utilized the logic outlined by Zhang et al.[6] and Steen et al.[8] to develop a simple LC−MS method for quantifying the degree of phosphorylation of proteins. Our method allows for differential cleavage due to the presence of phosphate and for differences in ionization efficiencies between peptides. We utilize a novel ^{2}H-labeling derivatization strategy that also increases the chromatographic retention of hydrophilic peptides on a C_{18} column. We illustrate the application of our method using ESI-LC−MS by quantifying the phosphorylation of troponin I treated with protein kinase A and protein kinase C.

EXPERIMENTAL SECTION

Materials. 1-[1,1,2,2-$^2H_4$]Propanol (abbreviated propanol-d_4) was purchased from CDN Isotopes (Pointe-Claire, Quebec) with >98% ^{2}H purity. Custom synthesized SSANYR and its phosphorylated analogue S*S*ANYR peptides were purchased from New England Peptide, Inc. Protein kinase A (PKA) was purchased from Sigma-Aldrich (St. Louis, MO), protein kinase C-α (PKC) was purchased from Calbiochem (San Diego, CA), and phospholipid activator was purchased from Upstate Biotechnology (Billerica, MA). Trypsin was purchased from Promega. HPLC grade water and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ). All other reagents including unlabeled 1-propanol (abbreviated propanol-d_0) were purchased from ThermoFisher Scientific (Waltham, MA).

ESI-LC−MS. All measurements were made by ESI-LC−MS. The liquid chromatography was performed using an Atlantis 1 mm × 150 mm i.d. column packed with 5 μm C_{18} (Waters Corporation, Milford, MA), with a Surveyor MS pump and autosampler (ThermoFisher Scientific). A 10 μL aliquot of sample was injected into 0.5% acetic acid in 5.0% acetonitrile at a flow of 25 μL/min. At 4 min, the flow was increased to 50 μL/min, and the gradient was ramped linearly to 0.5% acetic acid in 45% acetonitrile over 30 min and held isocratic for 10 min. The ramp was increased to 0.5% acetic acid in 60% acetonitrile for 6 min and then returned to 0.5% acetic acid in 5% acetonitrile. The column was allowed to re-equilibrate for 30 min prior to the next injection. The total run time was 86 min per analysis.

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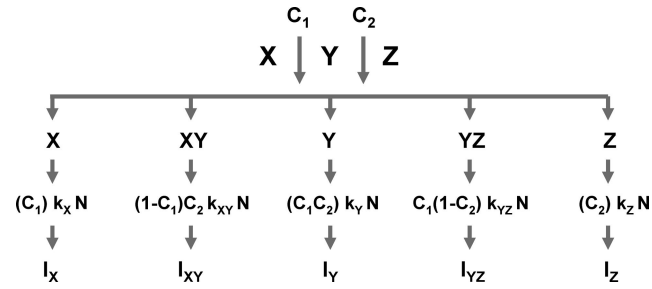


Figure 1. Cleavage of a hypothetical protein XYZ into peptides before measurement by mass spectrometry. Protein XYZ contains two cleavage sites that should generate peptides X, Y, and Z unless there is miscleavage producing peptides XY and YZ. C_1 and C_2 define the probability of cleavage at each site. The terms k_i represent the intrinsic ionization efficiency of each peptide (i), N represents the amount of XYZ prior to digestion, and I_i represent the ion current measured by the mass spectrometer for each peptide.

The LC eluant entered a LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corporation) connected to the LC by an ESI interface. The instrument was operated in positive electrospray ionization mode with a capillary temperature of 350 °C and a spray voltage of 4.5 kV. Spectra were collected by scanning from $m/z = 300-1800$ using automatic gain control. Data dependent MS/MS analyses were also performed on the same instrument using an isolation width of 1, collision energy of 42%, activation Q of 25, activation time of 30 ms, minimum MS signal 1×10^6, and a repeat count of 4 prior to a 0.75 s exclusion.

Mass Balance Approach Used to Quantify Phosphorylation. The ion current measured by the mass spectrometer for any peptide, i, from the digest of a single protein sample is related to the amount of protein prior to digestion:

$$I_i = \mu_i N \tag{1}$$

where I_i is the measured ion current, μ_i is the "flyability constant" of peptide i, and N is the amount of protein in moles.

The "flyability constant" is the product of two components

$$\mu_i = C_i k_i \tag{2}$$

where C_i is the probability of cleavage during protein digestion to form peptide i, and k_i is the intrinsic ionization efficiency of peptide i.

Figure 1 provides an example of eqs 1 and 2 for a hypothetical protein XYZ that contains two cleavage sites. The probability for cleavage at each site is defined as C_1 and C_2. The cleavage of protein XYZ should produce three tryptic peptides X, Y, and Z and the miscleaved peptides XY and XZ when the probability of cleavage at C_1 and C_2 are < 1.

The measured ion current of peptide X (I_X) will be a function of the probability of cleavage at the X−Y site (defined as C_1), the ionization efficiency of peptide X (k_X), and the protein amount (N):

$$I_X = C_1 k_X N \tag{3}$$

Similarly, the measured ion current of peptide XY (I_{XY}) will be a function of the probability of miscleavage at X−Y (i.e., 1-C_1), the probability of cleavage at Y−Z (defined as C_2), the ionization efficiency of peptide XY (k_{XY}), and the protein amount (N):

$$I_{XY} = (1 - C_1)C_2k_{XY}N \quad (4)$$

Similar equations can be written for the ion currents for peptides Y, Z, and YZ.

Now let us consider the case where two equal aliquots ($^1/_2N$) of protein XYZ are digested and one aliquot is derivatized to add an unlabeled propyl group (propyl-d_0) and the other is derivatized to add a deuterated propyl group (propyl-d_4). If the aliquots are mixed, the propyl-d_0 peptide ion currents should equal the propyl-d_4 peptide ion currents because each set of peptides should have the same cleavage probabilities and ionization efficiencies. For example, the ion current ratio for peptide X would be

$$\frac{I_{X(0)}}{I_{X(4)}} = \frac{C_1k_X\left(\frac{1}{2}N\right)}{C_1k_X\left(\frac{1}{2}N\right)} = 1 \quad (5)$$

where $I_{X(0)}$ is the ion current for the propyl-d_0 tagged peptides and $I_{X(4)}$ is the ion current for the propyl-d_4 tagged peptides.

Now consider a protein sample split into two unequal aliquots F and $1 - F$. Each aliquot is digested with trypsin, then the "F" fraction is derivatized with propyl-d_0, the "$1 - F$" fraction is derivatized with propyl-d_4. When these two aliquots are mixed and measured by MS, the ratio of the ion currents for peptide X will be

$$\frac{I_{X(0)}}{I_{X(4)}} = \frac{C_1k_XFN}{C_1k_X(1-F)N} = \frac{F}{1-F} \quad (6)$$

Similar measured ion current ratios will be found for all of the peptides:

$$\frac{I_{X(0)}}{I_{X(4)}} = \frac{I_{XY(0)}}{I_{XY(4)}} = \frac{I_{Y(0)}}{I_{Y(4)}} = \frac{I_{YZ(0)}}{I_{YZ(4)}} = \frac{I_{Z(0)}}{I_{Z(4)}} = \frac{F}{1-F} \quad (7)$$

The above discussion describes the case for a single homogeneous pool of protein XYZ. Now consider the case when there is a phosphorylated amino acid in peptide X but not in Y or Z. The fraction of peptide X that is phosphorylated (p) can be defined as

$$p = \frac{X_p}{X_0 + X_p} \quad (8)$$

where X_p is the amount of the peptide that is phosphorylated and X_0 is the amount of the peptide that is not phosphorylated. We cannot relate the measured ion currents of X_p and X_0 directly because they will have potentially different trypsin-cleavage and ionization efficiencies.

Next, let us dephosphorylate the $(1 - F)$ fraction with alkaline phosphatase, digest the fraction with trypsin, and derivatize the peptides to add propyl-d_4 groups. The ion current for peptides X and Z for this fraction will be

$$I_{X(4)} = C_1k_X(1 - F)N \quad (9a)$$

and

$$I_{Z(4)} = C_2k_Z(1 - F)N \quad (9b)$$

We will also digest protein fraction (F), but its peptides will be derivatized with unlabeled (d_0) propyl groups. The ion current for peptides X and Z for this fraction will be

$$I_{X(0)} = C_1k_XF(1 - p)N \quad (10a)$$

and

$$I_{Z(0)} = C_2k_ZFN \quad (10b)$$

Note that the "F" protein fraction has not been dephosphorylated and that the ion current of the unphosphorylated peptide X is reduced by $(1 - p)$. The two fractions (F and $1 - F$) are then combined and the peptides measured by mass spectrometry. With the combination of eqs 9a,b and 10a,b, the measured ion current ratios ($I_{(0)}/I_{(4)}$) of peptides X and Z are then

$$\frac{I_{X(0)}}{I_{X(4)}} = \frac{F}{1-F}(1 - p) \quad (11a)$$

and

$$\frac{I_{Z(0)}}{I_{Z(4)}} = \frac{F}{1-F} \quad (11b)$$

Substitution of $F/(1 - F)$ from eq 11b into eq 11a allows us to solve for the degree of phosphorylation

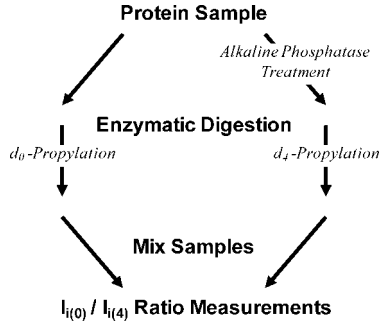
$$p = 1 - \frac{I_{X(0)}/I_{X(4)}}{I_{Z(0)}/I_{Z(4)}} \quad (12)$$

Equation 12 provides a simple means to quantify the degree of phosphorylation at a specific site (in this case peptide X) using a reference peptide that is not phosphorylated (in this case peptide Z) to adjust for differences in splitting the protein sample prior to digestion and the derivatization of the peptides. The splitting/dephosphorylation scheme follows the method of Zhang et al.,[6] but the splitting and dephosphorylation are performed on the whole protein prior to digestion (Scheme 1). This scheme accounts for differences in both peptide cleavage and ionization efficiency between the unphosphorylated and phosphorylated peptides by only measuring the unphosphorylated peptide signals (eq 12).

Overview of Experiments. Several experiments were carried out to assess the labeling strategy, evaluate the dynamic range of quantification, and demonstrate the application of the method for quantification of phosphorylation stoichiometry.

Optimization and Characterization of Derivatization Conditions. The synthetic peptides SSANYR and the doubly phosphorylated S*S*ANYR were used to assess the derivatization reaction efficiency and confirm that derivatization does not remove the phosphates from the peptide. Additionally, each peptide also contains an asparagine residue which helps to confirm that our labeling method does not deamidate the amide amino acids asparagine and glutamine. Once the reaction conditions were

Scheme 1. Analytic Scheme Used for Phosphorylation Analysis[a]



[a] The protein sample is split into two fractions. One protein fraction is treated with phosphatase and serves as an internal standard. Each fraction is digested with trypsin. The peptides in each fraction are differentially labeled with propylation reagent-d_0 or -d_4, and then the fractions are recombined. Ion current ratios are measured for each pair of labeled isotopologues.

optimized, the derivatization reaction was further assessed using peptides generated from the digestion of whole proteins.

Derivatization Protocol. The derivatization reagent was prepared by adding a 160 μL aliquot of acetyl chloride to 1 mL of either propanol-d_0 or propanol-d_4 on ice (abbreviated derivatization reagent-d_x). Propyl ester derivatives were prepared from peptides by adding a 100 μL aliquot of the derivatization reagent to 1 mL Eppendorf tubes containing dried peptides. The peptides were solublized by trituration with a mechanical pipet. The tubes were capped, and the esterification reaction was performed. The samples were dried in a speed vacuum device and then reconstituted in 0.1% formic acid in 25% acetonitrile.

This propylation procedure labels peptides by adding either 42 Da (d_0) or 46 Da (d_4) to carboxylic groups. All peptides gain one propyl group on the C-terminus. Peptides with aspartic and/or glutamic acid residues also gain propyl groups on these residues. Therefore, the minimum difference in mass between labeled and unlabeled peptides is 4 Da, which is sufficient to avoid natural abundance isotopic overlap from the d_0-peptides into the d_4-isotopologues.

Effect of Reaction Conditions. Aliquots (1 nmol) of synthetic SSANYR and S*S*ANYR peptides were reacted with derivatization reagent-d_0 at 25, 37, and 56 °C for 2 h. The samples were dried and reconstituted in 0.1% formic acid. Aliquots of each sample were analyzed via ESI-LC−MS/MS as described above.

Troponin protein complex containing troponin I, T, and C was isolated from beef cardiac tissue via column chromatography.[9] An aliquot (1.3 nmol) of protein was dried via speed vacuum and then digested by the addition of trypsin (~1:25 enzyme to substrate ratio) in 50 mM ammonium bicarbonate, incubated at 37 °C for 18 h. The resultant peptides were dried to completion in a speed vacuum device, and a 100 μL aliquot of derivatization reagent-d_0 was added to each tube and reacted at 25 °C for 18 h.

Identification of Peptides. SEQUEST searches were performed via the Thermo-Finnigan BioWorks software looking for bovine troponin I sequences. The presence of the propyl groups was accounted for by the addition of 42 or 46 Da to the C-terminus, aspartic acid and glutamic acid residues of each peptide. The phosphorylation was also accounted for by the variable addition of 79.96 Da to each serine, threonine, and tyrosine residue. In addition to the SEQUEST search, the peptide sequence for bovine cardiac troponin I[10] was downloaded from UniProtKB/Swiss-Prot (http://ca.expasy.org/uniprot/P08057) and a theoretical tryptic digestion with one miscleavage was performed using ExPASy Peptide Mass (http://ca.expasy.org/tools/peptide-mass.html).[11] The peptide mass list generated by Peptide Mass and modified by differentially adding 42 or 46 Da to the C-terminus of each peptide as well as to each aspartic acid and glutamic acid residue in each peptide to account for the addition of propyl-d_0 or propyl-d_4 groups to these sites. The mass list was compiled for $[M + H]^{+1}$, $[M + 2H]^{+2}$, and $[M + 3H]^{+3}$ ions. The measured ion currents corresponding to these ions were extracted from the filtered MS portion of each MS/MS chromatogram for each d_0- or d_4-propylated peptide. When ion profiles were found for these peptides, the associated data-dependent MS/MS spectra were manually compared to a library of theoretical product ions created using Protein Prospectors MS-Product (http://prospector.ucsf.edu/ucsfhtml4.0/msprod.htm)[12] to provide additional identification.

Assessment of the Derivatization Efficiency. For the SSANYR and S*S*ANYR peptides, a single propyl group is added to the C-terminus of each peptide. These peptides do not contain additional carboxylic functional groups that can be derivatized, and the abundance of the underivatized peptide was compared to the abundance of the derivatized species. However, many of the peptides resulting from the digestion of troponin I contain multiple potential derivatization sites. For a peptide that can accept n propyl groups, the fully or completely propylated species will have a fractional abundance of E^n where E is the efficiency (from 0−100%) of placing a propyl group on a carboxyl carbon. Conversely if we measure the fraction of the peptide that is completely propylated (F_n), then the derivatization efficiency will be $E = F_n^{1/n}$. Although we could also do this calculation for a partially derivatized peptide, e.g. a peptide with $n - 1$ propyl groups, these calculations become cumbersome.

Precision of Peptide Ratio Measurement. Aliquots of SSANYR were added to 1 mL Eppendorf tubes and derivatized with derivatization reagent-d_0 or -d_4. The samples were dried and reconstituted in 0.1% formic acid. The d_0- and d_4-labeled peptides were then mixed in mole fractions ranging from 0−50% propyl-d_0 peptide. A 10 μL aliquot of each sample was analyzed via ESI-LC−MS as described earlier and the MS data collected. Each extracted ion chromatogram contained a minimum of 25 data points across the peak. The MH^+ ion currents of the d_0- and d_4-labeled peptides (m/z = 739.3 and 743.3, respectively) were measured for SSANYR.

Aliquots of troponin protein-complex were dried via speed vacuum and then digested by the addition of trypsin at 37 °C for 18 h. The resultant peptides were dried to completion in a speed vacuum device, and a 100 μL aliquot of derivatization reagent (d_0 or d_4) was added to each tube, respectively, and reacted at 25 °C for 18 h. The d_0- and d_4-labeled peptides were mixed in mole fractions ranging from 0−50% d_0-labeled peptides. The ion currents

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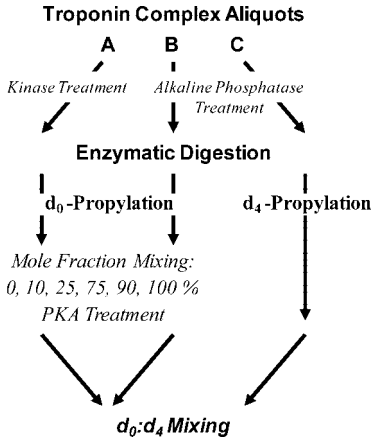
Scheme 2. Scheme Used to Create Protein Samples with an Increased Mole Fraction of Phosphorylation



of the d_0- and d_4-labeled peptides were then measured by ESI-LC−MS/MS.

Quantification of Troponin I Phosphorylation Using Protein Kinase A (PKA). To validate the scheme for the quantification of phosphorylation, protein mixtures with varying degrees of phosphorylation were created. Aliquots (90 μL) of 14.5 μM troponin protein-complex containing troponin I, T, and C were placed into 1 ml Eppendorf tubes labeled A, B, and C as illustrated in Scheme 2. The protein in tube A was exhaustively phosphorylated by the addition of 70.8 μL of 50 mM ammonium bicarbonate/10 mM magnesium chloride, 0.2 μL of 1 mM DTT, 0.7 μL (175 U) of PKA and was let to sit for 5 min at 30 °C. A 3.3 μL aliquot of 100 mM ATP was added to the tube, and the phosphorylation reaction was carried out at 30 °C for 18 h. The samples in tubes B and C were dephosphorylated using alkaline phosphatase by the substitution of the PKA and ATP with 4.0 μL of alkaline phosphatase. Samples A, B, and C were dried via speed vacuum and then incubated at 37 °C for 18 h with trypsin (~1:25 enzyme to substrate ratio) in 100 μL of 50 mM ammonium bicarbonate. The resultant peptides were dried to completion in a speed vacuum device. A 100 μL aliquot of derivatization reagent-d_0 was added to tubes A and B, while a 100 μL aliquot of derivatization reagent-d_4 was added to tube C. The samples where reacted at 25 °C for 18 h. The samples were dried, and a 180 μL aliquot of 0.1% formic acid in 25% acetonitrile was added to tubes A, B, and C and the peptides were solublized by trituration with a mechanical pipet. Aliquots of samples A and B (where A = PKA treated d_0-labeled peptides and B = alkaline phosphatase treated d_0-labeled peptides) were mixed in mole fractions ranging from 0−100% PKA-treated troponin protein-complex. An aliquot of sample C (containing d_4-labeled peptides) was added to each A−B mixture of d_0-labeled peptides. This scheme provided phosphorylated samples of known amounts that could be measured using eq 12. Two 10 μL aliquots of each of these samples where analyzed by ESI-LC−MS in both MS and data dependent MS/MS mode as described earlier.

Quantification of Troponin I Phosphorylation Using Protein Kinase C (PKC). Aliquots of troponin protein-complex were placed into 1 mL Eppendorf tubes labeled A and B. Sample A was exhaustively phosphorylated by the addition of 53.5 μL of 50 mM ammonium bicarbonate/10 mM magnesium chloride, 0.2 μL of 1 mM DTT, 1.5 μL of PKC (175 U), and 16.5 μL of PKC lipid activator and incubating for 5 min at 30 °C. A 3.3 μL aliquot of 100 mM ATP was added to the tube, and the phosphorylation reaction was carried out at 30 °C for 18 h. Sample B was dephosphorylated with alkaline phosphatase using conditions identical to those in the PKA experiment. After the incubation, the samples were dried and digested, the peptide pools were labeled by d_0- or d_4-propylation, the samples were mixed in a 2:1 ratio and analyzed as described in the PKA experiment.

RESULTS

Optimization and Characterization of Derivatization Conditions of the SSANYR Peptides. The derivatization reaction was performed under several conditions to optimize the efficiency of the reaction and to minimize unwanted side products. We evaluated the reaction at 25, 37, and 56 °C, for 2 h using the SSANYR and S*S*ANYR peptides. The ESI-LC−MS analysis of derivatized SSANYR peptide yielded several distinct peaks, and the intensity of each peak varied with respect to the conditions used during the reaction. Two distinct LC peaks were observed when the reaction was carried out at 25 °C for 2 h. These peaks corresponded to the unmodified SSANYR (17%, MH^+ = 697.3) and the propylated SSANYR (83%, MH^+ = 739.3) peptides. Deamidation and/or propylation of the asparagine were not observed. The ESI-LC−MS analysis of derivatized S*S*ANYR peptide produced results that were nearly identical to those for the unphosphorylated peptide under the above reaction conditions. Moreover, under these conditions, no dephosphorylation was observed.

Three LC peaks were observed when the temperature was increased to 37 °C for 2 h. These LC peaks corresponded to the unmodified SSANYR (2%), propylated SSANYR (84%), and propylated SSANYR peptide that was 17 Da lighter (14%, MH^+ = 722.3). Presumably, this compound had lost NH_3, which was confirmed by the MS/MS spectrum corresponding to this LC peak. Again, the S*S*ANYR peptide produced results that were nearly identical to those for the unphosphorylated peptide under the above reaction conditions. No dephosphorylation was observed.

Several LC peaks were observed when the temperature was increased to 56 °C for 2 h. These peaks corresponded to the unmodified SSANYR (1%), propylated SSANYR (42%), propylated SSANYR peptide that was 17 Da lighter (57%), as well as four other peaks that could not be identified.

On the basis of these results, we modified the reaction conditions and performed the derivatization reaction on peptides generated from the tryptic digestion of the troponin complex isolated from the bovine heart. We opted to use the lowest reaction temperature (25 °C) to prevent the formation of the side products and to increase the reaction time to 18 h. When these samples were measured by ESI-LC−MS/MS, only the propylated SSANYR (MH^+ = 739.3) peptide was observed in the total ion chromatogram.

Assessment of the Derivatization Efficiency for Each Troponin I Peptide. Table 1 shows the measured derivatization efficiency for each observed troponin I peptide propylated at 25 °C for 18 h. These peptides corresponded to 66% of the total protein by amino acid composition. The average derivatization efficiency was 87 ± 2%. The low standard deviation measured for the four preparations of the peptides indicates derivatization consistency and low variation among the samples. Despite some peptides having lower apparent derivatization efficiencies, the

Table 1. Efficiency of Derivatization of Peptides

	troponin I tryptic peptides	no. of propyl groups added	derivatization efficiency
1	EAEER	4	0.888 ± 0.004
2	CQPLELAGLGFAELQDLCR	4	0.835 ± 0.089
3	YDVEAK	3	0.923 ± 0.006
4	ETLDLR	3	0.871 ± 0.010
5	NIDALSGMEGR	3	0.699 ± 0.012
6	NITEIADLNQK	3	0.658 ± 0.029
7	IFDLR	2	0.990 ± 0.015
8	ISADAMMQALLGAR	2	0.954 ± 0.003
9	AYATEPHAK	2	0.936 ± 0.015
10	SGGSTAGDTVPAPPPVR	2	0.385 ± 0.017
11	SSANYR	1	1.000 ± 0.000
12	ISASR	1	1.000 ± 0.000
13	ALSTR	1	1.000 ± 0.000
14	FKRPTLR	1	0.954 ± 0.065
15	KLQLK	1	0.946 ± 0.076
16	TLMLQIAK	1	0.944 ± 0.003

ability to attach propyl groups to the peptides was reproducible. The poorest efficiency was observed for putting two propyl groups on the SGGSTAGDTVPAPPPVR peptide, which we ascribe to steric hindrance created by the four prolines located in the C-terminal region of this peptide.

Effect of Peptide Propylation on Peptide LC Retention. The peptides SSANYR and S*S*ANYR are indicative of a range of tryptic peptides that are small, polar, and hydrophilic. Phosphorylation does not significantly affect hydrophilicity.[13] The intrinsic hydrophilicity of these peptides complicates their measurement via ESI-LC−MS because they elute early from the C_{18} column, often in the void volume. However, the addition of a propyl group to the C-terminus of these peptides provides a hydrophobic handle and increases the interaction of these peptides with the C_{18} column significantly. Figure 2 illustrates the retention time of the unmodified SSANYR and propylated SSANYR peptides. The native SSANYR peptide is not retained on the column and appears in the void volume in less than 6 min, but the propylated SSANYR peptide is retained on the column for approximately 20 min. The LC retention times of the unmodified and modified S*S*ANYR were similar to those observed for the SSANYR peptides.

Effect of Deuterium Labeling on Chromatography. Replacement of hydrogen with deuterium in a molecule is known to decrease the GC and LC retention time, and the use of deuterated tags will therefore cause some chromatographic fractionation of deuterated and nondeuterated species. Figure 3 illustrates the shift in retention time between isotopologues for peptides NITEIADLNQK, ISADAMMQALLGAR, and FKRPTLR that contain multiple propyl-d_0 and -d_4 tags. The addition of each propyl group resulted in a 15% separation of peaks as shown in Figure 3. Minimal fractionation is observed between isotopologues containing multiple deuterium atoms.

Linearity and Precision of Peptide Ratio Measurement. The key element of this method is measuring d_0/d_4-labeled peptides that are not phosphorylated to determine phosphorylation. First, the linearity of the d_0/d_4 ratio measurement needs to be established. The SSANYR peptides were differentially labeled with propyl-d_0 and -d_4, mixed with a mole ratio ranging from 0:1 to 1:1 d_0-labeled peptides to d_4-labeled peptides, and measured by ESI-LC−MS to evaluate the precision and accuracy of the ratio measurement. A linear relationship $I_0/I_4 = (0.980 \pm 0.019)$ $(d_0/d_4) + (0.001 \pm 0.068)$ with $r^2 = 0.9968$ was observed, indicating precise and accurate measurements of the d_0- and d_4-peptide ratios for the SSANYR peptide.

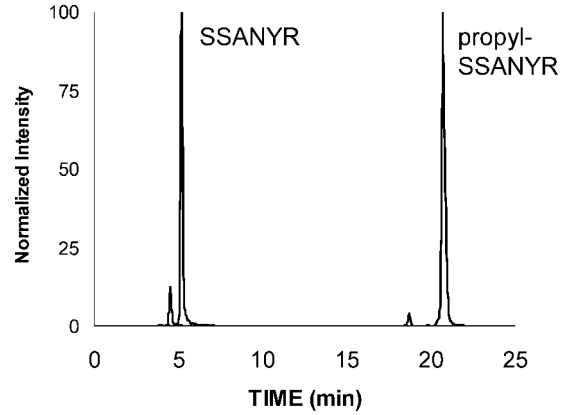


Figure 2. ESI-LC−MS chromatogram of underivatized SSANYR (m/z = 697.3) and derivatized propyl-SSANYR (m/z = 739.3) peptides. The addition of a single propyl group increased the retention time away from the void volume (~5 min) by ~15 min.

The peptides generated from the digestion of the isolated troponin protein-complex were differentially labeled with propyl-d_0 or -d_4 and then mixed with a mole ratio ranging from 0:1 to 1:1 d_0-labeled peptides to d_4-labeled peptides and measured by ESI-LC−MS/MS. Figure 4 shows the measured ion current ratios for each set of isotopologues listed in Table 1. This graph demonstrates linearity and precision of the d_0/d_4 peptide ratio measurement for a range of peptides.

Quantification of Troponin I Phosphorylation Using PKA. Troponin protein-complex samples were prepared over a range of phosphorylation values to test the method to quantify phosphorylation. Samples containing various amounts of phosphorylation were prepared by mixing peptides from alkaline phosphatase-treated (dephosphorylated) and PKA-treated (phosphorylated) troponin protein-complex. However, the specific sites of phosphorylation and/or the degree of phosphorylation in the PKA-treated sample were not known. Each phosphorylated sample was then mixed with an aliquot of d_4-labeled peptides generated from the digestion of alkaline phosphatase treated troponin protein-complex as illustrated in Scheme 2.

Figure 5 shows the measured ion current ratios (I_0/I_4) for the peptides listed in Table 1 in each of the PKA-treated samples. There is a notable decrease in the measured ion current ratio for peptides SSANYR and ISASR that corresponds to an increase in amount of PKA-treated sample in the mixture. This observation was anticipated because these two peptides contain sites that have previously been identified as targets for PKA phosphorylation.[14] The degree of phosphorylation of peptides SSANYR and ISASR was then determined in each sample using eq 12. However, eq 12 requires selection of a nonphosphorylated reference peptide or group of peptides for the quantification of phosphorylation.

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(14) Noland, T. A.; Guo, X. D.; Raynor, R. L.; Jideama, N. M.; Averyhartfullard, V.; Solaro, R. J.; Kuo, J. F. *J. Biol. Chem.* **1995**, *270*, 25445–25454.

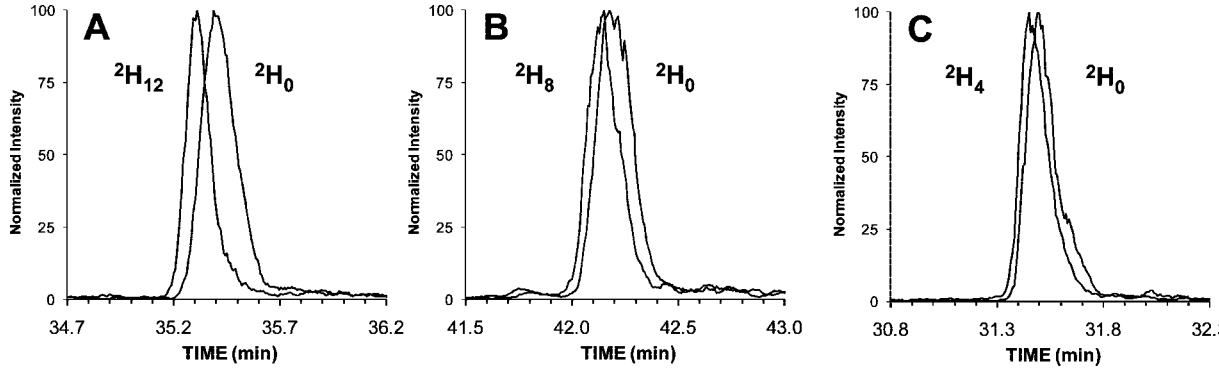

Figure 3. Peptide elution profiles of of d_0- and d_4-isotopologues with varying degrees of propylation. Shown are the $[M + 2H]^{2+}$ ion currents versus time for peptides: (A) NITEIADLNQK (m/z = 692.8 and 698.8), (B) ISADAMMQALLGAR (m/z = 766.4 and 770.4), and (C) FKRPTLR (m/z = 480.6 and 482.6). These peptides contain 3, 2, and 1 propyl groups and 12, 8, and 4 deuteriums, respectively. The total separation at the peak apexes are 5.5, 2.1, and 2.0 s, respectively, with an average peak width at $^1/_2$ height of 9.6 s.

There are five peptides in Table 1 that are not phosphorylated: EAEER, CQPLELAGLGFAELQDLCR, IFDLR, KLQLK, and TLMLQIAK. The mean value of the (I_0/I_4) ratios for this group of peptides was used as a mean reference peptide. The use of the mean value of multiple peptides reduces variability in the measurement introduced from the mass spectrometer, digestion, and labeling processes by the square root of the number of reference peptides used.

Figure 6 shows the degree of phosphorylation calculated for the SSANYR and ISASR peptides in each of the PKA-treated sample mixtures. We determined that 99% of the SSANYR peptide and 49% ISASR peptide were phosphorylated by PKA treatment. A linear decrease was observed for the degree of both SSANYR and ISASR phosphorylation when the PKA-treated sample was diluted with unphosphorylated peptides. These results demonstrate the linearity and precision of our method for determining phosphorylation.

Quantification of Troponin I Phosphorylation Using PKC. The troponin protein-complex was phosphorylated with PKC to test our ability to measure other phosphorylated peptides. As previously reported,[14] PKC is less selective than PKA and phosphorylates several peptides including SSANYR, ISASR, ISADAMMQALLGAR, FKRPTLR, and ALSTR. The measured degree of phosphorylation was 100% for SSANYR, 47% for ISASR, 37% for ISADAMMQALLGAR, 77% for FKRPTLR, and 82% for ALSTR.

Although the peptides CQPLELAGLGFAELQDLCR and YDVEAK do not contain a serine or threonine to be phosphorylated, PKC did reduce the abundance of these peptides, making them appear phosphorylated by our method (eq 12): CQPLELAGLGFAELQDLCR (95%) and YDVEAK (95%). This apparent error is due to differential cleavage caused by the phosphorylation of adjacent peptides that reduces the abundance of these peptides. This observation reminds us of the significant effect that phosphorylation may have on tryptic peptide miscleavage and the need for a method, such as ours, to compensate for this effect. The additional point is that reference peptides need to be selected carefully as well.

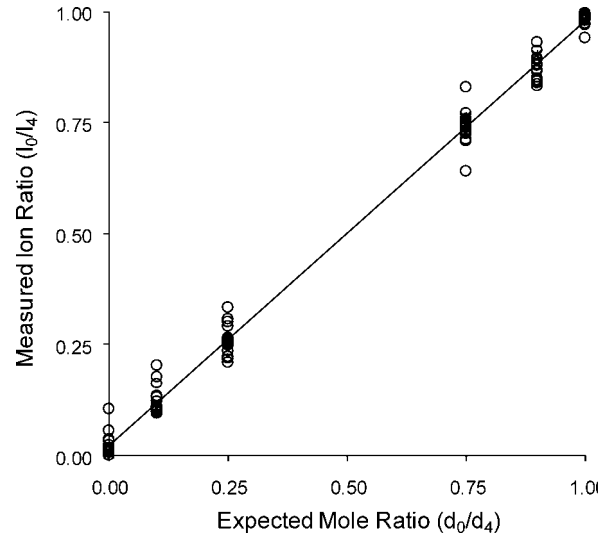

Figure 4. Linearity and precision of isotopologue ratio measurements. Data shown are the measured ion current (I_0/I_4) ratios for each peptide listed in Table 1 for samples prepared with defined mole d_0/d_4 ratios ranging from 0:1 to 1:1 d_0- to d_4-labeled peptides. A linear response in the measured ion current ratio, $I_0/I_4 = (0.956 \pm 0.005)(d_0/d_4) + (0.025 \pm 0.003)$ with $r^2 = 0.999$ was observed as a function of mole ratio of unlabeled (d_0) to deuterated (d_4) peptides.

DISCUSSION

We have developed a method for the quantification of protein phosphorylation utilizing C-terminal propyl esterification. The derivatization reaction used in this approach was adapted from what has commonly been used to derivatize carboxylic functional groups on amino acids in preparation for gas chromatography mass spectrometry.[15] The use of this derivatization strategy provides a cost-effective means of incorporating a minimum of four deuterium atoms into each peptide and is not only suitable for LC−MS analysis but also improves the chromatography of peptides.

The derivatization reaction (performed at 25 °C for 18 h) efficiently and reproducibly propylated peptide carboxyl groups. On average, 87% of each peptide carboxyl group was derivatized, with an average coefficient of variation between multiple derivatization reactions being 2.5%. Most importantly, the derivatization reaction did not alter other amino acids other than aspartic and glutamic acid, where additional propyl groups were added. The derivatization scheme did not deamidate asparagine or glutamine nor did it remove phosphate from phosphorylated peptides.

(15) Adams, R. F. *J. Chromatogr.* **1974**, *95*, 189–212.

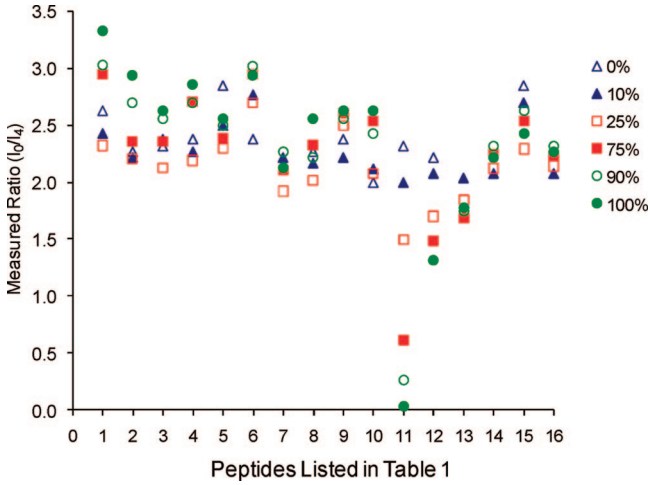

Figure 5. Measurement of d_0/d_4 isotope ratios as a function of dilution of PKA-treated peptides with dephosphorylated peptides. Dilution of PKA-treated peptides correlates to an increase in the isotopologue ratio for phosphorylated peptides. The *x*-axis lists the peptides in Table 1 by numbers, and the *y*-axis plots ion current ratios (I_0/I_4) measured for each peptide across a dilution range of PKA treatment. Symbols indicate the mole fraction of PKA treatment in each sample ranging from 0 to 100%. The measured I_0/I_4 ratios of peptides SSANYR and ISASR decrease with increased PKA treatment and resulting phosphorylation. The reference peptide ratio used was the mean ratio of the following five peptides: EAEER, CQPLELAGLGFAELQDLCR, IFDLR, KLQLK, and TLMLQIAK.

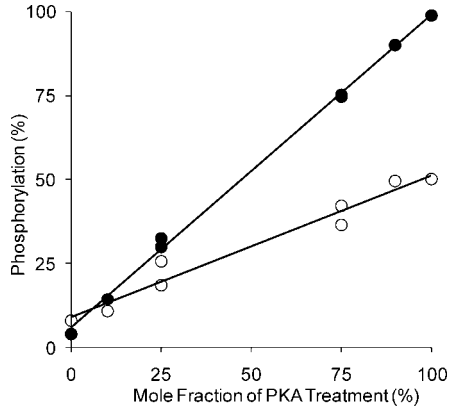

Figure 6. Demonstration of linearity of phosphorylation measurement. The degree of phosphorylation was determined for peptides SSANYR (●) and ISASR (○) from the samples shown in Figure 5. A linear response was observed for SSANYR, $p(\%) = (0.937 \pm 0.017)\ p_{Rx}(\%) + (5.6 \pm 6.4)$; $r^2 = 0.998$ and for ISASR, $p(\%) = (0.438 \pm 0.036)\ p_{Rx}(\%) + (8.7 \pm 9.5)$, $r^2 = 0.961$, where p is the fraction of peptide that is phosphorylated and p_{Rx} is the degree of PKA treatment.

A secondary advantage of the propylation method is that it reduces peptide hydrophilicity. A fundamental problem quantifying site-specific protein phosphorylation is that phosphorylation sites may be located near lysine and arginines such that short, polar peptides may be produced when using trypsin as the cleavage enzyme. These polar peptides have low affinity for a C_{18} column and, thus, often elute in the void volume. The addition of a propyl group increases peptide hydrophobicity and enhances retention of small, hydrophilic peptides that are not well retained on a C_{18} column. As evident in our study, trypsin cleavage of troponin I produces peptides that include SSANYR, ISASR, ALSTR, and FKRPTLR. All of theses peptides may be phosphorylated, and all elute in the void volume of the chromatogram. Neither the unphosphorylated nor the phosphorylated variants are readily detectable without the addition of the propyl group.

The incorporation of the stable isotope deuterium was selected for isotopic labeling rather than ^{13}C because of its greatly reduced cost and the fact that four deuteriums can be incorporated into the propyl. This gives a minimum of a 4 Da mass separation (compared to only 3 Da with a $^{13}C_3$-propyl) and reduces chromatographic fractionation (compared to a $^{2}H_7$-propyl). In addition to the C-terminus, both aspartic and glutamic acid contain carboxyl groups that are propylated during the derivatization reaction. While peptides that contained multiple propyl groups were observed, chromatographic fractionation remained acceptable. The addition of each propyl group resulted in a 15% separation of peaks (Figure 3). Moreover, ion currents measured for peptides with known propyl-d_0 to propyl-d_4 ratios were linear (Figure 4), indicating that there was no difference in ionization efficiency of peptides containing propyl groups with hydrogen versus deuterium. The ion current measurements were also not affected by the number of propyl groups added.

To validate the method for phosphorylation quantification, we used the muscle protein troponin I that is known to be phosphorylated by protein kinase A and protein kinase C. The troponin protein complex was treated with protein kinase A and mixed with known amounts of peptides generated from unphosphorylated proteins to produce a range of phosphorylation values. Protein kinase A phosphorylated two troponin I tryptic peptides, SSANYR and ISASR. The troponin I SSANYR and ISASR peptides each contain two potential phosphorylation sites. Because our method only measures the unphosphorylated peptides to determine the degree of phosphorylation, it cannot identify the specific amino acid location or the number of phosphorylated amino acids within the peptide. However, a strength of our method is that the phosphorylated peptide does not have to be observed. For example, no phosphopeptides containing the ISASR sequence were detected. However, we were able to determine that PKA treatment phosphorylated 49% of ISASR. On the basis of the quantification using the mass balance equation, the limit of detection of phosphorylation was 5% for SSANYR and 25% for ISASR when defined as 3 standard deviations from the mean measurements. The poorer limit of detection for ISASR phosphorylation is a result of a low ISASR "flyability constant" that corresponds to a greatly reduced ion current and thus precision of measurement compared to the other troponin I tryptic peptides.

To asses our ability to quantify the phosphorylation of other peptides, the troponin complex was treated with PKC and the phosphorylation of troponin I peptides was quantified. These included ISADAMMQALLGAR, SSANYR, ISASR, ALSTR, ALSTR, and FKRPTLR, which have all be previously identified through studies using ^{32}P labeling and two-dimensional peptide mapping.[14] We also used SEQUEST to qualitatively identify phosphopeptides contained in the mixture based upon MS/MS spectra. Several d_0-labeled phosphopeptides were identified including the IS*ADAMMQALLAGAR, VRIS*ADAMMQALLAGAR, RRS*S*ANYR, RSS*ANYR, and FKRPT*LR peptides. The later four peptides represent phosphopeptides with one miscleavage site near the phosphorylation site. As pointed

out by Steen et al.,[8] phosphorylation may produce a greater degree of miscleavage compared to the unphosphorylated peptide. While miscleavage of troponin I was observed, our method is not dependent on the measurement of phosphorylated peptides (cleaved or miscleaved) to determine phosphorylation, and thus, the quantification of protein phosphorylation is not affected by the varied miscleavage of peptides.

In contrast, the problem of increased/altered miscleavage with phosphorylation does affect reference peptide selection. If a reference peptide is adjacent to a phosphorylated peptide where miscleavage occurs, then the amount of measured reference peptide will be diminished, and this change will lower $I_{Z(0)}$ in eq 12 and artificially decrease the degree of measured phosphorylation. This appears to be the case for peptide CQPLELAGLGFAELQDLCR. Therefore, reference peptides must not be located next to potential phosphorylation sites. We can also use the multiplex advantage of averaging I_0/I_4 ratios of several reference peptides both to help define reference peptide anomalies and to improve precision. Although the phosphorylation eq 12 uses one reference peptide, multiple peptides can be used. The precision of the reference peptide I_0/I_4 ratio measurement then improves by $n^{-1/2}$ where n multiple reference peptides are measured and their I_0/I_4 ratios averaged.

Although Zhang et al.[6] using MALDI and Hegeman et al.[7] using ESI-LC−MS published previously similar schemes to ours, there were several limitations in these methods that are improved by our current scheme. First, we incorporate a longer ester (propyl) that improves chromatography of polar peptides versus use of a methyl ester.[7] The propyl group used also contains four deuteriums to increase the isotopic cluster separation between labeled and unlabeled species versus three deuteriums with a methyl but less than the maximum of seven deuteriums that could be placed in the propyl to reduce the isotopic fraction in the LC. The four deuteriums do not significantly increase LC peak separation of the deuterated/nondeuterated peptides over what has been previously reported for the methyl derivative.[7] Prior reports[6,7] also digested protein samples with trypsin then performed the phosphatase treatment. Any differential effect of phosphorylation upon peptide cleavage will induce a distortion in the distribution of peptides at this point and, therefore, a distortion in the measured degree of phosphorylation by the method.[7] By simply splitting our sample prior to the treatment of the protein with phosphatase and prior to digestion with trypsin, we remove any effects that differential miscleavage of phosphorylation may cause upon quantification of phosphorylation. Finally, prior reports[6,7] relied upon splitting of the samples into two equal halves. We realize that equal splitting is rarely achieved and added the measurement of reference peptides that are not phosphorylated (nor adjacent to a phosphorylated peptide) to obviate the need for equal splitting. This approach also removes errors induced by differential sample losses during processing of the samples.

All of these methods, including ours, depend upon complete removal of the phosphate group in the sample treated with phosphatase. This assumption is a natural limitation of the method and must be considered and characterized when using the method. Pflieger et al.[16] recently published an approach using commercial iTRAQ reagents both to assess and to obviate the problem of less than complete dephosphorylation by the phosphatase. The iTRAQ labels are attached after digestion by trypsin and after sample splitting but before dephosphorylation by the phosphatase. The degree of dephosphorylation by the phosphatase was determined by comparing the ion intensity ratio of the two iTRAQ ions in the phosphorylated peptide, and the degree of phosphorylation was calculated from the ion intensity ratio of the iTRAQ ions in the corresponding dephosphorylated peptide.[16] Because our method performs the labeling after dephosphorylation has occurred, our method does not provide this additional measurement but assumes complete dephosphorylation. Our method could be rearranged to make the same measurements as Pflieger et al.,[16] but then we would not be able to address the problem of variable miscleavage due to phosphorylation, which is anticipated to the more variable and important problem of the two. Therefore, we would prefer to minimize variable miscleavage due to phosphorylation and be unable to directly assess the degree of phosphatase efficiency.

In summary, our derivatization strategy provides a cost-effective way to efficiently insert multiple deuterium molecules into peptides for quantitative analysis by mass spectrometry. The addition of the C-propyl handle is also useful to increase the hydrophobicity of small polar peptides and their retention on a C_{18} column. The use of the mass balance approach to quantify phosphorylation is also beneficial in circumventing problems caused by differential cleavage in the presence of phosphate. This approach can also allow for the quantification of potential phosphorylation sites but should be followed up with phosphopeptides identification routines when interpreting in vivo data.

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