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Back to deuterium: Utility of ^{2}H-labeled peptides for targeted quantitative proteomics

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Dedicated to Professor Catherine Fenselau in honor of her original contributions to quantitative biological mass spectrometry.

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ABSTRACT

Multiple reaction monitoring mass spectrometry coupled with stable isotope dilution has become the method of choice for quantifying target proteins in complex biological samples. It quantifies signature peptides based on the sequential detection of peptide precursor ions and their fragments generated upon gas-phase collision. Heavy-isotope (^{13}C and/or ^{15}N) labeled peptides or proteomes are commonly used as internal reference standards for quantitation. However, use of these standards is becoming expensive when large numbers/amounts of reference peptides are needed. The search for low-cost, labeled references for proteomic quantitation such as those with ^{2}H-labels is an object of constant pursuit. In order to take the cost advantage of ^{2}H-labels, this work examines whether or not the known chromatographic separation of ^{2}H-based peptides from the native counterparts affects the peptide quantitation using multiple reaction monitoring mass spectrometry. Experimental results from model peptides and proteome digests indicate that targeted mass spectrometry quantitation of the ^{2}H- and ^{13}C/^{15}N-based peptides are comparable.

1. Introduction

Stable isotope labeling for quantitative biological mass spectrometry (MS) dates back to the pioneering utilization of deuterium-labeled compounds for quantifying cyclophosphamide and its metabolites in human body fluids [1]. Over years techniques for stable isotope labeling have been growing together with MS, continuously improving the quantitation of proteins and peptides [2,3]. The need for quantitative proteome analysis has further triggered a surge of efforts in developing new reagents and techniques for labeling peptides and proteins to be quantified by MS-based methods [4–11]. Soon after the introduction of initial isotope coded affinity tag (ICAT) reagents [4], a data processing challenge for relative quantitation of peptides with light and heavy stable isotope labels became clear. The differentially labeled peptides with H and ^{2}H atoms separate on reversed-phase high performance liquid chromatography (HPLC), a representative phenomenon for the chromatographic isotope effect [12]. Effects of this type of chromatographic separation on peptide quantitation have been systematically studied mainly in the context of quantitative proteomic profiling that involves the global quantitative proteome analysis [13,14]. The chromatographic effect depends primarily on the location of ^{2}H-labels and secondarily on the number of the labels and the size of labeled peptides. In contrast to the separation of the H- and ^{2}H-labeled peptide pairs, it is found that ^{13}C-, ^{15}N- and ^{18}O-labeled peptides minimally separate from their light counterparts [12–14]. These studies have led to the general consensus that minimizing chromatographic separation of differentially labeled peptides is essential to MS-based quantitative proteomics [13]. This consensus has since guided the development of new stable isotope labeling techniques and the use of ^{2}H-labels has thus been purposely avoided. New stable isotope labeling reagents exclusively use ^{13}C-, ^{15}N- and ^{18}O-labels. They are represented by new ICAT reagents [8] and so-called isobaric stable isotope labeling chemicals some of which are commercialized as isobaric tag for relative and absolute quantitation (iTRAQ) [9] and tandem mass tag (TMT) [10] reagents. The isobaric labeling chemicals allow MS quantitation of differentially labeled peptides based on special fragment ions or quantitation reporter ions.

In pursuit of low-cost strategies for labeling peptide and proteins, the potential of ^{2}H-labels for proteomic quantitation has continuously been investigated [15,16] with some exciting recent developments [17,18]. In two new types of isobaric stable isotope labeling reagents, ^{2}H-labels are used to partially replace more expensive ^{13}C-, ^{15}N- and ^{18}O-labels. Both types of reagents share the same reporter ion moiety and differ in the balance and

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reactive moieties [17,18]. However, the suitability of the ^{2}H-labels in the reagents is confined by the primary factor that controls the chromatographic separation: the location of the labels. When they reside in the hydrophilic domains on labeled peptides, ^{2}H-labels cause minimal chromatographic isotope effect [14]. Therefore, in the new reagents ^{2}H-labels are located by design on a tertiary amine group; the amine group is positively charged via protonation and thus highly hydrophilic under standard reversed-phase LC conditions for peptide separation.

The concept of minimizing chromatographic separation has also influenced two other important stable isotope labeling techniques for quantitative proteomics. One technique is the metabolic method of stable isotope labeling with amino acids in cell culture (SILAC) [7] and the other is the use of synthetic protein absolute quantitation (AQUA) peptides for targeted quantitation of proteins in proteome samples [19]. Evidently, the use of ^{15}N- and ^{13}C-labels in the SILAC-based quantitative proteomics methods [7] has overwhelmed the use of ^{2}H-labels [20]. For the AQUA-based quantitative proteomic measurements, when the absolute quantities of the synthetic peptide references are known, the absolute quantities of the native peptides for determination – with the same amino acid sequences as the reference peptides in complex proteome digests can be accurately determined. The large-scale use of ^{15}N- and/or ^{13}C-labeled AQUA peptides sets the foundation for focused proteomic quantitation of protein targets, enabling advanced applications in fundamental biological investigations [19] and biomarker development [21–23]. However, the great potential of AQUA-based quantitative proteomics is hindered by the prohibitively high cost for analyses that use large numbers and/or amounts of synthetic AQUA peptides.

AQUA peptides are often used with the MS techniques of multiple reaction monitoring (MRM) or selected reaction monitoring (SRM), which quantify peptides based on the sequential detection of peptide precursor ions and their fragments generated in the gas phase. The MRM methods detect and quantify peptides in a mixture independently, regardless of the labeling status of peptides. Therefore, we hypothesize that even though the ^{2}H-labeling of peptides could result in chromatographic separation, it would present minimal difficulty for MRM-MS quantitation of peptides. In fact, MRM-MS and deuterium-based standards have long been used in combination for quantifying small molecules in complex matrices. Herein, we use synthetic peptides and SILAC proteomes to examine the utility of ^{2}H-labeled peptides as quantitation standards for targeted proteomic quantitation.

2. Experimental

2.1. Materials

Peptide CFTR01 (sequence: NSILTETLHR), stable isotope labeled CFTR01-C13N15 (sequence: NSILTET[Leu-$^{13}C_6$,^{15}N]HR) and peptide CFTR02 (sequence: LSLVDSEQGEAILPR) were purchased from AnaSpec (San Jose, CA). Other stable isotope labeled peptides were named as CFTR02-H2 (LSLVDSEQGEAILPR with Leu-$^{2}H_{10}$) and CFTR02-C13 (LSLVDSEQGEAILPR with Leu-1,2-$^{13}C_2$). Stable isotope labeled amino acid leucine (L-Leu-$^{2}H_{10}$, 98% atom enrichment or L-Leu-1,2-$^{13}C_2$, 99% atom enrichment) and ethanol-$^{2}H_6$ were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents, L-leucine, L-lysine, L-arginine, methotrexate, anhydrous ethanol, chlorotrimethylsilane (ClTMS), iodoacetamide, protease inhibitor cocktail, sodium orthovanadate, trifluoroacetic acid, Tris–HCl 1 M solution, and phosphate buffer saline (PBS, 10× pH 7.4) were purchased from Sigma–Aldrich (St. Louis, MO). Formic acid (FA) was purchased from Fluka (Milwaukee, WI). Acetonitrile (ACN), acetic acid, dithioerythritol (DTE), and 2-mercaptoethanol (2-ME) were purchased from Fisher (Pittsburgh, PA). Trypsin (Roche Applied Sciences, Indianapolis, IN) was the sequence grade. DME/Low media that were deficient in L-arginine, L-leucine and L-lysine and fetal bovine serum were purchased from Thermo Scientific HyClone (Logan, UT). Dialyzed fetal bovine serum was purchased from Invitrogen (Carlsbad, CA). Deionized water was purified by a Direct-Q water purifying system (Millipore, Billerica, MA). Samples were dried either in a SpeedVac (Savant, Farmingdale, NY) or a lyophilyzer (Labconco, Kansas City, MO).

2.2. Carboxylate derivatization of CFTR01 peptides and LC–MRM-MS analysis of derivatized peptides

Total of nine aliquots were made, each containing 100 pmol of CFTR01 peptide and 100 pmol stable isotope labeled CFTR01-C13N15 peptide. Three aliquots containing the peptide mixture were dissolved in 200 μL of ethanolic HCl solution, which was prepared by mixing 432 μL of ethanol or ethanol-$^{2}H_6$ and 200 μL of ClTMS. Esterifications were allowed at 30 °C for 1 h. The reaction mixtures were dried in a SpeedVac, and the same esterification procedure was repeated. Following the second reaction, three extra additions of 200 μL ACN and SpeedVac drying were performed. The dried derivatization mixtures were dissolved with 20 μL of 30% (v/v) FA solution and further diluted with 80 μL 0.1%(v/v) TFA.

LC separation of peptides was run at a flow rate of 50 μL/min with a binary gradient: 1% B at 0 min → 5% B at 5 min → 35% B at 50 min → 80% B at 59 min → 90% B at 69 min → 1% B at 80 min, with 5 min equilibration. Exceptions were in the chromatographic resolution experiments, where different separation gradients were employed. In those cases, the ending Solvent B composition for the third separation step was changed to 30%, 40%, 45% or 50% Solvent B. Solvent A was 99% (v/v) of 0.1% (v/v) FA and 1% of ACN and Solvent B was 99% (v/v) ACN and 1% (v/v) of 0.1% (v/v) FA. Column temperature was set at 40 °C. The mass spectrometer used was a 4000 QTrap mass spectrometer (AB Sciex, Foster City, CA). Key instrument parameters were: IS 4300V, GS1 20, GS2 40, EP 10V, and CXP 30V. Transitions and optimized DP and CE values for the native and derivatized peptides were summarized in Table 1. Dwell time for each transition was 50 ms.

2.3. Cell culture and SILAC labeling

Baby Hamster Kidney (BHK) cells over-expressing wild-type cystic fibrosis transmembrane conductance regulator (CFTR) were obtained as a gift from Dr. J.R. Riordan at the University of North Carolina. The cells were grown in DME/Low deficient in L-lysine, L-leucine and L-arginine. Culture media were supplemented with 55 mM methotrexate, 0.40 mM L-arginine·HCl, 0.80 mM L-lysine·HCl, 0.80 mM L-leucine, and 10% fetal bovine serum. Stable isotope labeled cells were grown in similar conditions, except that dialyzed fetal bovine serum was used and L-leucine was substituted with stable isotope labeled L-Leu-$^{2}H_{10}$ (98%) or L-Leu-1,2-$^{13}C_2$ (99%). Cells grown in these labeled media were allowed for at least eight doublings prior to harvesting.

2.4. Cell lysis and SDS-PAGE

After washing cells with 1× PBS, lysis buffer (800 μL) containing 1% (v/v) Triton X-100, 150 mM NaCl, 20 mM Tris–HCl at pH 7.2 and 1% (v/v) protease inhibitors was used for lysis of about 12–14 millions of BHK wtCFTR cells, grown in normal or SILAC media. Protein extracts mixed at different volume ratios were subjected to short SDS-PAGE separation. Protein extracts were stacked by 3% (v/v) and separated by 10% (v/v) acrylamide gel. SDS-PAGE separation was run at constant 200 V for about 30 min. Gel slides were cut

Table 1
Transitions monitored for unesterified and esterified native CFTR01 and labeled CFTR01-C13N15.

Peptides	CFTR01		CFTR01-C13N15		CE (V)	DP (V)
	$[M+2H]^{2+}$	y_7	$[M+2H]^{2+}$	y_7		
CFTR 01	592.3	869.5	595.8	876.5	30.0	96.0
CFTR 01_OC_2H_5	620.4	925.6	623.9	932.6	31.5	97.5
CFTR 01_$OC_2{}^2H_5$	625.4	935.6	628.9	942.6	31.5	97.5

near the interface of stacking and separating gels and further sliced into small pieces (≈1 mm^3) for further sample preparation.

2.5. In-gel digestion

Gel pieces were washed with 200 μL of a mixture of 25 mM ammonium bicarbonate and ACN at a 1:1 (v/v) ratio for 30 min, and the washing step was repeated for a total of three times. This was followed by addition of 200 μL of ACN to completely shrink the gel pieces. Gel pieces were rehydrated with 400 μL of 10 mM solution of DTE, and incubated at 37 °C for 15 min. Washing of the gel pieces with 25 mM ammonium bicarbonate was followed by protein alkylation in 20 mM iodoacetamide solution at 37 °C for 30 min in dark. Gel pieces were washed three times with 200 μL of 25 mM ammonium bicarbonate solution before they were shrunk completely with 600 μL of ACN. Rehydration of gel pieces in 100 μL of 25 mM ammonium bicarbonate solution containing trypsin at 20 ng/μL was performed in ice for 1 h. The unabsorbed trypsin solution was removed, and 300 μL of 25 mM ammonium bicarbonate solution was added, followed by 16 h incubation at 37 °C. The resulting digestion solution was saved, and peptides were extracted twice with 200 μL (v/v) of 30% ACN/5% FA solution for 30 min each. Extraction and digestion solutions were combined and freezedried overnight. Peptides were dissolved in 20 μL of 30% formic acid solution and further diluted with 80 μL of 0.1% (v/v) TFA solution.

LC–MRM-MS analysis was done as mentioned above. Transitions for doubly charged ions to their y_{12} ions were monitored for peptide CFTR02, CFTR02-H2 and CFTR02-C13. Transitions included 862.5 → 1311.7 for native CFTR02, 865.5 → 1313.7 for CFTR02-C13 and 877.5 → 1321.7 for CFTR02-H2. DP and CE values were optimized accordingly. Quadrupole resolutions were set at unit resolution for both Q1 and Q3. Dwell time for each transition was 50 ms.

3. Results and discussion

3.1. Use of ^{2}H-based peptides for targeted mass spectrometry quantitation

Based on pre-determined gas-phase transitions, MRM-MS quantifies target peptides of known sequences in a highly specific and sensitive manner. It is important to appreciate that the MRM method measures peptides individually, regardless of sequences or stable isotope labeling status (i.e., the labeling status of a native target peptide or that of the added stable isotope peptide standard). Therefore, in principle the chromatographic separation of peptides of interest and their labeled reference counterparts adds minimal complications for data acquisition and analysis. In comparison, other MS methods used in quantitative proteomics simultaneously monitor a range of ions. Thus, it is critical for the non-MRM-based quantitation methods that peptides with differential stable isotopes co-elute [12–14], due to the massive sample complexity of proteome digests. The data acquisition and processing complications associated with the chromatographic separations of non-labeled and labeled peptides have been recognized early on during the development of quantitative proteomics technologies.

The cost advantage of the ^{2}H-based stable isotope labeling is commonly accepted. A semi-quantitative analysis, however, was conducted to compare the cost difference between the ^{2}H- and ^{13}C/^{15}N-based amino acids, the compounds for chemical peptide synthesis and metabolic labeling of proteins (Table 2). Prices of chemicals were obtained from the website of Cambridge Stable Isotope Laboratories in January 2011. The stable isotope compounds used in experiments of this work were purchased from the company. A price list for all of the available heavy amino acids with isotopes at different positions and of varying numbers was prepared for the cost comparison (Supplemental Information). The list was first sorted based on the amino acid type, and each type of amino acids was split into two subgroups: one with ^{2}H-labels and the other with ^{13}C- and/or ^{15}N-labels (Supplemental Information). Cost for stable isotope labeling was then calculated for one mass unit equivalence (i.e., how much it costs to increase mass by 1 a.m.u.). The calculated price was further normalized to the cost for the amino acid of the same type, but with a single ^{13}C-label (or ^{15}N-label, if a single ^{13}C-labeled amino acid was not available) and at the reagent package amount of 1 g. Finally, the average normalized cost for labeling at one unit mass equivalence was calculated for both of the subgroups for all available heavy amino acids and the ratios for the average normalized prices were calculated. These price ratios were used as the relative measures of the cost for the two subgroups of stable isotope labeling (Table 2). Significant differences between the cost for ^{2}H-labeling and that for ^{13}C/^{15}N-labeling were observed as expected. The ^{2}H-labeling was cheaper than the ^{13}C/^{15}N-labeling with cost differences ranging from 2- to 31-fold (Table 2), indicating that the ^{2}H-labeled peptides and proteins can be made at lower cost than the ^{13}C/^{15}N-labeled ones. The cost advantage for the ^{2}H-labeled peptide and protein standards becomes significant for large-scale, targeted quantitative investigations of proteome changes. These studies include focused analysis that follows changes of the involved proteins in a particular pathway and biomarker validation that requires quantitation of selected protein markers in large numbers of samples. In these large-scale studies, the use of synthetic ^{13}C/^{15}N-based peptide standards can be cost-prohibitive.

3.2. Effects of ^{2}H-labels on MRM-based peptide quantitation

A model system was designed using chemically-derivatized peptides. A synthetic peptide CFTR01 with a sequence of NSILTETLHR was used. This peptide has two carboxylate groups, one on the glutamic acid side chain and the other at the C-terminus.

Table 2
Price comparison of ^{13}C/^{15}N- to ^{2}H-based amino acids.

Amino acid	Normalized price ratio	Amino acid	Normalized price ratio
Glycine	21	L-Methionine	31
L-Alanine	10	L-Phenylalanine	13
L-Aspartic acid	2	L-Proline	2
L-Glutamic acid	2	L-Serine	5
L-Isoleucine	2	L-Tryptophan	9
L-Leucine	6	L-Tyrosine	9
L-Lysine	8	L-Valine	7

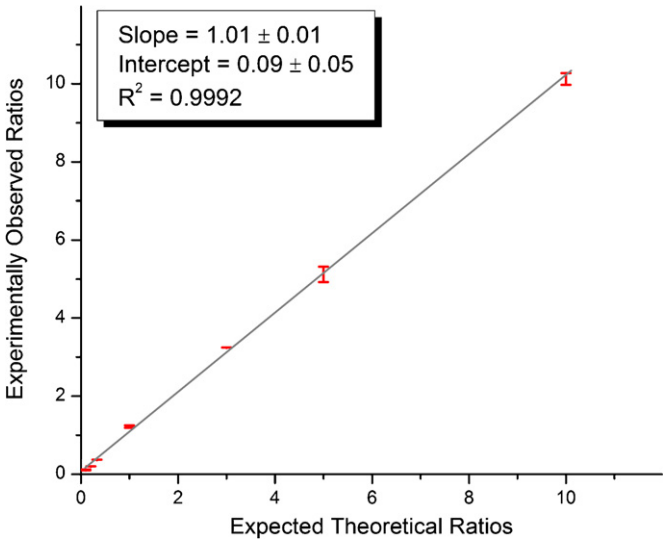

Fig. 1. Effect of ^{2}H-labeling on MRM-based peptide quantitation.

The peptidyl carboxylate groups were esterified using ethanol-$^{2}H_6$·HCl or ethanol·HCl. When the peptide was fully derivatized, the two esterifications of CFTR01 produced two model peptides with a stable isotope difference of ten ^{2}H-labels. Following the general sample preparation workflow for a method of ultrathroughput multiple reaction monitoring (UMRM) MS [24], experimental variations attributed to the esterification were controlled by the use of a master 1:1 mixture of peptides CFTR01 and CFTR01-C13N15. CFTR01-C13N15 was labeled with Leu-$^{13}C_6$,^{15}N at the second leucine residue and thus 7 Da higher in mass than the native CFTR01. Aliquots of the master mixture were used for the esterifications with ethanol-$^{2}H_6$·HCl or ethanol·HCl. The derivatizations were allowed to reach the completion; therefore, primary and secondary kinetic isotope effects on the esterification can be ignored. Both the labeled and unlabeled peptides in the mixture concurrently underwent the same derivatization reaction (and side-reactions). Thus the quantity ratio of CFTR01 and CFTR01-C13N15 should remain the same before and after the derivatization and variations observed in the ratio quantitation for CFTR01 and CFTR01-C13N15 peptides in the different esterification mixtures should only attribute to variations caused by MS measurements and/or potential chromatographic isotope effects [24].

Two sets of experiments were performed to compare the quantitation accuracy and precision for native peptides and peptides with ^{2}H-labels. The first experiment was the quantitation of mixtures of the esterification products of the master 1:1 mixture, using ethanol or ethanol-$^{2}H_6$. The mixtures were prepared at a series of ratios: 10:1, 5:1, 3:1, 1:1, 1:3, 1:5, and 1:10. The observed ratios were plotted against the theoretical ratios and a linear regression analysis using the Origin 8 software (OriginLab, Co., Northampton, MA) was conducted, giving a slope of 1.01 ± 0.01, an intercept of 0.09 ± 0.05, and R^2 of 0.9992 (Fig. 1). The unit slope demonstrates that ^{2}H-labels cause no bias over H-labels for peptide quantitation using MRM methods.

A second set of experiments encompassed the triplicate derivatization of aliquots of the 1:1 master mixture using ethanol or ethanol-$^{2}H_6$. The ethanol and ethanol-$^{2}H_6$ derivatization products were mixed at a ratio of 1:1 to prepare three samples (Table 3). Each mixture was run in triplicate LC–MRM-MS measurements. Because both the native CFTR01 and the labeled CFTR01-C13N15 were esterified concurrently, the esterification should not cause differential variations in the quantitation of the original ratio of CFTR01 and CFTR01-C13N15. The ethanol or ethanol-$^{2}H_6$ derivatizations of the master mixture gave comparable results and the peptides with ^{2}H-labels gave an average ratio of 0.95 and a CV of 6.5%, compared to the control peptides esterified with ethanol that gave an average ratio of 0.94 and CV of 8.4% (Table 3). Both data sets were indistinguishable with the measurements for the master mixture that gave an experimental CFTR01 to CFTR01-C13N15 ratio of 0.95 with a CV of 2.0%. Therefore, ^{2}H-labels causes no compromise in the quantitation accuracy for the MRM-based analysis.

Table 3
Effect of ^{2}H-labels on quantitation accuracy and precision.

	Run #	Area ratio (CFTR01/CFTR01-C13N15)	
		OC_2H_5	$OC_2{}^{2}H_5$
Sample #1	Run #1	1.01	0.93
	Run #2	1.07	0.95
	Run #3	1.02	0.93
	Average	1.03	0.94
	CV (%)	3.2	1.4
Sample #2	Run #1	0.93	0.82
	Run #2	0.84	0.95
	Run #3	0.92	0.98
	Average	0.90	0.92
	CV (%)	5.8	9.4
Sample #3	Run #1	0.83	0.93
	Run #2	0.95	0.99
	Run #3	0.92	1.05
	Average	0.90	0.99
	CV (%)	6.7	5.9
Total average		0.94	0.95
Total CV (%)		8.4	6.5

3.3. Chromatographic isotope effect on LC–MRM-MS quantitation

Four different HPLC gradient methods were set to observe the differential migration of ^{2}H-based peptides and their native counterparts on the reversed-phase stationary phase, a phenomenon that is particularly significant when ^{2}H-labels reside at nonhydrophilic moieties of labeled peptides [12–14]. The sample used was a 1:1 mixture of the ethanol and ethanol-$^{2}H_6$ esterification products of the master mixture. Thus this sample contained four different peptides (Fig. 2): the ethanol esterified CFTR01, CFTR01-$(OC_2H_5)_2$; the ethanol esterified CFTR01-C13N15, CFTR01-C13N15-$(OC_2H_5)_2$; the ethanol-$^{2}H_6$ esterified CFTR01, CFTR01-$(OC_2{}^{2}H_5)_2$; the ethanol-$^{2}H_6$ esterified CFTR01-C13N15, CFTR01-C13N15-$(OC_2{}^{2}H_5)_2$. Peptides with ^{2}H-labels [CFTR01-$(OC_2{}^{2}H_5)_2$ and CFTR01-C13N15-$(OC_2{}^{2}H_5)_2$] eluted earlier than the counterpart peptides without the ^{2}H-labels [CFTR01-$(OC_2H_5)_2$ and CFTR01-C13N15-$(OC_2H_5)_2$] (Fig. 2). In comparison, $^{13}C/^{15}N$-based peptides coeluted with their counterparts; CFTR01-$(OC_2H_5)_2$ and CFTR01-C13N15-$(OC_2H_5)_2$ eluted together and so did CFTR01-$(OC_2{}^{2}H_5)_2$ and CFTR01-C13N15-$(OC_2{}^{2}H_5)_2$ (Fig. 2). In order to quantitatively evaluate the peak resolution for the peptides, the full width at half maximum (FWHM) values for chromatographic peaks were measured. Because all peaks had similar peak heights and peak symmetry (Fig. 2), peak resolutions for the ^{2}H-based and H-based peptides (Table 4) were calculated according to the equation, $R_s = \Delta t_R / w_b$, as approximation. Δt_R was the elution time difference between the ^{2}H-based and ^{1}H-based peptides and w_b was the average peak base width for the ^{2}H-based and ^{1}H-based peptides. Peak base widths were calculated based on the FWHM values assuming the peaks were Gaussian.

The differentially-esterified peptides were separated with resolutions of 0.40(±0.06) for the ending solvent composition of 30% Solvent B in the third separation step of a gradient method (see Section 2), 0.24(±0.01) for 40%, 0.21(±0.07) for 45%, and 0.24(±0.01) for 50%, respectively. This was expected because when fully ester-

Table 4
Chromatographic isotope effect on peptide quantitation.

Gradient Ending			Area ratio		Elution time (min)		FWHM (min)		Resolution
			OC_2H_5	$OC_2{}^2H_5$	OC_2H_5	$OC_2{}^2H_5$	OC_2H_5	$OC_2{}^2H_5$	
30%	Run #1	CFTR01	0.97	0.97	44.79	44.57	0.27	0.27	0.48
		CFTR01-C13N15			44.78	44.55	0.29	0.29	0.47
	Run #2	CFTR01	0.96	0.99	43.5	43.33	0.28	0.27	0.36
		CFTR01-C13N15			43.49	43.33	0.27	0.27	0.35
	Run #3	CFTR01	0.95	0.95	42.69	42.52	0.27	0.26	0.38
		CFTR01-C13N15			42.68	42.5	0.27	0.27	0.39
40%	Run #1	CFTR01	0.93	0.97	35.43	35.34	0.23	0.22	0.24
		CFTR01-C13N15			35.42	35.33	0.23	0.23	0.23
	Run #2	CFTR01	0.92	0.97	35.51	35.41	0.24	0.24	0.25
		CFTR01-C13N15			35.51	35.41	0.24	0.24	0.25
	Run #3	CFTR01	0.96	0.95	35.53	35.43	0.24	0.25	0.24
		CFTR01-C13N15			35.52	35.42	0.24	0.25	0.24
45%	Run #1	CFTR01	0.95	0.93	34.00	33.93	0.24	0.23	0.18
		CFTR01-C13N15			34.00	33.92	0.24	0.25	0.19
	Run #2	CFTR01	0.95	0.94	34.41	34.35	0.24	0.23	0.15
		CFTR01-C13N15			34.41	34.35	0.24	0.24	0.15
	Run #3	CFTR01	0.93	0.94	33.64	33.52	0.23	0.24	0.30
		CFTR01-C13N15			33.63	33.52	0.23	0.23	0.28
50%	Run #1	CFTR01	0.97	0.96	31.59	31.48	0.27	0.25	0.25
		CFTR01-C13N15			31.58	31.47	0.26	0.26	0.25
	Run #2	CFTR01	0.93	0.94	31.62	31.51	0.25	0.26	0.25
		CFTR01-C13N15			31.62	31.52	0.26	0.26	0.23
	Run #3	CFTR01	N/D	N/D	N/D	N/D	N/D	N/D	N/D
		CFTR01-C13N15			N/D	N/D	N/D	N/D	N/D

ified, the derivatized peptides carry ten ^{2}H-labels. The average resolution values and the associated standard deviations were calculated for CFTR01 and CFTR01-C13N15 peptides as a group, because the esterification products of both peptides were not resolved when comparing peptides esterified with the same type of ethanol reagent (Table 4). Therefore, the steeper the gradient was the less the separation between ^{2}H-based peptides and the ^{1}H-based counterparts. It has been reported that when a separation gradient is fast and steep enough, no distinguishable separation can be detected between a native peptide and the added internal standard peptide with six ^{2}H atoms on two alanine residues [25].

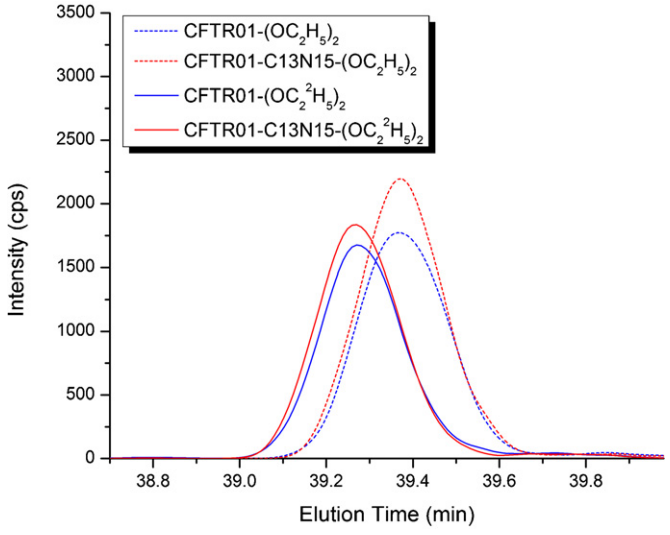

Fig. 2. Chromatographic isotopic effects of peptides. The sample was a 1:1 mixture of the ethanol and ethanol-$^2H_6$ esterification products of a master mixture (at a measured ratio of 0.95 ± 0.02) of CFTR01 and CFTR-C13N15 that carried six ^{13}C-labels and one ^{15}N-label. Blue dotted line for the ethanol esterified CFTR01, CFTR01-$(OC_2H_5)_2$; red dotted line for the ethanol esterified CFTR01-C13N15, CFTR01-C13N15-$(OC_2H_5)_2$; blue solid line for the ethanol-$^2H_6$ esterified CFTR01, CFTR01-$(OC_2{}^2H_5)_2$; red solid line for the ethanol-$^2H_6$ esterified CFTR01-C13N15, CFTR01-C13N15-$(OC_2{}^2H_5)_2$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The MRM-MS quantitation of ethanol-esterified peptides gave an average ratio of 0.95(±0.01) for CFTR01 to CFTR01-C13N15 in the master mixture and the ethanol-$^2H_6$-esterified peptides gave a ratio of 0.96(±0.02) (Table 4). Therefore, although the chromatographic isotope effect was significant under the experimental conditions, the large chromatographic separations did not affect the quantitation of the peptides in comparison and the results for the ethanol and ethanol-$^2H_6$ products were not distinguishable. The determined ratios for CFTR01 to CFTR01-C13N15 in the master mixture were highly consistent to the original ratio of 0.95(±0.02).

3.4. *Quantitation comparison of membrane protein CFTR in ^{13}C- and ^{2}H-based proteomes*

The utility of ^{2}H-labeled peptides as internal standards for targeted quantitative proteomics was further studied using transfected BHK cells that express CFTR. Accurate quantitation of the protein is critical for examination of potential drugs for treating cystic fibrosis [26]. In this work, stable isotope-labeled proteome samples were prepared by culturing cells in heavy amino acid-containing media with Leu-1,2-$^{13}C_2$ or Leu-$^2H_{10}$, respectively. The absolute quantities of the CFTR protein in individual, differentially-labeled cell samples were not known. Cells prepared with native amino acids-containing medium were used as the quantitation normalization standard. Membrane preparations of these cells were mixed at different volume ratios: 5:1:1 (native to the ^{13}C to the ^{2}H), 1:1:1, 1:3:3, and 1:5:5. The mixed membrane samples were subjected to run a short SDS-PAGE separation to enrich CFTR protein near the interface of the stacking and separating gels. The in-gel digestion of the CFTR-enriched samples produced peptide mixtures that contained CFTR peptides.

A signature peptide CFTR02 of the protein, which produced a stronger signal than CFTR01, was used for quantitation comparison. CFTR02 in each resulting peptide mixture was quantified based on triplicate LC–MRM-MS experiments. The coefficients of variance ranged from 0.6% to 4.4% (average CV = 2.2%) for the quantitation

Table 5
Chromatographic separation of CFTR02 peptides.

Volume ratio	Sample #	FWHM (min)			Elution time change (min)		Peak resolution	
		CFTR02	CFTR02-C13	CFTR02-H2	CFTR02-C13	CFTR02-H2	CFTR02-C13	CFTR02-H2
5:1:1	1	0.29	0.29	0.31	-0.01	0.20	-0.02	0.39
	2	0.32	0.31	0.32	0.02	0.31	0.04	0.58
	3	0.31	0.32	0.32	-0.02	0.27	-0.04	0.50
1:1:1	1	0.31	0.33	0.31	0.00	0.35	0.00	0.64
	2	0.30	0.32	0.32	0.00	0.34	0.00	0.63
	3	0.31	0.30	0.31	0.02	0.24	0.04	0.46
1:3:3	1	0.31	0.31	0.32	0.02	0.38	0.04	0.71
	2	0.32	0.32	0.33	0.02	0.35	0.04	0.63
	3	0.31	0.31	0.32	0.01	0.29	0.02	0.54
1:5:5	1	0.34	0.33	0.33	0.00	0.21	0.00	0.37
	2	N/D	N/D	N/D	N/D	N/D	N/D	N/D
	3	0.31	0.31	0.32	0.00	0.26	0.00	0.49
Average		0.31	0.31	0.32	0.01	0.29	0.01	0.54
StDev		0.01	0.01	0.01	0.01	0.06	0.03	0.11

of labeled CFTR02 peptides in individual samples, relative to the native CFTR02 peptide as the quantitation reference. The ratios for the ^{2}H-based CFTR02-H2 peptide to the native CFTR02 were plotted against the ratios for the ^{13}C-based CFTR02-C13 to the native one (Fig. 3). A linear correlation was found with a R^2 value of 0.9999 indicating the comparable quantitation precisions for the ^{2}H- and ^{13}C-based peptides. The linear regression had a slope of 1.27 ± 0.01, giving that the CFTR concentration in cells cultured in the ^{13}C-containing media was 1.27-fold higher than that in cells cultured in ^{2}H-containing media. The minimum intercept (0.05 ± 0.06) for the linear regression suggested the comparable background signals (virtually zero in this experiment) that were experienced by the partially-resolved chromatographic peaks for the ^{2}H- and ^{13}C-labeled CFTR02 peptides. Therefore, quantitation performance for the two types of labeled peptides was comparable (also see Fig. S1 in Supplemental Materials), regardless of the very large chromatographic separation ($R_s = 0.54 \pm 0.11$, Table 5) of CFTR02-H2 from CFTR092 and CFTR02-C13. A caveat should be noted, however, that the sample matrix may affect the ionization efficiency of native and labeled peptides differently, if they are separated, and the possibility of such differential effect should increase with the sample complexity. It has yet to be experimentally determined if the ^{2}H-labeled peptides are appropriate for targeted quantitative proteomics of complex samples like undepleted human plasma digests.

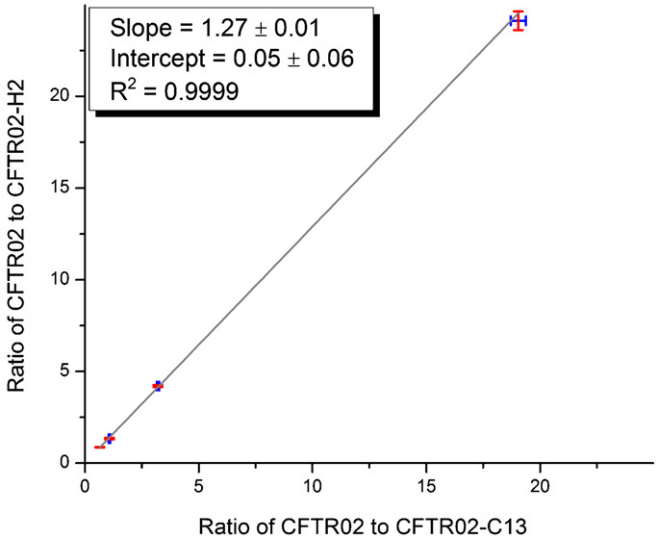

Fig. 3. Quantitation comparison of ^{2}H- and ^{13}C-based peptides.

4. Conclusion

The cost benefit for ^{2}H-labeled peptides for targeted quantitative proteomics is two-fold. Firstly, ^{2}H-based amino acids used for producing labeled peptides either chemically or metabolically are less expensive than ^{13}C/^{15}N-based ones. Secondly, the applicability of labeled peptides with large numbers of ^{2}H-labels, e.g., thirty as demonstrated in this work, reduces the isotopic purity requirement (and thus cost) of ^{2}H-based amino acids. This is because the native and labeled peptide pairs are separated by large mass differences. The reduced cost for producing stable isotope labeled peptides as quantitation references makes it practically attractive for proteomic quantitation that requires the use of large amounts of one or several labeled reference peptides or the use of large numbers of reference peptides with different sequences. Focused proteomic quantitation of proteins involved in particular pathway or network opens great opportunities for in-depth or hypothesis-driven biological investigations using LC–MRM-MS. Considering that significant numbers of proteins are involved and multiple signature peptides are preferred for quantifying a specific protein, ^{2}H-based quantitation references allow for exploring these opportunities at the more affordable cost. Another major application for the cost-effective ^{2}H-based quantitation references is in the development and validation of biomarkers, during which either large numbers of labeled reference peptides are needed at the early stages of a biomarker development pipeline or a small number but a large amount of labeled peptides are required for validating biomarkers in many patient and control samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijms.2011.05.006.

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