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Minimizing Resolution of Isotopically Coded Peptides in Comparative Proteomics

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Stable isotopes are now widely used to quantify concentration changes in proteomics. This paper focuses on the resolution of isotopically coded peptides and how isotope effects occurring during chromatographic separations can be minimized. Heavy isotope derivatizing agents used in this work were the commercially available $^{2}H_{8}$-ICAT reagent and $^{13}C_{4}$-succinic anhydride. The ICAT reagent derivatizes cysteine-containing peptides, whereas the succinic anhydride reacts with primary amine groups in peptides. It was observed during reversed-phase chromatography of peptides from a BSA tryptic digest differentially labeled with the $^{2}H_{0}$- and $^{2}H_{8}$-ICAT reagents that resolution of the isoforms exceeded 0.5 with 20% of the peptides in the digest. Three-fourths of the peptides in this group contained two cysteine residues and were doubly labeled. Only 23% of the peptides labeled with a single ICAT residue had a resolution greater than 0.4. The resolution of peptides differentially labeled with ^{13}C- and ^{12}C-succinate never exceeded ± 0.01, even in the case of peptides from the BSA digest labeled with 2 mol of succinate. Because this value is within the limits of the method used to determine resolution, it was concluded the ^{13}C- and ^{12}C-coded isoforms of labeled peptides did not resolve. The isotope ratio in the case of $^{13}C/^{12}C$ coding could be determined from a single mass spectrum taken at any point in the elution profile. This enabled isotope ratio analysis to be completed early in the elution of a peptide from chromatography columns.

Keywords: comparative proteomics • quantification • GIST • ICAT • isotope effects • isotope ratio • labeling • signature peptides • mass spectrometry

Introduction

Two approaches to proteomics are being pursued today. One focuses on cataloging all the proteins in a biological system, the cellular components with which each of these proteins interact, the pathways of which they are a part, and the location in which they reside. The other strategy is based on comparison. Disease and a wide range of stimuli cause biological systems to pass into a new, chemically distinct state distinguished by changes in the occurrence and amount of specific proteins. Comparing samples taken from organisms in the normal and an altered state can be used to recognize proteins involved in the transition. The means by which components that vary are recognized in this comparative proteomics is the subject of this paper.

Stable isotope coding strategies are of great value for distinguishing changes in comparative proteomics.[1−11] These coding techniques may be broadly classified as internal standard methods in which components from control samples are derivatized with an isotopically distinct labeling agent, and after mixing with experimental samples, they are used as standards for determining the relative concentration of components in experimental samples derivatized with a different isoform of the labeling agent. Most of the coding agents used today are labeled with deuterium, and relative concentration measurements are based on isotope ratio determinations with either matrix-assisted laser desorption ionization−mass spectrometry (MALDI−MS) or electrospray ionization−mass spectrometry (ESI−MS). As with all internal standard methods, it is important that the behavior of analytes and standards be as nearly alike as possible before the final step of isotope ratio measurement.

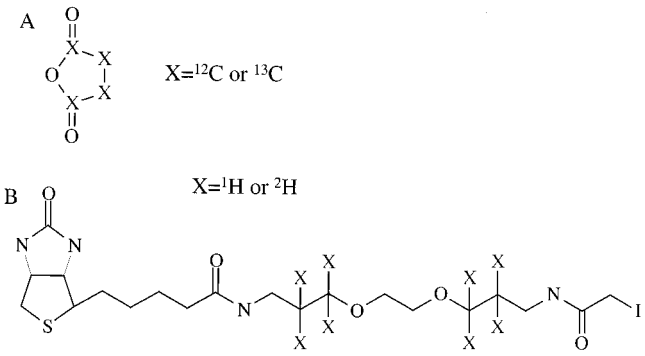

Figure 1. Structures of derivatization agents: (A) succinic anhydride-$^{13}C_{0}$ and -$^{13}C_{4}$; (B) ICAT-$^{2}H_{0}$ and -$^{2}H_{8}$.

The problem with current stable isotope coding methods for proteomics is that as the number of deuterium atoms is increased to enlarge the mass difference between isotopically

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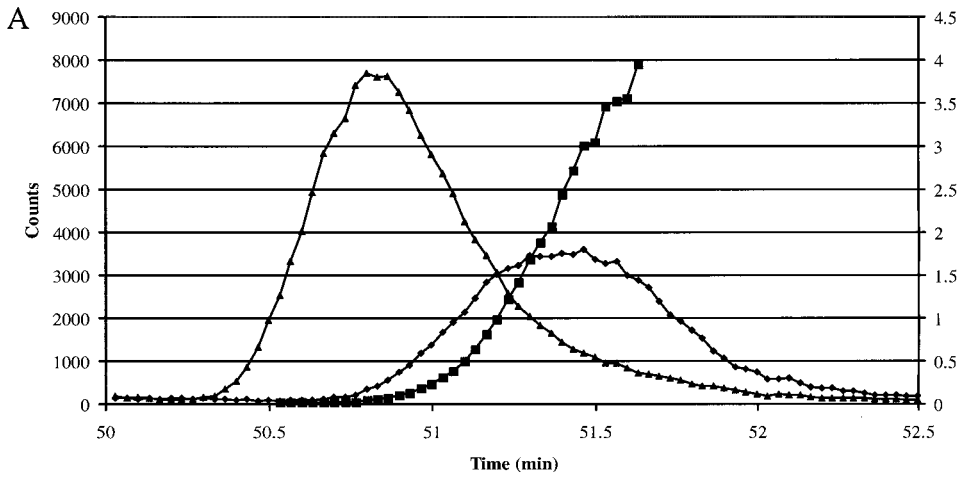
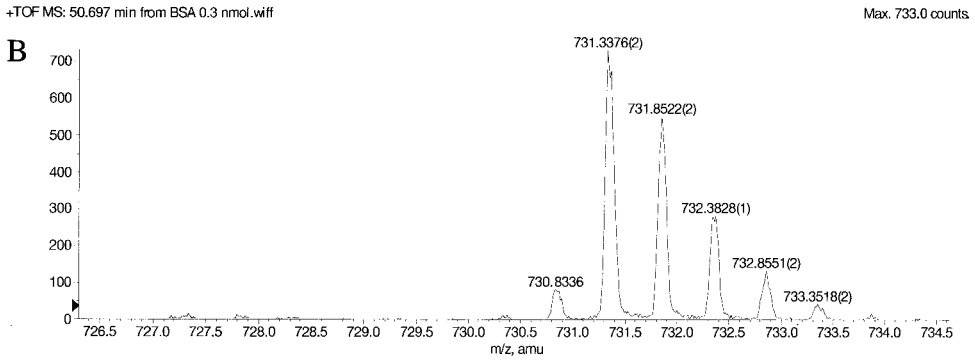

A
Counts
Time (min)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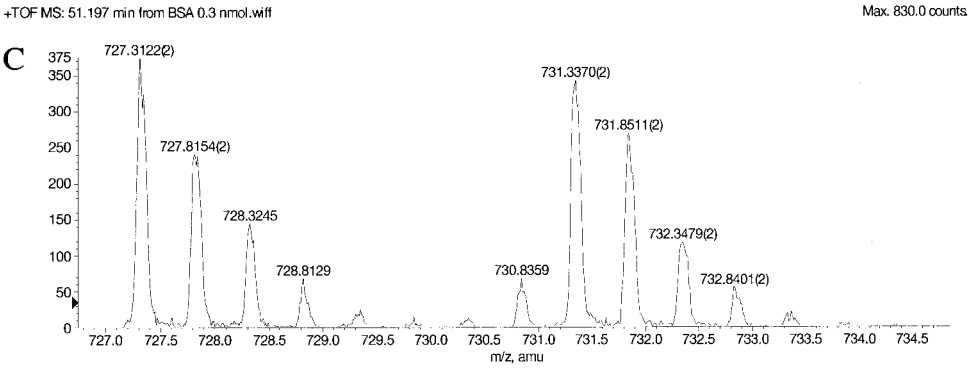
m/z, amu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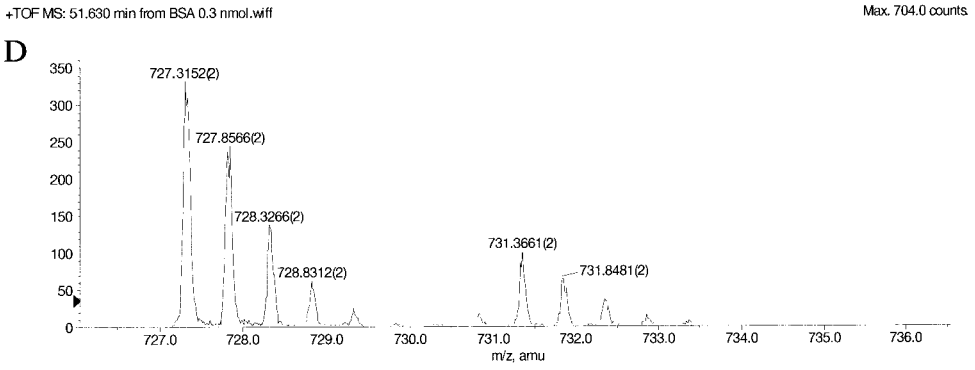
m/z, amu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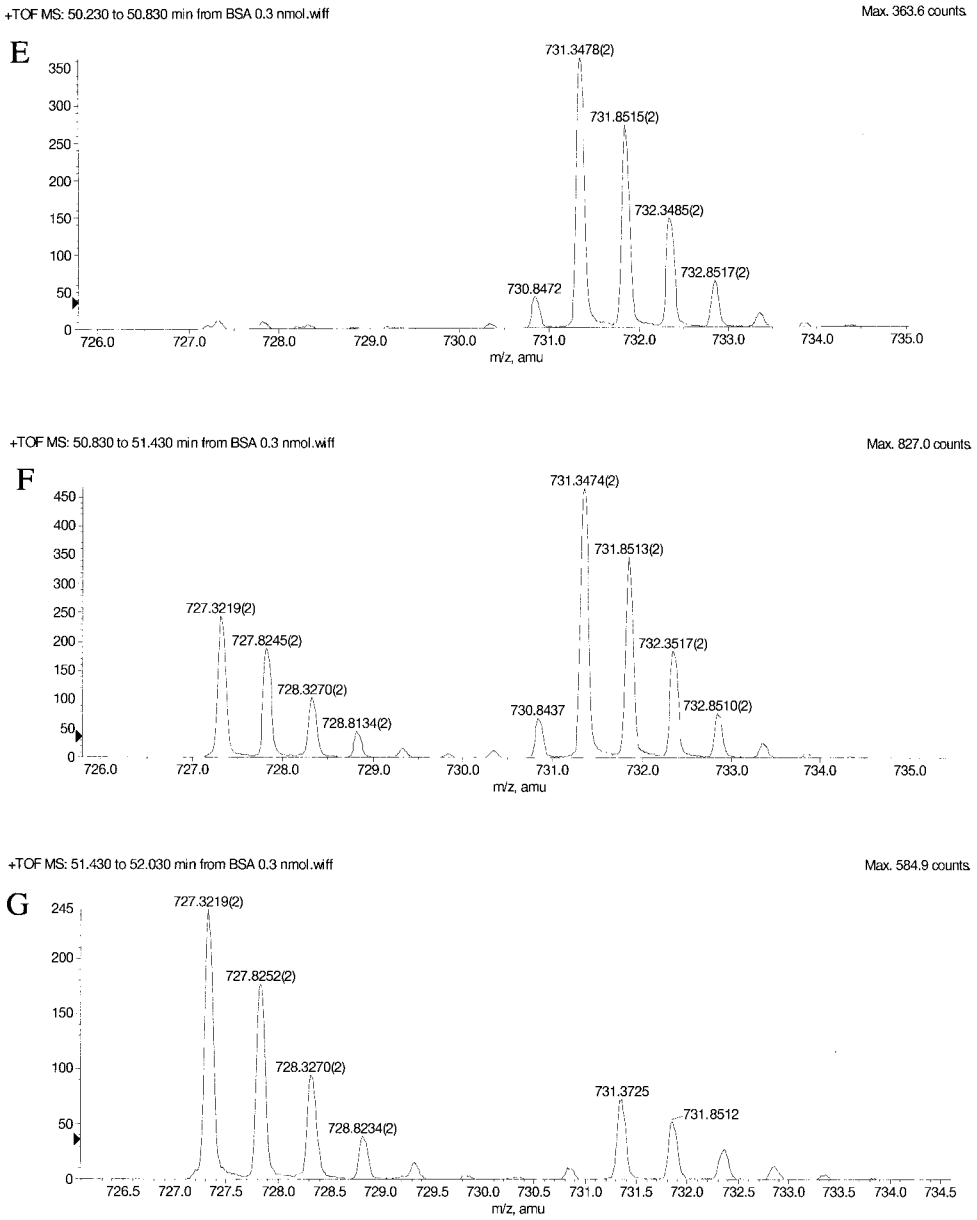

Figure 2. Fractionation of a pair of ICAT-$^2H_0$- and -$^2H_8$-labeled peptides in reversed-phase chromatography. The peptide sequence is QN**C**DQFEK. Eight deuteriums were incorporated into the deuterated peptide. Resolution equals 0.74. Key: (A) extracted ion chromatogram of the deuterated peptide (▲), nondeuterated peptide (◆), and ratio between them (■); (B) mass spectrum at 50.7 min; (C) mass spectrum at 51.2 min; (D) mass spectrum at 51.6 min; (E) mass spectrum of fraction collected at 50.2−50.8 min; (F) mass spectrum of fraction collected at 50.8−51.4 min; (G) mass spectrum of fraction collected at 51.4−52.0 min.

coded standards and analytes, there is a corresponding increase in chromatographic resolution of the isotopic isoforms, particularly in the case of reversed-phase chromatography.[12] When this isotope effect occurs, the concentration ratio of isoforms varies continuously across the elution profile of the two components. Determination of the relative concentration of components from a single mass spectrum is precluded in this case. Instead, relative concentration must be obtained by a comparison of area measurements between integrated extracted ion chromatograms in the case of ESI−MS or from eluent fractions with MALDI−MS. Further complications can arise when ionization efficiency of the isoforms varies with time in ESI−MS or between fractions in MALDI−MS. Integrating peak areas is difficult and inaccurate when isotopically labeled peptides are fractionated in one or more of the early steps in a multidimensional separation experiment. For example, isotopically labeled peptides could be separated in ion-exchange chromatography followed by reversed-phase chromatography[13] or reversed-phase chromatography followed by ion mobility separation.[14]

The objective of the work reported here was to (1) determine the magnitude of the isotope effect with a commercial deuterated labeling agent, (2) assess the degree to which isotope effects can be minimized with a ^{13}C-labeled derivatizing agent, and (3) evaluate the prospect of real-time isotope ratio analysis for intelligent data acquisition (IDA) in proteomics.

Materials and Methods

Materials. Bovine serum albumin (BSA), HPLC-grade acetonitrile (ACN), succinic anhydride, acetyl chloride, phosphorus

pentaoxide dithiothreitol (DTT), iodoacetic acid (IAA), urea, tris-(hydroxymethy)aminomethane (Tris base), tris(hydroxymethyl)-aminomethane hydrochloride (Tris acid), and calcium chloride were purchased from Sigma−Aldrich (St. Louis, MO). Trifluoroacetic acid (Sequanal Grade) was obtained from Pierce (Rockford, IL). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). Butanedioic acid-$^{13}C_4$ was supplied by Isotec (Miamisburg, OH). The ICAT kit was purchased from Applied Biosystems (Framingham, MA). A C18 column (2.1 mm × 250 mm) was obtained from Vydac (Hesperia, CA). Double-diionized water (ddI H_2O) was produced by a Milli-Q gradient A10 system from Millipore (Bedford, MA).

Proteolysis of BSA. BSA (5 mg) was reduced in 1 mL of 0.2 mol/L Tris buffer (pH 8.5) containing 8 M urea and 10 mM DTT. After a 2 h incubation at 37 °C, iodoacetic acid was added to a final concentration of 20 mmol/L and incubated in darkness on ice for an additional 2 h. After dilution with 0.2 mol/L Tris buffer to a final urea concentration of 2 M, modified sequencing grade trypsin was added to the sample at a 50:1 BSA/trypsin mass ratio and the solution was incubated for 8 h at 37 °C. Digestion was stopped by freezing the mixture in liquid nitrogen for 10 min.

Synthesis of Succinic Anhydride-$^{13}C_4$. Butanedioic-$^{13}C_4$ acid (2 g) and acetyl chloride (5 mL) were heated under reflux for 1.5 h. After reaction, the clear solution was cooled to room temperature and kept at 0 °C overnight. The crystals formed upon cooling were further purified from dry diethyl ether and dried in vacuo over P_2O_5.

Derivatization of Peptides. A 50-fold molar excess of succinic anhydride and succinic anhydride-$^{13}C_4$ were added individually to tryptic peptides from experimental and control samples. The reaction was allowed to proceed for 2 h at room temperature. Peptides were also derivatized with ICAT and ICAT-$^2H_8$ as described in the instructions for the ICAT kit.

Reversed-Phase Chromatography of Isotopically Labeled Peptides. Isotopically labeled peptide mixtures were separated by gradient elution from a Vydac C18 column (2.1 mm × 250 mm) on an Integral Micro-Analytical Workstation (Applied Biosystems, Framingham, MA). The C18 column was equilibrated using 100% mobile phase A (0.01% TFA in ddI H_2O) at a flow rate of 250 μL/min for two column volumes (CV). Isotopically labeled peptide mixtures (2 nmol) were injected and eluted at a flow rate of 250 μL/min in a linear gradient ranging over 60 min from 100% mobile phase A to 60% mobile phase B (95% ACN/0.01% TFA in ddI H_2O). At the end of this period, a second linear gradient was applied in 10 min from 60% B to 100% B at the same flow rate. The gradient was then held at 100% mobile phase B for an additional 10 min. Throughout the analysis, an on-line UV detector set at 214 nm was used to monitor separation of the peptide mixtures. The peptides were simultaneously monitored by ESI−MS by directing 10% of the flow into the mass spectrometer.

ESI−MS Analysis. Mass spectral analyses were performed using a QSTAR workstation (Applied Biosystems, Framingham, MA) equipped with an Ionspray source. All spectra were obtained in the positive-ion TOF mode at a sampling rate of one spectrum every 2 s. During LC−MS data acquisition, masses were scanned from m/z 300 to 1800.

Results and Discussion

Assessing the impact of heavy isotopes on chromatographic behavior requires quantification of small changes in resolution (R). Moreover, peaks are not always Gaussian, and it is important to deal with peak asymmetry in computing resolution. A "Δ *tuning*" method described in a recent study[12] is capable of determining resolution in the case of both symmetrical and asymmetric peaks. It was chosen for the studies outlined below for this reason. This method is based on shifting integrated plots of isoform elution profiles until they overlap. Resolution (R) from the shifting in time, or "Δ *tuning*" required to cause overlap is give by the expression

$$R = \frac{\Delta}{W_{1/2}} \quad (1)$$

where Δ is the time shift required to cause the heavy-isotope-labeled analyte and the nonisotope-labeled analyte elution curves to overlap and $W_{1/2}$ is the average of full peak width at half-maximum (fwhm) derived from extracted ion chromatograms of the isoforms. Even the peak shapes of the isotopically labeled peptides are sometime very different when resolution is large. In this case, "Δ" simply equals the differences in retention times and "$W_{1/2}$" still equals the average peak widths.

The possibility that isotope effects could vary between peptides must also be considered. For example, peptides with multiple derivatizable functional groups would be expected to show larger isotope effects. The location of the derivatizing agent relative to hydrophobic groups in the peptide could also play a role. Rather than select specific peptides to test these hypotheses, a tryptic digest of BSA was chosen for study.

Isotope Effects with ICAT. The isotope coded affinity tag (ICAT) reagent is offered commercially for differentially labeling peptides. This reagent is a sulfhydryl-directed alkylating agent composed of iodoacetate attached to biotin through a coupling arm. Isotopic isoforms of this reagent are differentially labeled in the coupling arm (Figure 1B). During the normal course of reducing and alkylating proteins in preparation for proteolysis, the ICAT reagent is used to alkylate cysteine residues in proteins. After differential labeling of control and experimental samples with the $^2H_0$ and $^2H_8$ versions of the ICAT reagent, respectively, the samples are mixed and digested with a proteolytic enzyme. Biotinylated, cysteine-containing peptides were selected from digests and the relative concentration of the isotopic isoforms of peptides quantified by mass spectrometry. Approximately 10−20% of all peptides derived from the proteome of eukaryotes contain one or more cysteine residues. This means that the ICAT reagent can only be used to quantify changes in a proteome that involve cysteine-containing peptides.

An example of isotopic fractionation of ICAT-$^2H_0$- and -$^2H_8$-labeled peptides is seen in the case of QN***C***DQFEK. Derivatization was at the single (bold) cysteine residue in the peptide. The $^2H_8$-labeled peptide eluted approximately 28 s earlier than the nondeuterated peptide, causing an enormous variation in isotope ratio across the elution profile of the isoforms (Figure 2A−D). Resolution of the isoforms was calculated to be 0.74. Even the chromatographic peak shapes of the deuterated and nondeuterated peptides can differ (Figure 2A). It is readily seen that the only accurate way to quantify the isotope ratio in the initial sample is through a comparison of the integrated extracted ion chromatograms of the isoforms.

Resolution of tryptic peptides from a digest of bovine serum albumin is seen in Figure 4. Twenty percent of the peptides examined in this limited study showed a resolution of greater than 0.5. Of this number, three-fourths were derivatized with two molecules of ICAT reagent. In fact, half the peptides

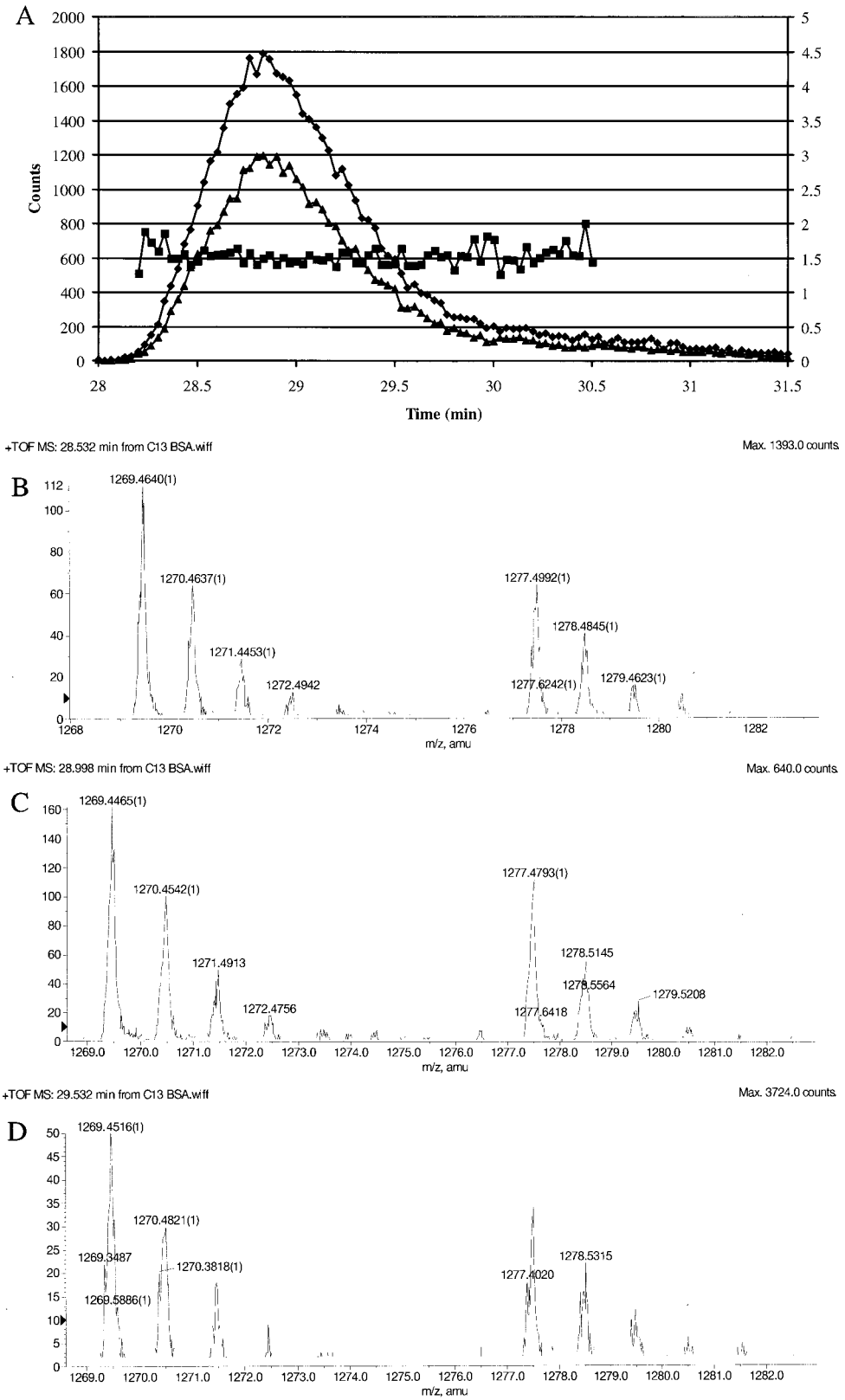

Figure 3. Coelution of a pair of succinic anhydride-$^{13}C_0$- and -$^{13}C_4$-labeled peptides in reversed-phase chromatography. The peptide sequence is QN**C**DQFEK. Eight ^{13}C were incorporated into the heavy-isotope-labeled peptide. Key: (A) extracted ion chromatogram of the ^{13}C-labeled peptide (▲), non-^{13}C-labeled peptide (◆), and ratio between them (■); (B) mass spectrum at 28.5 min; (C) mass spectrum at 29.0 min; (D) mass spectrum at 29.5 min.

derivatized with two molecules of ICAT reagent showed a resolution of greater than 0.5. In contrast, only 23% of singly labeled peptides had a resolution greater than 0.4. It is also seen in Figure 4 that isotope effects tend to become smaller with increasing molecular weight of the peptide, particularly with those that are singly derivatized.

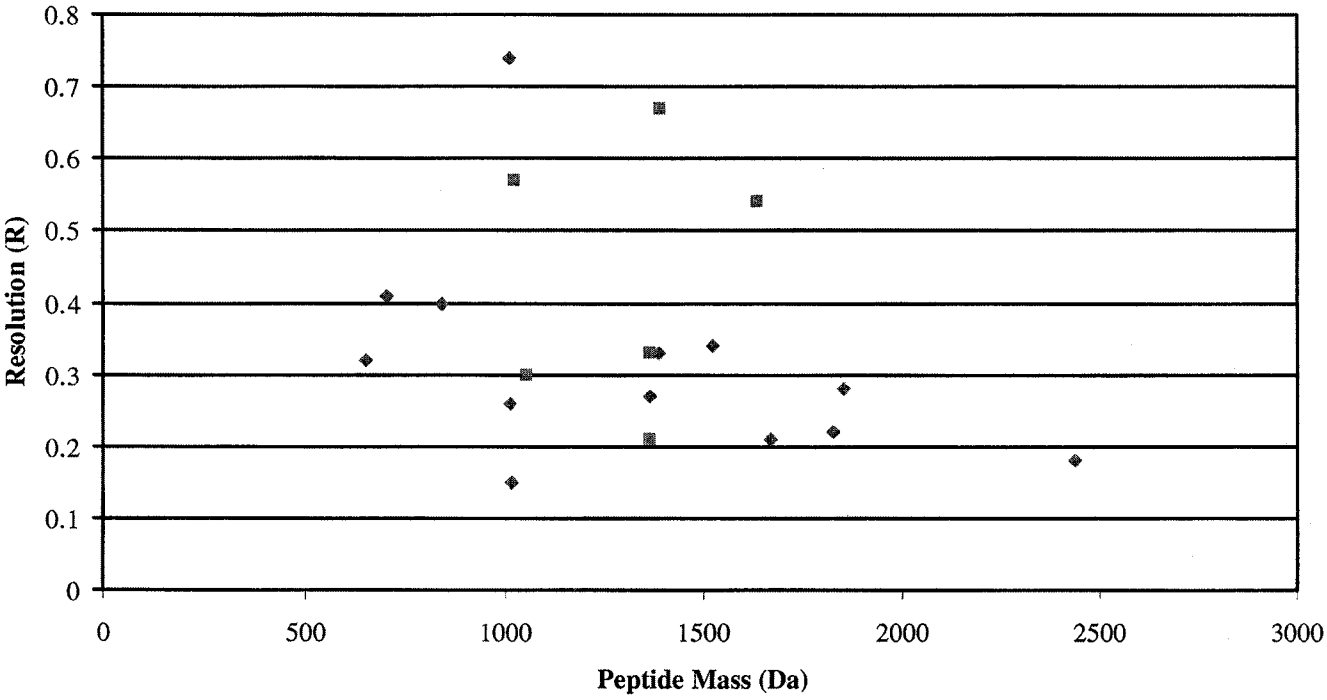

Figure 4. Resolution (R) caused by ICAT-$^2H_8$ on tryptic peptides of BSA. Thirteen peptides contain one cysteine and have eight deuteriums incorporated (◆). Six peptides contain two cysteines and have 16 deuteriums incorporated (■).

Resolution of peptide isoforms is an important issue for a number of reasons. One is the matter of ionization efficiency across the elution profile of the isoforms. It is probable that in a complex mixture the peptides eluting in the leading and tailing parts of a peak are different. If sample matrix components diminish ionization efficiency more in one part of an analyte peak than another does, there is a problem in ESI−MS. Integrated peak areas will not be accurate in cases where the ionization efficiency of a compound varies during peak elution. Moreover, ionization efficiency could vary between the isoforms when they are partially resolved for the same reason.

A similar problem occurs with MALDI−MS. Large numbers of peaks will overlap in the reversed-phase elution profile of complex tryptic digests and there will be no distinct peaks. This means that fraction collection for MALDI−MS from chromatography columns will be time based, and there is a high probability that components will inadvertently be split into multiple fractions. When isotopic isoforms are differentially resolved in these fractions they will differ substantially in isotope ratio as the simulation in Figures 2E−G shows. The problem is in knowing the portion of the analyte present in each fraction that is needed to calculate the true isotope ratio in the initial sample.

Still another problem is that it is necessary to wait until both isoforms elute before extracted ion chromatograms can be constructed and peak areas can be calculated to determine isotope ratio. When the objective is to identify only those components in the mixture that have been up- or down-regulated and there is a need for MS/MS data to identify a peptide that has changed in concentration, the isoforms will have eluted before it is known that MS/MS sequence data is needed. This means that either MS/MS data must be acquired on all components as they elute, which is difficult to do, or the sample must be run a second time to acquire the necessary MS/MS data, which costs more time and sample.

Isotope Effects with ^{13}C-Succinate. Succinic anhydride (Figure 1A) derivatizes primary amine groups in peptides at their N-termini and in lysine residues. This means that each peptide produced during proteolysis can be derivatized. The exception would be rare peptides (1) that do not contain a lysine residue and (2) are derived from the amino terminus of an amino terminally blocked protein. Differential labeling of tryptic digests from control and experimental samples with $^{13}C_0$- and $^{13}C_4$-succinic anhydride, respectively, produces peptide isoforms that vary by four and eight atomic mass units (amu). Peptides with a C-terminal arginine appear in mass spectra as a double cluster of ions separated by 4 amu, whereas C-terminal lysine-containing peptides are separated by 8 amu. In the case of the peptide QNCDQFEK, 2 mol of succinate was added when it was derivatized with succinic anhydride. The reversed-phase chromatography elution profile of the $^{13}C_0$- and $^{13}C_4$-succinate-labeled isoforms of QNCDQFEK is seen in Figure 3A. Resolution of the isoforms was calculated to be −0.0043. This is within the measurement error of the method. The fact that there is no detectable resolution of the isoforms means that the isotope ratio will be constant across the elution profile (Figure 3A−D). Resolution of all the peptide isoforms from a tryptic digest of BSA falls between +0.01 and −0.01 (Figure 5), again within experimental error.

It is important to know that there was no evidence of peptide fractionation based on the content of natural heavy isotopes in peptides. In view of the fact that all peptides contain small amounts of ^{13}C and ^{2}H from the occurrence of these isotopes in nature, it is interesting that they were not observed to resolve in reversed-phase chromatography. This is probably because the isotope peaks of unlabeled peptides are due mainly to ^{13}C. The natural abundance of ^{13}C is 1.10%, whereas that of ^{2}H is 0.015%. Although peptides have roughly twice as many hydrogen as carbon atoms, few peptides would contain more than one or two deuterium atoms. The lack of isotope effects in the

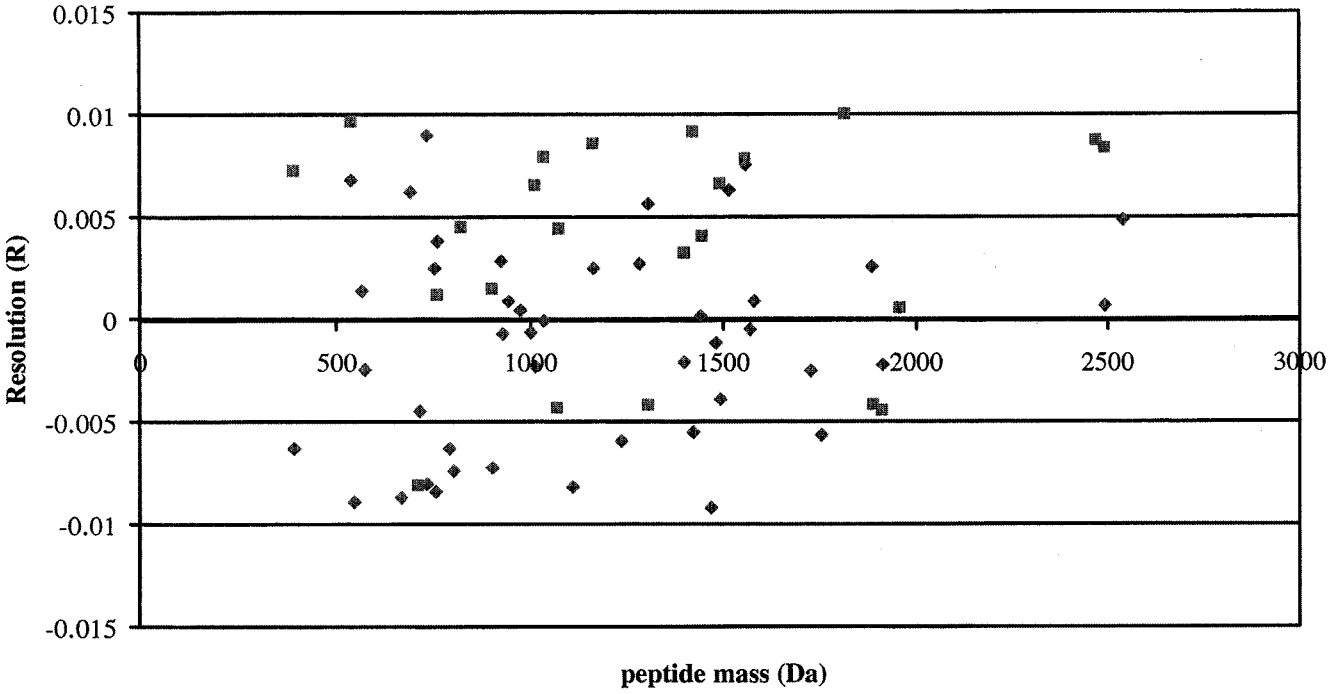

Figure 5. Resolution (R) caused by succinic anhydride-$^{13}C_4$ on tryptic peptides of BSA. Forty-four peptides contain no lysine and have four ^{13}C incorporated (◆). Twenty-three peptides contain lysine and have eight ^{13}C incorporated (■).

Table 1. Comparison of Resolution (R) Caused by Succinic Anhydride-$^{13}C_4$ and ICAT-$^2H_8$

peptide sequence	R (succinic anhydride-$^{13}C_4$)	no. of ^{13}C	R (ICAT-$^2H_8$)	no. of ^2H
CASIQK	−0.0081	8	0.32	8
GACLLPK	0.0012	8	0.41	8
LCVLHEK	0.0015	8	0.4	8
QNCDQFEK	−0.0043	8	0.74	8
SHCIAEVEK	0.0044	8	0.15	8
SLHTLFGDELCK	0.0091	8	0.27	8
YICDNQDTISSK	0.004	8	0.33	8
LFTFHADICTLPDTEK	−0.0045	8	0.28	8
GLVLIAFSQYLQQCPFDEHVK	0.0083	8	0.18	8
MPCTEDYLSLILNR	−0.0025	4	0.21	8

fractionation of ^{13}C-labeled peptides and the corresponding natural monoisotopic peptides means they could be added together to calculate the ratio between experimental and control samples to improve the precision of isotope ratio measurement.

A direct comparison of peptide resolution caused by succinic anhydride-$^{13}C_4$ and ICAT-$^2H_8$ in the same group of peptides is seen in Table 1. ICAT-$^2H_8$ caused significant resolution between the isotopically labeled peptides, while succinic anhydride-$^{13}C_4$ did not. Clearly, coding labeling agents with ^{13}C is vastly superior to ^2H coding.[13] A second major advantage of succinate labeling is that the number of peptides labeled is much broader. All peptides in tryptic digests are labeled except those that are N-terminally blocked and contain no lysine.

Real-Time Intelligent Data Acquisition and Analysis (IDA). The discussion above has outlined all the problems associated with isotope effects in isotopically based, internal standard methods for quantifying relative concentrations of peptides. Having shown that this problem is eliminated with ^{13}C-labeled derivatizing agents and that real-time analysis is possible, there is the question of whether real-time isotope ratio analysis could have any other benefits.

It is known in the multidimensional chromatography approach to proteomics used here that proteolysis of a proteome produces very large numbers of peptides and that identifying all these peptides by MS/MS is a lengthy, formidable task.[15] However, studies have shown that only a small percentage of all the proteins in a proteome change significantly in concentration as a result of some stimulus.[16] When the objective it to identify only those proteins that have undergone change, throughput and data quality could be greatly improved by fragmenting only those peptides of interest. Real-time isotope ratio analysis would allow a mass spectrometer (MS) and data system to select peptides for MS/MS sequencing based on changes in relative concentration that exceed preset values. Although isotope ratio would be the primary selection criterion, difference in mass between ion clusters could also be used. For example, peptides labeled with succinate in this study varying by 4 amu contained a C-terminal arginine while those varying by 8 amu contained a C-terminal lysine. As more coding agents become available, it is likely that different types of derivatization will be coded by this mass shift technique.

Real-time IDA could probably be achieved in several ways. Although isotopically labeled isoforms of peptides are not resolved in the case of ^{13}C coding, large random errors in isotope ratio will seen at both the leading and tailing edges of eluting peaks where concentration and counts are low. The is very different than in the middle of a peak where counts are high. One way to deal with this problem would be in the following steps. First, recognize pairs of isotopically labeled peptides. Second, trace accumulative counts of both isotopically labeled peptides. Third, average the ratio between the accumulated counts of subsequent measurements when they reach a preset level. Finally, perform an MS/MS analysis when the ratio exceeds the preset threshold. An example is seen in Figure 6C. The ratio could be determined accurately at 28.5

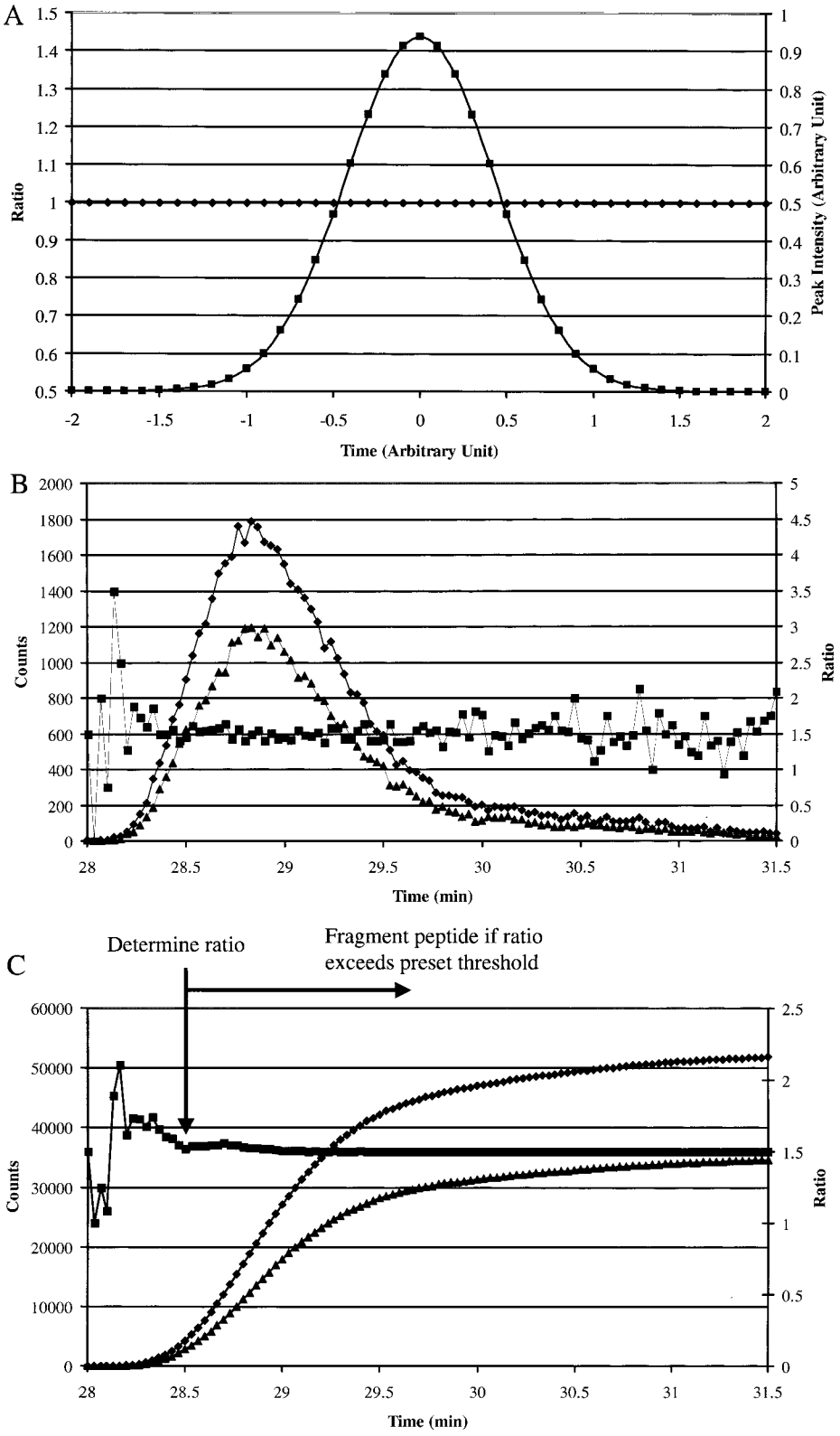

Figure 6. Real-time intelligent data acquisition (IDA) when resolution is negligible. (A) A simulation of isotope ratio (◆) as a function of elution time in a chromatographic separation of isotopically labeled peptides. R = 0 and ratio (◆) does not vary with time. (B) A pair of succinic anhydride-$^{13}C_0$- (◆) and -$^{13}C_4$-labeled (▲) peptides coelute in reversed-phase chromatography. Ratio (■) is constant in the middle of elution peak where counts are high. Large random errors in the ratio (■) exist at both ends of the elution peak where counts are low. (C) Ratios settle quickly when ratios are calculated based on cumulative counts, instead of counts in individual mass spectrum. In this case, it reaches an accurate ratio at approximately 28.5 min, which leaves approximately 1 min (28.5−29.5 min) to perform MS/MS analysis if the ratio exceeds preset threshold.

min, which leaves approximately 1 min for further analysis of the peptides if the ratio exceeds the preset threshold. The ratio could be determined even earlier if the intensities of the peptides are higher than in Figure 6.

Real-time IDA will probably increase throughput, reduces sample consumption, and improve the quality of MS/MS data because more time can be spent on the peptides of interest. Isotopically labeled peptides can be fragmented together when resolution is small, which could help to identify the y and b ion series. For example, y ions are paired with mass differences of 4 Da and b ions are unpaired when the carboxyl terminus of the tryptic peptides are labeled by ^{18}O during trypsin digestion.[17]

Conclusions

It is concluded that isotopic fractionation can be minimized in isotope-based, internal standard methods for quantifying relative concentrations of peptides by using coding reagents containing ^{13}C instead of ^{2}H. As a result of eliminating isotope effects in reversed-phase chromatography, quantification can be achieved much faster and with greater accuracy. This will improve throughput and potentially allow real-time monitoring and intelligent data analysis.

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