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Isotopologue Distributions of Peptide Product Ions by Tandem Mass Spectrometry: Quantitation of Low Levels of Deuterium Incorporation¹

Benlian Wang[‡], Gang Sun^{‡,†}, David R. Anderson[‡], Minghong Jia^{‡,#}, Stephen Previs[¶], and Vernon E. Anderson^{‡,*}

[‡]Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, OH 44106

[¶]Department of Nutrition, Case Western Reserve University School of Medicine, Cleveland, OH 44106

Abstract

Protonated molecular peptide ions and their product ions generated by tandem mass spectrometry appear as isotopologue clusters due to the natural isotopic variations of carbon, hydrogen, nitrogen, oxygen and sulfur. Quantitation of the isotopic composition of peptides can be employed in experiments involving isotope effects, isotope exchange, isotopic labeling by chemical reactions, and studies of metabolism by stable isotope incorporation. Both ion trap and quadrupole-time of flight mass spectrometry are shown to be capable of determining the isotopic composition of peptide product ions obtained by tandem mass spectrometry with both precision and accuracy. Tandem mass spectra obtained in profile-mode of clusters of isotopologue ions are fit by non-linear least squares to a series of Gaussian peaks (described in the accompanying manuscript) which quantify the M_n/M_0 values which define the isotopologue distribution (ID). To determine the isotopic composition of product ions from their ID, a new algorithm that predicts the M_n/M_0 ratios is developed which obviates the need to determine the intensity of all of the ions of an ID. Consequently a precise and accurate determination of the isotopic composition a product ion may be obtained from only the initial values of the ID, however the entire isotopologue cluster must be isolated prior to fragmentation. Following optimization of the molecular ion isolation width, fragmentation energy and detector sensitivity, the presence of isotopic excess (²H, ¹³C, ¹⁵N, ¹⁸O) is readily determined within 1%. The ability to determine the isotopic composition of sequential product ions permits the isotopic composition of individual amino acid residues in the precursor ion to be determined.

Keywords

isotopologue distribution; mass isotopomer distribution; tandem mass spectrometry; deuterium incorporation; isotopic excess; isotope quantitation; H/D exchange; protein turnover

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* Corresponding Author: Vernon E. Anderson, Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, OH 44106-4935, Phone: 216-368-2599 (VEA) Fax: 216-368-3419, Email: vernon.anderson@case.edu.

#Current Address: General Clinical Research Center, Miller Health Science Center, University of Florida, 1600 Archer Road, Gainesville, FL 32610-022

[†]Current Address: Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110

³The analysis of the ID presented here should not be confused with the mass isotopomer distribution analysis (MIDA) developed by Hellerstein [22] which relies on specific features of the ID.

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Stable isotopic labels can be incorporated randomly into proteins and peptides through chemical exchange, metabolic labeling with tracers, and through chemical modifications used for quantitation in proteomic studies. The quantitation of isotopically modified proteins and peptides is necessary for all of these applications. Tandem mass spectrometry results in higher specificity and sensitivity for many analytical purposes. As routinely applied, information on the isotopic composition of the peptides is routinely lost due to the isolation of only the base ion during tandem mass spectrometry. We demonstrate that the complete molecular isotopologue ion cluster (IIC)¹ can be isolated both in ion trap and quadrupole time-of-flight mass spectrometers. Further, the isotopologue distributions (IDs)² of the product IICs obtained following fragmentation of the molecular ion accurately reflect the isotopic composition of the product ions. The accuracy and precision of the experimental IDs of product ions can be better than that of the molecular precursor ion, or of the ID obtained by matrix assisted laser desorption ionisation (MALDI) - time of flight (TOF) mass spectrometry.

Methods to calculate the IDs of biological macromolecules based on their isotopic compositions have been developed [1,2] and implemented in numerous freeware and web-based applications, e.g. IsoPro [3], Isotopica [4], MS-Isotope [5], ChemCalc [6], Isotopident in ExPASy [7] and the Sheffield Chemputer [8]. These implementations, while useful, cannot be incorporated into a statistical analysis program to determine how well an experimental ID corresponds to that calculated from an assumed isotopic composition. This problem was initially solved by matrix algebra [9] and more recently updated to accommodate H/D-exchange data using a fast Fourier transform analysis [2] which has been adapted to H/D exchange experiments [10]. We have developed a simplified algorithm applicable to oligopeptides, oligonucleotides and oligosaccharides that calculates the ID normalized to the monoisotopic mass (equation 1A) rather than to the fraction of the total intensity as given by equation 1B.

$$\text{monoisotopic normalization} = \frac{M_n}{M_0} \quad (1A)$$

$$\text{fractional intensity} = \frac{M_n}{\sum_{j=0}^i M_j} \quad (1B)$$

The sum in the denominator in 1B is routinely truncated when M_j becomes arbitrarily small. Our simplified algorithm provides the analytically correct ratio of M_i/M_0 and is validated against calculations for $n \geq 2$. The algorithm permits the variation of experimental IDs to be quantified by a non-linear least squares fit of the observed to the predicted IDs with a single parameter that describes the presence of excess isotope. This algorithm has the additional benefit of not requiring the entire ID to be acquired, as is necessary for algorithms requiring the determination of the denominator of equation 1B. We compare the precision and accuracy of MALDI-TOF, and electrospray ion trap mass spectrometry of peptide ions with the precision and accuracy of the product ions obtained from ion trap and from quadrupole-time of flight mass spectrometers. Surprisingly, the accuracy and precision of the product ions in the tandem mass spectra can surpass that determined for the precursor ion. The precision of the

¹Abbreviations used: HCCA, α -cyano-4-hydroxy cinnamic acid; IIC, cluster of isotopologue ions; ID, isotopologue distribution; MALDI, matrix assisted laser desorption ionisation; TOF, time of flight; Trp-Cage, tryptophan cage protein

²Isotopologue distribution is used specifically to mean the relative intensities (areas) of the individual ions in a cluster of isotopologue ions where M_0 denotes the contribution of the monoisotopic ion and M_n denotes the intensity of any isotopic peak containing n additional neutrons. M_n/M_0 is the ratio intensities of the M_n peak and the monoisotopic peak. An isotopologue distribution can be represented by a series of n M_n/M_0 ratios. Isotopic composition requires the identification of the mol fraction of each isotope present in an ion/molecule.

determinations of M_1/M_0 for successive product ions can be better than ± 0.01 , making it possible in favorable cases to determine the excess isotope present individual amino acid residues within a peptide.

MATERIALS

The 20-residue tryptophan cage protein (Trp-Cage) [11] (purity > 90%) was synthesized by Biomer Technology (Hayward, CA). Reversed phase HPLC (Vydac C18 column) was used to obtain the purified protein with an acetonitrile (0.075% trifluoroacetic acid)/water (0.1% trifluoroacetic acid) gradient from 5% to 65% in 30 min. The concentration was determined by UV at $\epsilon_{278} = 6760 \text{ cm}^2 \text{ mmol}^{-1}$. Angiotensin I, α -cyano-4-hydroxy cinnamic acid (CHCA), and [Glu 1]-fibrinopeptide B (human) and Kemptide were obtained from Sigma, and used without further purification. Trifluoroacetic acid and formic acid were from Aldrich. Water and acetonitrile were from Burdick & Jackson.

$^2\text{H}_2\text{O}$ Administration for ^2H -incorporation into serum albumin

Male C57BL/6J mice ($n = 15$, $22.9 \pm 0.3 \text{ g}$, Jackson Laboratories) were given a “primed-infusion” of $^2\text{H}_2\text{O}$. A bolus of $^2\text{H}_2\text{O}$ -saline was given on day 0 ($30 \mu\text{L g}^{-1}$ body weight) and mice were then provided 6% ^2H -labeled drinking water. As we previously demonstrated, this approach will maintain a steady state labeling of body water [12]. Mice were allowed to eat and drink *ad libitum*. Animals ($n = 3$ per day) were sacrificed at various times after the bolus of $^2\text{H}_2\text{O}$ was administered. Blood and heart tissue were collected at the time of sacrifice. This protocol was approved by the CWRU institutional review board. Plasma proteins were precipitated using trichloroacetic acid ($5 \mu\text{L}$ of 10 % acid per μL plasma). The pellet was washed 3 times with 5% trichloroacetic acid. Albumin was extracted into 100% ethanol. Following isolation the albumin, solution tryptic digestion of the albumin was accomplished by addition of sequencing grade trypsin (Promega) and the resultant peptides desalted for MALDI-TOF mass spectrometry using C_{18} ZipTips (Millipore).

Mass spectrometry

MALDI-TOF mass spectra were acquired on a Bruker BiFlex III MALDI-TOF mass spectrometer equipped with a nitrogen laser in the positive ion mode. C_{18} -ZipTips (Millipore) were used to desalt the sample. Freshly prepared matrix solution consisting of 10 mg/ml CHCA in 70% acetonitrile/0.1% trifluoroacetic acid was used to elute the sample from the ZipTip and immediately spotted on the target plate. The reflectron mode was used with a laser power attenuation range from 70-80 and an average of 500 scans acquired so that the base peak had an intensity in excess of 20,000 counts. MALDI data were converted to ASCII files using the *m over z* [13] export window function and the data fit in Origin [14] as described in the accompanying paper [15].

ESI-quadrupole mass spectrometry was carried out on either a Finnigan TSQ 7000 or Micromass Quattro II in the SIM mode, while tandem mass spectrometry was performed with a Thermo-Finnigan LTQ linear ion trap or an Applied Biosystems Q-STAR XL hybrid quadrupole-TOF mass spectrometer. All IICs detected by ion trap were acquired in the Zoom Scan mode. Most of the samples were directly infused into the ESI source and micro-electrosprayed through a $10 \mu\text{m}$ i.d. PicoTip nanospray emitter (New Objective), of a solution containing $0.1 \mu\text{M}$ - $10 \mu\text{M}$ analyte in 0.1% formic acid, acetonitrile /water (50/50). Tandem mass spectra were collected in either zoom scan or profile mode. Typical experimental conditions using the LTQ were: spray voltage 1.9 kV; capillary temperature 200°C ; ion isolation width $10 m/z$; collision energy 25%. For quadrupole-TOF tandem mass spectra, the ionspray voltage was set at 3 kV, collision energy 50%. The quadrupole was run in the low resolution mode, with a routine setting of 1.8 when isolating singly or doubly charged ions with $m/z < 1000$.

THEORY

The presence of 0.018% ^2H , ~1.11% ^{13}C , 0.45% ^{15}N and 0.20% ^{18}O results in every peptide analyzed by high resolution mass spectrometry appearing as a multiplet comprised of separate isotopic peaks denoted M_n . The IIC that comprise the mass spectrum of Trp-cage, a typical twenty-residue peptide, determined by MALDI-TOF MS is shown in Figure 1. The protonated molecular monoisotopic ion, $[\text{M}+\text{H}]^+$, whose area corresponds to M_0 appears as the lowest m/z peak. The areas of the successive ions in the IIC comprise the ID and are denoted M_n . The isotopologues contributing to the intensity of the M_1 peak will have a single heavy atom substitution, exactly one ^2H , ^{13}C , ^{15}N or ^{17}O atom substituted for the more common isotope (with the higher resolution of ion cyclotron resonance mass spectrometry it is possible to separate the different isotopologues, but not with the typical resolution of commercial TOF instruments).

The ID of an ion is determined by its isotopic composition, i.e. the number of atoms of each element, c, n, o, h and s for carbon, nitrogen, oxygen, hydrogen and sulfur, respectively, and the mol fraction of each isotope; e.g. x_{12} and x_{13} for the mol fractions of ^{12}C and ^{13}C ; x_{14} and x_{15} for the mol fractions of ^{14}N and ^{15}N ; x_{16} , x_{17} and x_{18} for the mol fractions of ^{16}O , ^{17}O and ^{18}O , respectively. With these definitions, the M_0 and M_1 components of the fractional ID (i.e. as defined by equation 1B) can be calculated exactly from probability theory:

$$M_0 = x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot x_{16}^o \cdot x_{32}^s \quad (2)$$

$$M_1 = h x_2 x_1^{h-1} \cdot x_{12}^c \cdot x_{14}^n \cdot x_{16}^o \cdot x_{32}^s + c x_{13} x_{12}^{c-1} \cdot x_1^h \cdot x_{14}^n \cdot x_{16}^o \cdot x_{32}^s + \\ n x_{15} x_{14}^{n-1} \cdot x_1^h \cdot x_{12}^c \cdot x_{16}^o \cdot x_{32}^s + o x_{17} x_{16}^{o-1} \cdot x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot x_{32}^s + \\ s x_{33} x_{32}^{s-1} \cdot x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot x_{16}^o \quad (3)$$

Since it is the direct result of probability theory, most calculations of IDs use equations (2) and (3), that the expressions for M_n can be simplified by normalizing to M_0 is shown in equations 6, 10 and 14. This normalization of the ID to M_0 has the theoretical and experimental advantage that the complete ID does not need to be calculated or measured and hence only the initial peaks in the IIC need to be acquired and/or analyzed (particularly valuable in practice when there are overlapping IICs in the mass spectrum). Normalizing M_1/M_0 yields equation 4.

$$M_1/M_0 = h x_2 / 1! x_1 + c x_{13} / 1! x_{12} + n x_{15} / 1! x_{14} + o x_{17} / 1! x_{16} + s x_{33} / 1! x_{32} \quad (4)$$

$$\text{defining : } m_I = \sum_{\text{elements}} e (x_{eI} / x_{e0}) \quad (5)$$

$$\text{yields : } M_1/M_0 = m_I / 1! \quad (6)$$

By defining m_I as the sum of the products of the number of atoms of each element, e , and its isotopic mol fraction ratio, x_{eI} / x_{e0} , the very simple form of equation 6 is generated. The $1!$ is included in equation 6, as the general form for M_n/M_0 has $n!$ as a factor in the denominator. At natural abundance, m_I can be calculated readily from the elemental composition and tabulated values for the mol fraction ratios [16]. For biological macromolecules the ^{13}C term, $c (x_{13}/x_{12})$ makes the most significant contribution to m_I . In compounds containing an isotopic label, m_I contains a contribution from the product of the number of labeled sites and the mol fraction ratio of the label. To obtain a general form, consider the evaluation of M_2/M_0 ; the complete expression for M_2 is provided by equation 7 and the simplification provided by normalization to M_0 appears as equation 8.

$$\begin{aligned}
M_2 = & [h(h-1)x_2^2x_1^{h-2} \cdot x_{12}^c \cdot x_{14}^n \cdot x_{16}^o \cdot x_{32}^s + c(c-1)x_{13}^2x_{12}^{c-2} \cdot x_1^h \cdot x_{14}^n \cdot x_{16}^o \cdot x_{32}^s \\
& + n(n-1)x_{15}^2x_{14}^{n-2} \cdot x_1^h \cdot x_{12}^c \cdot x_{16}^o \cdot x_{32}^s + o(o-1)x_{17}^2x_{16}^{o-2} \cdot x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot x_{32}^s \\
& + s(s-1)x_{33}^2x_{32}^{s-2} \cdot x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot x_{16}^o] / 2! \\
& + hx_2x_1^{h-1} [cx_{13}x_{12}^{c-1} \cdot x_{14}^n \cdot x_{16}^o \cdot x_{32}^s + x_{12}^c \cdot nx_{15}x_{14}^{n-1} \cdot x_{16}^o \cdot x_{32}^s + \\
& x_{12}^c \cdot x_{14}^n \cdot ox_{17}x_{16}^{o-1} \cdot x_{32}^s + x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot sx_{33}x_{32}^{s-1}] \\
& + cx_{13}x_{12}^{c-1} [x_1^h \cdot nx_{15}x_{14}^{n-1} \cdot x_{16}^o \cdot x_{32}^s + x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot ox_{17}x_{16}^{o-1} \cdot x_{32}^s \\
& x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot x_{16}^o \cdot sx_{33}x_{32}^{s-1}] \\
& + nx_{15}x_{14}^{n-1} [x_1^h \cdot x_{12}^c \cdot ox_{17}x_{16}^{o-1} \cdot x_{32}^s + x_1^h \cdot x_{12}^c \cdot x_{16}^o \cdot sx_{33}x_{32}^{s-1}] \\
& + ox_{17}x_{16}^{o-1} [x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot sx_{33}x_{32}^{s-1}] \\
& x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot ox_{18}x_{16}^{o-1} \cdot x_{32}^s + x_1^h \cdot x_{12}^c \cdot x_{14}^n \cdot x_{16}^o \cdot sx_{34}x_{32}^{s-1}
\end{aligned} \tag{7}$$

$$\begin{aligned}
M_2 / M_0 = & h(h-1)x_2^2 / 2! x_1^2 + c(c-1)x_{13}^2 / 2! x_{12}^2 + n(n-1)x_{15}^2 / 2! x_{14}^2 + o(o-1)x_{17}^2 / 2! x_{16}^2 \\
& + s(s-1)x_{33}^2 / 2! x_{32}^2 \\
& + hx_2 / x_1 [cx_{13} / x_{12} + nx_{15} / x_{14} + ox_{17} / x_{16} + sx_{33} / x_{32}] \\
& + cx_{13} / x_{12} [nx_{15} / x_{14} + ox_{17} / x_{16} + sx_{33} / x_{32}] \\
& + nx_{15} / x_{14} [ox_{17} / x_{16} + sx_{33} / x_{32}] + ox_{17} / x_{16} [sx_{33} / x_{32}] \\
& + ox_{18} / x_{16} + sx_{34} / x_{32}
\end{aligned} \tag{8}$$

Approximating $h(h-1) \approx h^2$, $c(c-1) \approx c^2$, $n(n-1) \approx n^2$ for each element and defining $\mathbf{m}_2$ in a manner analogous to $\mathbf{m}_1$:

$$\mathbf{m}_2 = \sum_{elements} e^{(x_{e2} / x_{e0})} \tag{9}$$

where x_{e2} / x_{e0} is the mol fraction ratio of the +2 isotope of element e . This approximation and definition allow the exact expression to be simplified to:

$$M_2 / M_0 = m_1^2 / (2! 0!) + m_2 / (0! 1!) = m_1^2 [1 / (2! 0!) + m_2 / m_1^2 / (0! 1!)] \tag{10}$$

Further assuming for each element, e , that

$$\prod_{i=0}^{n-1} (e-i) \approx e^n \tag{11}$$

it can be shown that:

$$M_3 / M_0 = m_1^3 [1 / (3! 0!) + m_2 / m_1^2 (1! 1!)] \tag{12}$$

$$M_4 / M_0 = m_1^4 [1 / (4! 0!) + m_2 / m_1^2 (2! 1!) + m_2^2 / m_1^4 (0! 2!)] \tag{13}$$

and in general:

$$M_n / M_0 = m_1^n \sum_{i=0,1,2,\dots}^{n/2} (m_2)^i / m_1^{2i(n-2i)} i! \quad (14)$$

In these expressions the two combinatorial factors in the denominator arise from the number of +1 and +2 isotopic atoms, respectively. The approximation, $\prod_{i=0}^{n-1} (e - i) \approx e^n$, made to simplify equation 8 is required to reduce the number of expansion terms which rapidly exceed 100 when the M_r of the analyzed peptide is > 2000 and the analysis is extended to $n = 6$ and beyond. These approximations place constraints on the use of equation 14 to cases where the number of isotopically substituted atoms is greater than the number of isotopologue ions analyzed⁴, and where the mol fractions x_{e1} and $x_{e2} < 0.25$. To determine the isotopic composition from the ID in cases with a small number of highly enriched sites, the method of the accompanying paper may be used [15].

The approximations of equation 11 result in a small overestimation of M_n/M_0 for $n \geq 2$ which increases with n . For molecules at or near natural abundance, this overestimation can be empirically corrected by a fudge factor, $f (\leq 1)$, as shown in equation 15. Using $n-1$ as the exponential power of f accounts both for the fact that M_1/M_0 is exactly correct (so the correction is unity for $n=1$) and subsequently increases with n .

$$M_n / M_0 = m_1^n f^{n-1} \sum_{i=0}^{n/2} (m_2)^i / m_1^{2i(n-2i)} i! \quad (15)$$

To determine f , we compared the IDs calculated using the general formula for average peptides [17] at 500, 1000, 1500, 2000 and 2500 Da to the correct ID determined with an average value calculated from IsoPro 3.0, MS-Isotope, ChemCalc and IsotopIdent. For each ID an optimum value of f could be determined by non-linear least squares. The important result of this exercise was that f showed minimal variation, from 0.985 and 0.990, as a function of peptide mass. Using a value for f of 0.99, the values for each M_n/M_0 ratio predicted by this algorithm varied by less than 0.002 from the average of the values calculated with the other programs, and fell within the range of values calculated by these various programs, i.e. the error introduced by the assumption of equation 11 when corrected by an f of 0.99 in equation 15 is less than the spread in calculated values from “exact” calculations. This variation derives from differences in the values assumed for x_{13} , ranging from 0.0110 to 0.0111, although other computational differences also contribute. The variation in x_{13} is recapitulated in the biosphere, as x_{13} in metabolites, including proteins can vary from <0.0098 to > 0.0111 depending on the dietary carbon source [18-20]. Thus the potential inaccuracy of equation 15 is significantly less than the uncertainty introduced by potential variations in the natural abundance of ¹³C.

Equation 15 shows that an experimentally determined ID can be characterized by two parameters, m_1 and m_2 that are characteristic of the number of +1 and +2 isotopic atoms. At natural abundance, the best fit values of these parameters can be compared to those calculated by equations 5 and 9, respectively. This allows the precision and accuracy of different mass spectrometric determinations of IDs to be quantified. With molecules labeled with +1 isotopes, the ID is fit with m_1 as an unconstrained parameter and m_2 constrained to the value calculated from the elemental composition. Isotopic excess introduced by a label at low abundance is defined by equation 16:

⁴When the number of atoms of a given element, e , with more than one +1 or +2 isotope is small, the running product becomes 0 when $n + 1 = e$, or $2n + 2 = e$, respectively, invalidating the assumption. This problem is minimized when the mol fraction ratio is < 0.1, as the higher order terms become negligible. The methods of [15] must be used for Cl and Br containing compounds and/or molecules with a small number of highly enriched atoms.

$$m_1(\text{label}) = m_1(\text{obsd}) - m_1(\text{nat.abund.}) = \sum_{\text{labeled sites}}^N x_{\text{lab}} / (1 - x_{\text{lab}}) \quad (16)$$

where x_{lab} is the mol fraction of label in excess of natural abundance at each site containing label. If x_{lab} is the same at each site, the number of labeled sites, N , is given by $m_1(\text{label}) / [x_{\text{lab}} / (1 - x_{\text{lab}})]$ while if the number of sites, N , is known, x_{lab} can be determined as $(m_1(\text{label}) / N) / (1 + m_1(\text{label}) / N)$.

Experimental quantitation of IDs

As described in the accompanying manuscript, IICs have been quantified by non-linear least squares fits to a series of Gaussian peaks of identical peak shape spaced at $1/z$ intervals. For these analyses the mass spectrometric data are acquired in profile mode, converted to ASCII character files and imported into ORIGIN 7.5[14]. The characteristic results generated a series of Gaussian peaks, with m/z separations of 1.00 ± 0.02 and peak widths that varied by less than 5%. Occasionally, the last peak corresponding to the heaviest isotopologue detected would diverge from these expected characteristics, and would have to be constrained to the correct peak center and peak width, chosen as the average of the M_1 and M_2 peak widths, using the advanced fitting tool in ORIGIN. The areas of the Gaussian peaks were normalized to M_0 , yielding $M_1/M_0 \dots M_n/M_0$. Only peaks where $M_n/M_0 > 0.01$ were used in the non-linear least squares fitting procedure to determine m_1 from the ID by encoding the algorithm of equation 15 in an Origin 7.5 function definition file available from the corresponding author (VEA) or an Excel worksheet "ID calc and m1 fit based on elemental composition.xls" made available online as supplementary material.

RESULTS AND DISCUSSION

Accuracy and precision of IDs by MALDI-TOF

Analyses of peptides by MALDI-TOF in the reflectron mode readily generate IDs of the peptides present. As an example, the molecular IIC in the MALDI-TOF spectrum of Trp-Cage, $M_r = 2168$, is shown in Figure 1. The fit of the data to a series of peaks, as described in the accompanying manuscript, is also shown yielding M_0 to M_5 . After normalization, the ratios M_1/M_0 through M_5/M_0 are fit to equation 15 by non-linear least squares using m_1 as the sole variable parameter and m_2 set by natural abundance. To characterize IDs from MALDI-TOF spectra, i.e. determine m_1 , with precisions of better than 5%, intensities of the base peak of 20,000 counts or greater are desirable. Further the data must not be acquired too rapidly or the most intense peaks are diminished due to counts missed in the dead time of the detector [21]. On the Bruker BiFlex this phenomenon was qualitatively recognized when the observed intensity decreased below the baseline between the largest peaks in the IIC. In practice fulfilling this requirement is difficult as the data acquisition rate can vary dramatically as the laser focuses on different regions of the sample and the temptation is to concentrate on regions where data are acquired most rapidly. This feature coupled with the higher backgrounds at lower m/z may be the limiting features of ID analyses by MALDI-TOF. Both problems are minimized when M_n/M_0 is near unity. The m_1 value for Trp-cage was 1.28 ± 0.04 compared to the value calculated for natural abundance of 1.22, respectively. This standard deviation is typical for numerous peptides we have studied by MALDI-TOF, i.e. in the absence of significant background interference, the m_1 value describing the ID can be determined with a precision of ~3%. We have noted a slight tendency of our MALDI-TOF data to overestimate m_1 , particularly on larger peptides.

Accuracy and precision of IDs by electrospray-ion trap and quadrupole detection

We performed an extensive analysis on the precision and accuracy of a Finnigan ESI-quadrupole operating in the selected ion monitoring (SIM) mode and an ion trap mass spectrometer to determine the ID of Kemptide along with the ability to quantify small increases in ^2H . A minimally deuterated Kemptide generated by alkyl H/D exchange [22,23] was used for serial isotope dilution to generate a series of Kemptide solutions of known isotopic composition. These solutions were analyzed with MALDI-TOF, ESI-ion trap (Zoom Scan) and ESI-quadrupole mass spectrometers and the results reported in Table 1. In these results, to our surprise, the ion trap instrument performed better than the SIM quadrupole determination which had greater precision than the MALDI-TOF analyses. The performance of the quadrupole analysis contributed to our more thorough evaluation of the potential effects of mass granularity reported in the accompanying paper [15]. Based on the results reported there, the accuracy of the quadrupole could have been improved if the data had been acquired and analyzed in profile scan rather than SIM mode.

Accuracy and precision of IDs by tandem mass spectrometry

IDs can be obtained by tandem mass spectrometry if the entire IIC is included during fragmentation. Tandem mass spectra acquired for Trp-cage and Glu-fibrinopeptide are shown in Figures 2A and 2B acquired with the Finnigan LTQ ion trap and the ABI Q-STAR, respectively. Changes in the IDs can be easily observed as the M_1/M_0 ratio increases with increasing mass of the product ions as expected from the increasing number of carbon atoms. To determine the IDs of product ions with the greatest precision, we have found that acquiring data in the zoom-scan profile mode with the ion trap, or the profile mode with Q-TOF instruments is essential. Further, to generate both accurate and precise IDs in the product ions generated in tandem mass spectrometry, it is essential that there not be a significant discrimination against either the higher or lower isotopologues during the isolation of the molecular ion. Both the ion trap and Q-TOF instruments allow control of the isolation width of precursor ions. This phenomenon is illustrated in Figure 3, where the change in the ID is dramatically affected by the isolation width of the molecular ion. Figure 4A shows the variation of the accuracy of the observed value of m_1 vs. the isolation width while Figure 4B shows the variation of m_1 as a function of intensity. Similar results were obtained with the Q-STAR where the tandem mass spectra were acquired in the profile mode and varying the settings of the low-resolution mode (data not shown). For peptides $< 2500\text{ }m/z$, isolation widths of 10 for the ion trap and a low-resolution setting of 1.8 for the Q-STAR proved to be sufficiently large.

The IDs of each of the product ions for the Trp-cage peptide were analyzed to determine m_1 by non-linear least squares. This was repeated on three separate days with the results plotted in Figure 5. The agreement of the experimental m_1 values with those calculated from equation 5 indicates that many of the product ions have sufficient intensity to permit their isotopic composition to be determined with precision and accuracy. An Excel spreadsheet is available as Supplementary Material ("Tryptic Digest ID calc.xls") which generates the theoretical tryptic digest of a protein, the natural abundance ID for each tryptic peptide, and subsequently tabulates the elemental composition along with natural abundance values of m_1 and m_2 for the b and y" series of ions for every tryptic peptide.

If the ID for two successive y" or b ions can be determined, the isotopic composition of the individual amino acid residue that differentiates the two product ions can be determined by difference. To evaluate the incorporation of ^2H into the Trp-cage molecule by alkyl H/D exchange the ID of the y" and b series of ions were determined and the results are also plotted in Figure 5. The results indicate significant alkyl H/D exchange into the amino-terminal residues as evidenced by the large increases in m_1 between y_{15}'' and y_{16}'' , and between y_{16}'' and y_{18}'' .

Determination of ^2H incorporation into proteins from $^2\text{H}_2\text{O}$

One potential application of the ability to quantify isotope incorporation via analysis of IDs comes from metabolic studies utilizing $^2\text{H}_2\text{O}$. Low mol fractions of ^2H can be introduced into animals (including humans) resulting in the metabolic incorporation into amino acids and subsequently into *de novo* synthesized proteins [24]. The methodology described here permits the quantitation of the incorporated ^2H into peptides. This is shown in Figure 6A where changes in the ID of a tryptic peptide of serum albumin are detected. The peptides were generated by isolating serum albumin at the indicated times following a bolus of $^2\text{H}_2\text{O}$ being administered to the rodent. The incorporation of ^2H into three separate albumin tryptic peptides as a function of time is shown in Figure 6B. While these data are obtained on the intact peptide, the demonstration that product peptides obtained by tandem mass spectrometry can be utilized could eliminate difficulties when peptides have overlapping IICs. The potential for the application of this procedure to the analysis protein turnover on a proteomic basis and its primary advantage of obviating the need for pure protein has been presented in preliminary form [25].

SUMMARY

In these two contributions we have established the means to detect by tandem mass spectrometry the incorporation of low levels of a heavy isotope into both molecular and, importantly, product ions obtained by tandem mass spectrometry. Additionally, the benefits of acquiring the data in profile mode and subsequently fitting the IIC to a series of sequential identically-shaped peaks has been employed to obviate inaccuracies inherent to SIM data acquisitions due to mass granularity. A new rapid method of calculating IDs normalized to the monoisotopic peak was developed. This method eliminates the requirement for the complete cluster of isotopologue ions to be acquired and has been incorporated into standard non-linear least squares routines to permit the quantitation of incorporated isotopic label (in the case of low level isotope incorporation) or the determination of mol fraction of an enriched isotopically-substituted compound. These combined methods have proven useful in quantifying isotope incorporation into peptides for biophysical and metabolic studies and for the study of kinetic isotope effects on reactions employing polynucleotide substrates. Excel spreadsheets implementing these methods have been made available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

ID of Trp-cage obtained by MALDI-TOF mass spectrometry shown as the solid black line. The fit to 6 sequential identically shaped peaks is indicated by the gray (green) line with the each separate peak shown by the dashed line. The relative amplitudes predicted by determining m_1 and m_2 from the elemental formula are shown as sticks at the mass centroid of each peak.

Figure 2.

(A) LTQ linear ion trap tandem mass spectrum of Trp-cage from fragmenting the doubly charged precursor ion of m/z 1086 with an isolation width of 10 and the product ion spectrum obtained in the zoom scan mode. The sequence of Trp-cage is shown with the y and b product ions consistent with the sequence labeled. The IDs of several of the product ions are shown as insets.

(B) Q-TOF tandem mass spectrum of [Glu 1]-fibrinopeptide B from fragmenting the doubly charged parent ion of m/z 785 with low resolution mode. 0.1 μ M sample was infused directly into the ESI-sMS at a flow rate of 1 μ L/min.

Figure 3.

Two typical product ion mass spectra of Trpcage b_{16}^+ (m/z of 1772.9) and y_{14}^+ (m/z of 1351.7) obtained with isolation widths of 2 (A) and of 10 (B).

Figure 4.

Root mean square mass errors obtained from analysis of the IDs of y_{11} – y_{17} ions from LTQ linear ion trap tandem mass measurement are shown as a function of parent ion isolation width (A) with Intensity $\geq 10^3$, and daughter ion intensity (B) at an isolation width of 10.

Figure 5.

Plot of the experimentally determined m_1 compared to the calculated m_1 for the successive y'' ions of Trp-Cage at natural abundance and following alkyl H/D exchange. ($\square$) The y'' ions of Trp-Cage at natural abundance were analyzed on three separate days. The error bars for each product ion were determined from the analysis of m_1 , and represent the reproducibility, i.e. the precision of the measurement. The vertical deviation from a line with slope 1 and intercept at the origin, shown on the graph, represents the inaccuracy of the determination of m_1 for each product ion. ($\circ$) Plot of experimentally determined m_1 values for Trp-Cage that has been labeled with a small mol fraction of ^2H by hydroxyl radical mediated alkyl H/D exchange. The incorporation of ^2H into each product ion is given by the vertical distance above the natural abundance line. The data indicate that the greatest H/D exchange occurred in the five N -terminal residues.

Figure 6.

(A) The incorporation of ^2H -into a tryptic peptide from mouse serum albumin as a function of time of exposure to $^2\text{H}_2\text{O}$ is shown by changes in the IDs obtained by of MALDI-TOF mass spectrometry. (B) The ^2H incorporation calculated as $(\text{excess } m_1) / x^2\text{H}_2\text{O}$ determined from the IDs is plotted as a function of time of exposure to $^2\text{H}_2\text{O}$ for the three different tryptic peptides indicated by their sequences. As expected the time courses can be fit by with a single rate constant but with different amplitudes. Each peptide's amplitude reflects the different number of metabolically equilibrated hydrogens present.

Table 1

ID analysis of [²H]Kemptide

Method	Ratio ^I	Day-1 <i>m</i> _I (S.D.)	Day-2 <i>m</i> _I (S.D.)	Day-3 <i>m</i> _I (S.D.)	Day-4 <i>m</i> _I (S.D.)	Daily variation <i>m</i> _I (S.D.)
ESI-IT	HxD	119.49 (0.69)	119.0 (0.28)	117.06 (0.24)	118.27 (0.45)	118.46 (0.42)
	1:3	85.73 (0.95)	85.74 (0.59)	85.60 (0.32)	85.59 (0.61)	85.66 (0.62)
	1:1	63.08 (0.93)	63.37 (0.15)	63.14 (0.20)	63.56 (0.38)	63.29(0.41)
	3:1	46.76 (1.15)	48.40 (0.11)	48.30 (0.09)	48.63(0.29)	48.02 (0.41)
	9:1	43.12 (1.65)	42.71 (0.13)	42.57 (0.25)	42.36 (0.14)	42.69 (0.54)
	81:1	40.12 (0.55)	39.72 (0.05)	39.97 (0.15)	39.68 (0.29)	39.87 (0.26)
	Std	39.25 (0.10)	39.39 (0.19)	39.00 (0.05)	38.51 (0.26)	39.04 (0.15)
	Slope (r ²)	1.01 (0.995)	1.03 (0.999)	1.03 (0.998)	1.01 (0.9993)	1.02 (1.000)
MALDI TOF	HxD	120.99 (1.63)	121.5 (1.05)	118.9 (1.45)	120.7 (1.15)	120.52 (1.32)
	1:3	88.41 (1.36)	88.06 (3.47)	82.28 (3.82)	87.95 (1.28)	86.67 (2.48)
	1:1	62.09 (1.52)	64.71 (1.88)	63.20 (0.70)	64.05 (1.78)	63.51 (1.47)
	3:1	50.43 (3.22)	49.83 (1.62)	51.85 (3.71)	47.00 (2.42)	49.78 (2.74)
	9:1	42.58 (2.26)	41.94 (1.51)	43.95 (0.17)	42.18 (2.23)	42.66 (1.54)
	81:1	41.80 (1.25)	39.13 (0.88)	45.58 (3.03)	41.04 (1.57)	41.89 (1.68)
	Std	40.25 (1.33)	40.54 (2.13)	43.33 (1.72)	40.60 (0.74)	41.18 (1.48)
	Slope (r ²)	1.03 (0.990)	1.06 (0.9996)	0.88 (0.989)	1.06 (0.992)	1.01 (0.9926)
ESI-Q	HxD	118.42 (1.28)	113.76 (2.14)	112.20 (0.92)	113.29 (0.93)	114.42 (1.32)
	1:3	82.27 (0.54)	82.96 (0.95)	77.96 (0.99)	82.17 (2.29)	81.34 (1.19)
	1:1	60.29 (0.51)	61.97 (1.27)	60.67 (1.94)	61.06 (2.94)	61.00 (1.67)
	3:1	46.33 (0.69)	46.93 (0.47)	48.82 (1.43)	46.33 (1.58)	47.10 (1.04)
	9:1	39.76 (1.34)	41.01 (0.40)	39.22 (0.73)	40.16 (0.81)	40.04 (0.82)
	81:1	38.83 (1.03)	39.19 (1.37)	38.62 (0.77)	39.17 (2.50)	38.95 (1.42)
	Std	38.68 (1.10)	37.54 (0.94)	37.48 (1.39)	39.24 (0.08)	38.23 (0.88)
	Slope (r ²)	0.99 (0.997)	1.05 (0.999)	0.95 (0.993)	1.04 (0.997)	1.01 (0.996)

^I
HxD and Std are samples of H/D exchanged and standard without exchange