Determination of the uncertainties in the theoretical mass isotopomer distribution of molecules.

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Abstract

A procedure for the determination of the uncertainties in the theoretical mass isotopomer distribution of molecules due to natural variations in the isotope composition of their constituting elements is described here for the first time. For this purpose, a Visual Basic macro for Microsoft Excel was written by adapting the direct stepwise calculation algorithm published by Kubinyi (Anal. Chim. Acta **1991**, *247*, 107-119, Fig. 1). In our procedure no pruning threshold factors were used to eliminate round up errors for large molecules. Then, the Kragten procedure of uncertainty propagation (Analyst **1994**, *119*, 2161-2165) was applied taking into account the correlation coefficients between the isotope abundances of the corresponding atoms. For bi-isotopic elements (C, H, N, Cl, Br) the correlation coefficients were given the value of –1. For tri- and tetra-isotopic elements the correlation coefficients were calculated using the mass dependent fractionation law used in stable isotope geochemistry and values of

+1 or -1 were obtained depending on the isotope system considered. It was observed that for small organic molecules of natural isotope abundances, such as phenol or polybrominated diphenylethers, the method provided relatively small propagated uncertainties similar in magnitude to those measured experimentally. For ^{13}C-labelled molecules the calculated uncertainties were mainly due to the uncertainties in the isotope enrichment of ^{13}C and were much larger than the experimental uncertainties. For large molecules of natural isotope abundances, such as peptide $C_{68}H_{107}N_{17}O_{25}$ (NIST 8327 RM), the uncertainties in their mass isotopomer distributions were much larger and their source could be assigned mainly to the uncertainty of the natural isotope composition of carbon. When the size of the molecule was even larger, such as bovine insulin ($C_{254}H_{377}N_{65}O_{75}S_{6}$), Kragten procedure provided a good estimate for the uncertainty when the most probable isotope composition of carbon in mammals was used in the calculations.

Keywords

Mass Isotopomer Distributions; Uncertainty propagation; Mass Spectrometry; Labelled compounds.

Introduction

The term “uncertainty” is perhaps one of the most employed in modern Analytical Chemistry after the widespread introduction of quality management systems in the analytical laboratory. It has been described in the Vocabulary of basic and general terms in Metrology [1] as “A parameter associated with the result of a measurement that characterize the dispersion of the values that could reasonably be attributed to the measurand”. Therefore, when an analytical procedure is implemented, a full uncertainty budget should be included as a part of method development and validation. The calculation of uncertainty budgets should take into account all possible sources of uncertainty in the measurements including also “the uncertainties in reference data such as atomic and molecular weights or concentrations of calibrants” [2]. In our laboratory we are working on an alternative procedure for organic isotope dilution analysis which requires the use of the theoretical mass isotopomer distributions, both for natural abundance and isotopically labelled molecules, in the calculations. According to EURACHEM [2], the uncertainties in the theoretical mass isotopomer distributions of the molecules will need to be computed for the estimation of the uncertainty budgets in this new procedure.

To the best of our knowledge uncertainty estimations for mass isotopomer distributions of molecules have never been described in the literature. However, natural variations in the isotope abundances of the elements will change slightly the isotope composition of a molecule. For example, Hellerstein and Neese [3] indicated that natural variations in the isotope composition of carbon in mammals (typically from 1.08% to 1.11% ^{13}C relative abundance [3]) may have a small influence in Mass Isotopomer Distribution Analysis (MIDA) calculations but the effects were considered negligible in their calculations and natural variations were not taken into account. Additionally, theoretical isotope compositions have been used also in qualitative analysis, e.g. for the confirmation of the chemical formula of organic compounds [4] using automatic recognition algorithms. Nevertheless, the uncertainties in the mass isotopomer distribution of candidate molecules have never been implemented in these recognition algorithms.

In many other mass spectrometric applications, such as the optimisation of culture isotope labelling conditions [5], the simulation of isotopomer mass distribution experiments [3] and during the development of isotope dilution analysis procedures for peptides and proteins [6], theoretical mass isotopomer distributions will need to be calculated both at natural abundances and at different isotopic enrichments. If those theoretical isotope patterns are used to obtain quantitative results their uncertainties will need to be calculated.

Current isotope dilution procedures for organic and biological compounds do not require the computation of theoretical isotope distributions. In these procedures the labelled compound is used only as an internal standard added to samples and calibrants so its concentration or its isotope composition does not need to be known precisely. However, in the last few years, alternative calculation procedures for isotope dilution analysis, which involved the measurement of the isotope compositions by Mass Spectrometry, have been developed for elemental analysis [7, 8]. The evaluation of such procedures required the calculation of uncertainty budgets in which the uncertainties in the isotope composition of the natural abundance elements must be included [9]. In our laboratory we are trying to extrapolate such methods for the isotope dilution analysis of organic molecules so it was required to calculate both the theoretical mass isotopomer distribution of the natural abundance and labelled molecules and their uncertainties.

For the determination of molecular mass isotopomer distributions several algorithms have been published [10-12] and most computer programs provided by manufacturers of mass spectrometers include a “theoretical isotope peak distribution” calculator. Unfortunately, none of these algorithms or computer programs includes an uncertainty estimate for the calculated mass isotopomer distributions. In this paper we try, for the first time, to develop a simple procedure, based on a Microsoft Excel spreadsheet, to estimate the uncertainties in the theoretical mass isotopomer distribution of molecules.

There are several practical difficulties in the calculation of the uncertainties in the isotope composition of molecules. If we take the general equation for uncertainty propagation for a function *y* whose value depends on the parameters x_1, x_2,…x_n , the uncertainty *u(y)* is expressed by [2]:

$$u(y(x_1,x_2...x_n)) = \sqrt{\sum_{i=1,n}\left(\frac{\partial y}{\partial x_i}.u(x_i)\right)^2 + \sum_{i,k=1,n}\left(\frac{\partial y}{\partial x_i}\cdot\frac{\partial y}{\partial x_k}.u(x_i,x_k)\right)} \qquad (1)$$

where $u(x_i)$ are the standard uncertainties for each parameter in the equation and $u(x_i,x_k)$ are the covariances. The values of the covariances can be estimated from the standard uncertainties for each parameter and the corresponding correlation coefficients, r_{ik}, as [2]:

$$u(x_i, x_k) = r_{ik}.u(x_i).u(x_k) \qquad (2)$$

The mathematical algorithms used for the calculation of isotope distributions apply either a polynomial expansion [10], a direct stepwise combination of the isotope composition of the elements [11] or a Fourier transform convolution procedure [12]. None of these algorithms admit differentiation so the direct application of equation (1) is not possible. An alternative would be to use calculation procedures which do not require differentiation of the function such as Kragten's method of uncertainty propagation [13] or Monte Carlo simulations. In our approximation we have selected Kragten method for two main reasons. First, the number of times the mass isotopomer distribution needs to be calculated (*n+1* times, where *n* is the number of isotopes involved) is much lower in comparison with Monte Carlo simulations, where between 100 and 1000 simulations need to be performed depending on the number of parameters involved. Second, Kragten's procedure is easily implemented using spreadsheet software and the validity of the results can be tested using positive and negative values of the estimated uncertainties. Kragten's method uses the following linear approximation:

$$\frac{\partial y}{\partial x_i} \approx \frac{y(x_i + u(x_i)) - y(x_i)}{u(x_i)} = \frac{u(y, x_i)}{u(x_i)} \qquad (3)$$

assuming that higher order terms in the MacLaurin series are negligible. The uncertainty in the function *y* due to the uncertainty in the parameter x_i, $u(y,x_i)$, is estimated as the difference in the values of the function *y* when the value of x_i is substituted by x_i+$u(x_i)$. So, using Kragten procedure equation (1) is transformed into:

$$u(y(x_1,x_2...x_n)) = \sqrt{\sum_{i=1,n}\left(u(y,x_i)\right)^2 + \sum_{i,k=1,n}\left(r_{ik}.u(y,x_i).u(y,x_k)\right)} \qquad (4)$$

where the covariances are substituted by the correlation coefficients and standard uncertainties using equation (2). It is claimed [2, 13] that, when the relative uncertainties of the parameters are small, this procedure provides acceptable accuracy for practical purposes. To check this assumption the uncertainties in the function y, $u(y,x_i)$, can be calculated both for positive and negative values of the standard uncertainties, $u(x_i)$. Then, the propagated uncertainty values calculated by both procedures should be similar [13]. As indicated in Kragten's procedure [13], "changes in a few per cent in $u(y)$ are not important regarding the uncertainties that standard deviations usually have".

For the determination of the mass isotopomer distribution of a given molecule, the parameters x_i will be the isotope abundances of the constituting atoms and their uncertainties $u(x_i)$ their tabulated natural variability. We have taken these data from the last compilation of the representative isotope composition of the elements and their uncertainties due to natural variations by the IUPAC [14]. It is clear that for certain elements, such as carbon, the isotope composition and uncertainties tabulated in the IUPAC table may not be adequate for animal or plant derived compounds where ^{13}C isotope enrichment compared to inorganic carbon is well documented. In this paper we have decided to use the IUPAC values for all elements but the database could be easily modified to suit particular circumstances (e.g. carbon isotope abundances for materials of biological origin such as bovine insulin).

Meija and Mester [15] reflected recently on the effect of isotope abundance correlation on the uncertainties of elemental atomic weights. The isotopic abundances of the elements are always correlated because the sum of all abundances is 1. The correlation coefficients for bi-isotopic elements such as carbon or hydrogen are always $r = -1$ [15]. However, for tri-, tetra- or poly-isotopic elements the correlation coefficients will need to be calculated in order to apply equation (4). In this work, the correlation coefficients between the isotope abundances of a given poly-isotopic element were calculated applying the mass dependent fractionation law [16] used in stable isotope geochemistry. The final procedure is applied here for the determination of the isotope composition and its uncertainty for small organic molecules, metallic chelates, peptides and proteins of current interest in our laboratory.

Procedures

Calculation algorithm for the isotope peak distribution

A Visual Basic programme was written as a macro for Excel by adapting the calculation algorithm described by Kubinyi [11]. To avoid round up errors no pruning thresholds were used and the whole mass isotopomer distribution was calculated. Additionally, and in contrast to Kubinyi's procedure, no normalization of the intermediate mass isotopomer distributions was done during the calculations so the sum of the whole isotopomer pattern was always 1. Data on the natural isotopic composition of the elements [14] and the exact mass of the isotopes [17] were introduced in the Excel spreadsheet and were read from the Visual Basic programme. Finally, the resulting isotopic composition for the first 20 consecutive masses was returned to the spreadsheet as output. The macro was tested by calculating the isotopic composition of bovine insulin ($C_{254}H_{377}N_{65}O_{75}S_6$) using exactly the same elemental isotope abundances than those employed by Kubinyi. The results were identical to those reported in his paper [11] indicating that no computing errors were present in the programme. Figure 1 shows the code employed in the Visual Basic macro. The actual version of the macro programme is prepared for 18 different types of atoms and the distribution is calculated for a maximum of 2000 consecutive masses. However, the number of atom types and the number of masses can be incremented easily. Data on number of atoms of each type in the molecule is read from column B in the worksheet “calculation”. Data in column C of the worksheet “calculation” provides the number of stable isotopes for each atom type. For ease of calculation, elements such as Cl or Br were given 3 isotopes each with abundance of 0.0000 for masses 36 and 80 respectively. The elemental isotope abundances are read from the worksheet “database” and the first 20 consecutive masses of the final distribution are then written in the worksheet “calculation”.

The worksheet “database” contained isotopic information, standard uncertainties and exact masses for natural abundance H, B, C, N, O, F, Si, P, S, Cl, Fe, Br, Se and I [14]. Thus, the mass isotopomer distribution and its uncertainty for molecules containing only these elements could be calculated in the actual version of the programme. In addition, isotopically enriched forms of C, Cl, Br and Fe were included to calculate the isotope pattern and its uncertainty for isotopically labelled molecules.

Kragten procedure for the calculation of mass isotopomer distribution uncertainties

The Kragten [13] procedure requires the isotope composition of the target molecule to be calculated *n+1* times being *n* the number of isotopes present in the molecule. For example, for phenol C_6H_6O, the number of isotopes is $n=7$ (^{1}H, ^{2}H, ^{12}C, ^{13}C, ^{16}O, ^{17}O and ^{18}O). The isotope composition is first calculated using the nominal isotope abundances given in the database and then it is calculated another *n* times by adding (or subtracting) sequentially the standard uncertainty in the isotope composition of every isotope. To illustrate how Kragten's procedure works, Table 1 shows the input data and the intermediate results obtained for phenol. Please note that the mass isotopomer distributions will be always given as absolute abundances with a constant sum of 1. As can be observed in part A of the Table, the isotope peak distribution at masses 94, 95, 96, 97 and 98 needs to be calculated 8 times by modifying sequentially the input isotope composition of each isotope as indicated in Table 1A. The results obtained for the isotopomer distributions calculated are shown in Table 1B. Finally, the standard uncertainties $u(y,x_i)$ for each isotopomer *i* (Table 1C) are calculated by subtracting the result obtained at the modified abundances from those calculated using the nominal elemental isotope abundances as shown also in Table 1. For example, the standard uncertainty for ^{13}C at nominal mass 95 of phenol, u(y,^{13}C), is 0.002267 (Table 1C).

Then, the combined standard uncertainties for each element *j* in the molecule were calculated using the equation:

$$u(y(Element_j)) = \sqrt{\sum_{i=1,n} \left(u(y,x_i)\right)^2 + \sum_{i,k=1,n} \left(r_{ik}.u(y,x_i).u(y,x_k)\right)} \qquad (5)$$

Equation (5) takes into account the correlation coefficients between the different isotopes of the element. The final total combined uncertainty was then calculated by the square sum of all the uncertainties due to each element using:

$$u(y) = \sqrt{\sum_{j=1,m} \left(u(y(Element_j)\right)^2} \qquad (6)$$

where *m* is the number of elements in the molecule. This calculation procedure allows to compute the contribution of each element in the molecule to the total uncertainties for each nominal mass (in %).

For validation purposes, the procedure was applied always twice by adding and subtracting the standard uncertainties in two separate calculations as recommended in the original paper by Kragten [13]. The final abundance uncertainties are indicated in this paper as u(A)(+) and u(A)(-) with two significant figures.

Results and discussion

Calculation of the correlation coefficients for different elements

As already indicated by Meija and Mester [15], the isotope abundances of di-isotopic elements are perfectly anti-correlated. The correlation coefficient for di-isotopic elements is always r = -1. That means that, for carbon for example, equation (5) will be simplified to:

$$u(y(Carbon)) = \sqrt{\left(u(y,{}^{12}C)\right)^2 + \left(u(y,{}^{13}C)\right)^2 + 2.(-1).u(y,{}^{12}C).u(y,{}^{13}C)} \qquad (7)$$

Similar equations will be given for hydrogen, boron, nitrogen, chlorine or bromine. However, for poly-isotopic elements, the calculation of the correlation factors is not trivial. Meija and Mester [15] proposed a method for the calculation of the correlation factors for tri-isotopic elements based on the stated isotope abundance uncertainties. However, their method could not be extended to any poly-isotopic element. In this paper we propose an alternative method which could be applied to any poly-isotopic element. This alternative method is based on the application of the mass dependent fractionation law [16], used in stable isotope geochemistry, to predict the correlation factors between the different isotopes of an element.

For an element in two possible isotope states *A* and *B*, the fractionation factor $\alpha_{A\text{-}B}$ between both states for two given isotopes *a* and *b* is given by the following equation [16]:

$$\alpha_{A-B}^{a/b} = \frac{R_A^{a/b}}{R_B^{a/b}} \qquad (8)$$

where $R_A^{a/b}$ and $R_B^{a/b}$ are the isotope ratios *a/b* in the states *A* and *B,* respectively. The mass dependent fractionation law calculates the fractionation factor for any other pair of isotopes of the same element, for example isotopes *c* and *b*, using:

$$\alpha_{A-B}^{c/b} = \left(\alpha_{A-B}^{a/b}\right)^{z} \qquad (9)$$

where $z = \frac{m_a}{m_c}\left(\frac{m_c - m_b}{m_a - m_b}\right)$ and m_a, m_b and m_c the exact masses of the corresponding isotopes [16].

For the calculation of the correlation coefficients we have assumed that state *A* is the nominal IUPAC isotope composition and state *B* is an altered composition but within the stated natural variation range [14]. Then, if we express equations (8) and (9) in terms of isotope abundances, *A*, instead of isotope ratios (e.g. $R^{a/b} = A^a/A^b$) we end up with:

$$\left(\frac{A_A^c}{A_A^b}\right) \cdot \left(\frac{A_B^a}{A_B^b}\right)^{z} = \left(\frac{A_B^c}{A_B^b}\right) \cdot \left(\frac{A_A^a}{A_A^b}\right)^{z} \qquad (10)$$

If we group all terms in state A (IUPAC values) as a single constant K_A we obtain:

$$\left(A_B^a\right)^z . \left(A_B^b\right)^{1-z} = K_A . A_B^c \qquad (11)$$

Equation (11) relates the isotope abundance of a given isotope c of a poly-isotopic element with the abundances of two other isotopes of the element, a and b, assuming a mass dependent fractionation of the isotopes a, b and c.

1. <u>Tri-isotopic elements</u>

For tri-isotopic elements, such as oxygen, magnesium or silicon, the relationship between the abundances of all isotopes will be given by equation (11) if the mass dependent fractionation law is obeyed. If we consider that the sum of all abundances is 1 we can give tentative values to one of the abundances (e.g. ^{30}Si) to calculate the other two. Equation (11) will provide always two solutions for the isotope abundances but only one of the solutions provided positive isotope abundances within the stated elemental uncertainties. For example, Figure 2 shows the calculated isotope abundances for ^{28}Si and ^{29}Si giving tentative values for ^{30}Si within the stated natural variation range (e.g. ^{30}Si abundances varied from 0.03081 to 0.03103) and assuming the mass dependent fractionation law. As can be observed, the variation in the isotope abundances follows a straight line of positive (^{29}Si vs. ^{30}Si) or negative (^{28}Si vs. ^{30}Si) slope. The calculated correlation coefficients were $r_{30/29}$ = 1, $r_{30/28}$ = -1 and $r_{29/28}$ = -1. For the case of oxygen the correlation coefficients found were $r_{18/17}$ = 1, $r_{18/16}$ = -1 and $r_{17/16}$ = -1. For magnesium the correlation coefficients were:

$r_{26/25}$ = 1, $r_{26/24}$ = -1 and $r_{25/24}$ = -1. As can be observed, all these correlation coefficients are values of 1 or -1 indicating a linear trend of positive or negative slope. For all three isotope systems tested, the slope is negative when comparing a minor and the major isotope and it is positive when the two minor isotopes are compared. In all cases the major isotope is the lower in mass (^{16}O, ^{24}Mg or ^{28}Si) and the minor isotopes are 1 or 2 masses higher. These results may be relevant for elemental isotopic studies as, for example, the isotope ratio ^{30}Si/^{28}Si (negative correlation) will change in nature relatively more than the ratio ^{30}Si/^{29}Si (positive correlation).

2. Tetra-isotopic elements.

For a tetra-isotopic element, such as sulfur, iron or chromium, the system of equations is more complex. First, we can establish two independent equations similar to equation (11) using three of the four isotopes of the element. For example, for iron we can establish two equations using isotopes 54, 56 and 58 or 56, 57 and 58. At the end we obtain two non-lineal equations with two unknowns which would provide 4 different solutions. We observed that only one of the solutions gave meaningful results with positive isotope abundances within the stated natural variability. As for the three-isotope systems, there was a linear variation between the isotope abundances with values of the six possible correlation coefficients of 1 or –1. The actual values found for the six correlation coefficients for iron were: $r_{54/56}$ = -1, $R_{54/57}$= -1, $R_{54/58}$ = -1, $R_{56/57}$ = 1, $R_{56/58}$ = 1 and $R_{57/58}$ = 1. These calculations were performed also for sulfur and chromium. The correlation coefficients obtained for sulfur were: $r_{33/32}$ = -1, $R_{33/34}$ = 1, $R_{33/36}$ = 1, $R_{32/34}$ = -1, $R_{32/36}$ = -1 and $R_{34/36}$ = 1 while for chromium: $r_{50/52}$ = 1, $r_{50/53}$ = -1,

$r_{50/54}$ = -1, $r_{52/53}$ = -1, $r_{52/54}$ = -1 and $r_{53/54}$ = 1. The correlation coefficients between the two major isotopes for each element are always negative (^{54}Fe-^{56}Fe, ^{53}Cr-^{52}Cr and ^{34}S-^{32}S) while for the minor isotopes the sign of the correlation coefficient depends on the corresponding isotope abundances and on their relative mass. No calculations were performed for other higher poly-isotopic elements. Once the correlation factors were calculated, the application of equation (5) for each element is straightforward.

Example 1: the mass isotopomer distribution of phenol

First, phenol was selected as a proof of concept to test the validity of this approach as this molecule has been used in our laboratory to develop a new procedure for isotope dilution analysis of organic molecules based on minimal labelling and Isotope Pattern Deconvolution [18]. The procedure was applied for natural abundance, singly $^{13}C_1$-labeled and fully $^{13}C_6$-labeled phenol. The nominal enrichment of the labelled compounds was given as 99% ^{13}C by the manufacturer [18]. Table 2A shows the final results obtained for natural abundance phenol. These results were obtained from the data shown in Table 1. Relative abundances higher than 0.0001 are given in the tables. The validity of the Kragten procedure was evaluated by calculating the propagated uncertainties both after adding or subtracting the standard uncertainties for each isotope. The uncertainty values calculated by adding the standard uncertainties are indicated as u(A)(+) and those calculated by subtracting the standard uncertainties as u(A)(-). The uncertainty results were identical to the 5th decimal place for all masses. For example, the uncertainty for mass 94 changed only from 0.002279 to 0.002275 validating the uncertainty calculation procedure for this compound. Table 2 also shows the distribution of the uncertainty between the different elements after calculating the relative contribution from equation (6). It was observed that for the natural abundance compound, more than 99% of the uncertainty at masses 94 and 95 was due to the uncertainty in the isotope composition of carbon.

For the calculation of the isotope distribution of the labelled phenols and its uncertainty, we needed to establish the uncertainties for the isotope composition of ^{13}C in both labelled compounds. The isotope enrichment of the labelled compounds was indicated as 99% by the manufacturer [18] so the uncertainty was calculated assuming a rectangular distribution [2] and dividing the maximum range (±1%) by the square root of 3. The results obtained for the singly ($^{13}C_1$) and fully labelled ($^{13}C_6$) phenol are summarized in Tables 2B and 2C respectively. For the singly labelled phenol the uncertainty values were almost the same by both calculation procedures (adding or subtracting the standard uncertainties). However, for the fully labelled phenol these uncertainties differ after the 3rd decimal figure and mainly for low abundance masses. For the main peak at mass 95 of $^{13}C_1$-phenol the abundance and its uncertainty would be given as 0.9358 ± 0.0055 while for the main peak of $^{13}C_6$-phenol at mass 100 it would be 0.939 ± 0.033 with only three significant figures. As can be observed, the uncertainties increased with the number of ^{13}C atoms in the molecule due to the high uncertainty in the ^{13}C isotope enrichment. Table 2 also includes the sources of uncertainty for all masses in the isotope distribution in % for each element. For the labelled compounds, the uncertainty was dominated by the ^{13}C isotope enrichment uncertainty. When the isotope composition of the labelled compounds is used in the isotope dilution calculations [18], it is clear that certificates of ^{13}C isotope enrichment with more significant figures will be needed to reduce this uncertainty source.

Table 2 also shows the experimental uncertainties [18] obtained for the measurement of the mass isotopomer distribution of natural abundance phenol and both ^{13}C labelled compounds by GC-MS. Experimental details can be found elsewhere [18]. As can be observed the experimental uncertainties (standard deviations from n=3 independent experiments) are smaller that the theoretical uncertainties particularly for the ^{13}C labelled phenols. So, the isotope enrichment for these labelled compounds could be better certified by the experimental measurement of its mass isotopomer distribution.

Example 2: the mass isotopomer distribution of brominated diphenyl ethers

We have synthesized in or laboratory a group of brominated diphenyl ethers (PBDEs) labelled with ^{81}Br for their use as standards for the determination of PBDEs in environmental samples [19] using Isotope Pattern Deconvolution procedures. For our purposes, the mass isotopomer distribution and its uncertainty both for the natural abundance and labelled compounds had to be determined. In this case, the bromine isotope composition of the labelled PBDEs could be measured in our laboratory [19]. By using GC-ICP-MS coupling the isotope composition of bromine was determined to be 0.9953 for ^{81}Br and 0.0047 for ^{79}Br with standard uncertainties of 0.0001 for both isotopes.

These uncertainties are lower than those tabulated for the natural variation expected for bromine [14], which are of 0.00035 for both bromine isotopes. The results obtained for the tetrabrominated diphenyl ether BDE-47 ($C_{12}H_6OBr_4$) are summarized in Table 3 for natural abundance BDE-47 (A), for the $^{81}Br_4$-labelled analogue (B) and for the commercially available $^{13}C_{12}$-labelled compound (C). As can be observed, the uncertainty values u(A)(+) and u(A)(-) are very similar in all cases validating the Kragten approximation. For the most abundant peaks in the mass spectrum of both natural and labelled compounds the uncertainty source is dominated by the carbon isotope composition with some contribution by the natural or enriched bromine isotope composition depending on the selected nominal mass. For the ^{13}C-labelled compound the only source of uncertainty was the isotope composition of carbon which was given as 99% enriched.

For the most abundant peak in the isotope distribution of natural $C_{12}H_6OBr_4$, nominal mass 486, the abundance would be indicated as 0.3307 ± 0.0015 while for the most abundant peak of the $^{81}Br_4$-labelled compound, nominal mass 490, the abundance would be 0.8600 ± 0.0042. For the $^{13}C_{12}$-labelled compound the abundance of the most abundant peak, nominal mass 498, was 0.333 ± 0.022. As can be seen in Table 3, the theoretical uncertainties increased drastically for the $^{13}C_{12}$–labelled compound in comparison with the natural abundance and the $^{81}Br_4$-labelled.

The experimental uncertainties (standard deviations of n=5 measurements [19]) are also given in Table 3. As can be observed the experimental uncertainties are similar in magnitude to the theoretical ones for the natural abundance compound and the $^{81}Br_4$-labelled. However, for the $^{13}C_{12}$-labelled compound the theoretical uncertainties are up to 20 times higher than the experimental ones. This means, as in the case of $^{13}C_6$-labelled phenol, that better certificate of the isotope enrichment could be obtained by the experimental measurement of the mass isotopomer distribution.

Example 3: the mass isotopomer distribution of $Fe_3Citrate_3$

The study of the molecule which transports iron in Fe-deficient plants [20] required the comparison of the experimental isotope profile with different theoretical profiles calculated both using natural abundance iron and isotopically enriched iron. When enriched ^{54}Fe was used for iron re-supply in Fe-deficient plants, the transporting molecule resulted to be a trinuclear iron-citrate of formula $(Fe_3C_{18}H_{15}O_{22})^{2-}$ as measured by LC-MS with electrospray ionisation [20]. The comparison of the theoretical and experimental isotope abundances both using natural abundance iron and ^{54}Fe-enriched iron confirmed the structure of the molecule [20]. Figure 3 shows the theoretical mass isotopomer distribution and its uncertainty for natural abundance $(Fe_3C_{18}H_{15}O_{22})^{2-}$. The u(A)(+) and u(A)(-) values were identical for this molecule to the 4th decimal place. The sources of uncertainty distributed for each element (in %) for several selected masses are also indicated in Figure 3. As can be observed, the uncertainty is dominated also here by the uncertainty in the isotope composition of carbon and, for some masses, with a small contribution from iron or oxygen. In summary, the calculation of the uncertainties of the theoretical mass spectra will improve the metrological quality of current formula assignment methods based on matching scores [4].

Example 4: the mass isotopomer distribution of peptide NIST 8327 RM

The preparation of isotopically labelled peptides for their use in the isotope dilution analysis of proteins after trypsin digestion is an area of growing interest. For this purpose, labelled peptides with one or several ^{13}C will need to be prepared and, perhaps, certified in isotope composition and concentration depending on the isotope dilution procedure applied. Anyway, both for the calibration of isotope dilution procedures, using the labelled peptide as internal standard, or for the certification of the concentration of labelled peptides using reverse isotope dilution analysis a certified natural abundance peptide standard will be required. Ideally, natural abundance peptide standards should be certified reference materials. Peptide NIST 8327 RM ($C_{68}H_{107}N_{17}O_{25}$) is one of the few peptides which can be obtained nowadays certified in purity on a weight basis. The proposed procedure has been applied to this peptide and the final results are summarized in Table 4. In this table we have reduced the number of significant digits to 4 because of the increased relative uncertainty. The main peak in the isotope distribution of this peptide occurs at nominal mass 1561 (exact mass 1561.76) with an abundance of 0.420 ± 0.012. The Kragten procedure shows slightly different values for the u(A)(+) and the u(A)(-) uncertainties. The main source of uncertainty in this molecule is again the isotope composition of carbon as can be extracted from Table 4. If this peptide was of natural origin (animal or plant material) its isotope composition and uncertainties would be different from the data shown in Table 4. First, biological carbon has higher ^{13}C isotope enrichment (between 1.08 to 1.11% for mammals [3]) in comparison to the IUPAC value (1.07% enrichment) and, second, the variation range is smaller. Obviously, for biological applications the isotope composition of carbon and its uncertainty used in the database would need to be changed.

Another important use of peptides in modern quantitative proteomics is in the study of the differential expression of proteins between control and altered physiological states. Several relative quantitation procedures using isotopically coded tags have been published in the literature [21]. One of those procedures, reductive dimethylation [22], involves the derivatisation of NH_2 groups in the peptide with H_2CO (formaldehyde). The result of the reaction is the introduction of two methyl groups in the N-terminal amino acid and in the ε-amino units of Lysine residues [22]. For differential expression proteomics control states would be derivatised with natural abundance formaldehyde while altered states would be derivatised with isotopically labelled formaldehyde and then both samples would be mixed before LC-MS(MS) analysis [22]. The use of D_2CO was recommended in the literature [22] but other labelling alternatives exist. We have calculated the isotope composition of peptide NIST 8327, whose amino acid sequence is DAEPDILELATGYR, after derivatisation with natural abundance and ^{13}C labelled formaldehyde enriched at 99% ^{13}C. As no Lysine is present in the peptide the derivatisation will introduce only two methyl groups in the molecule and the mass shift between the natural abundance and the isotopically labelled peptide would be of only two mass units. Traditionally [22], this mass shift is considered insufficient for relative protein quantitation because of overlap in the mass spectra, but it serves its purpose for this study. Figure 4 shows the calculated mass isotopomer distribution both for the natural abundance and the isotopically labelled derivatised peptide. The error bars on the relative abundances are the calculated propagated uncertainties. As can be observed, the base peaks at exact masses of 1589.8 and 1591.8 for the natural abundance and isotopically labelled peptide respectively show quite large propagated uncertainties while the peaks at M+1 mass units for both compounds show relatively low uncertainties with only a little lower isotope abundances. For example, for the natural abundance peptide the relative uncertainty reduced from 2.9% at mass M (base peak) to 0.6% at mass M+1 while for the labelled peptide the reduction is from 3.1% to 1.4%. That means that lower uncertainty in the differential expression proteomic studies would be provided if the ratio of labelled to unlabelled peptides was performed at the M+1 ions instead of at the M ion. This fact would have never been anticipated if no uncertainty calculations were performed.

Example 5: the mass isotopomer distribution of bovine insulin

Bovine insulin ($C_{254}H_{377}N_{65}O_{75}S_6$) has been till now the molecule of choice for the evaluation of isotope distribution calculation programs [4, 11]. However, no uncertainty values have ever been given for this protein. Thus, the validity of Kragten procedure for larger molecules will be tested here. For bovine insulin we have taken into account that the most probable isotopic composition of carbon in mammals is 1.09% ^{13}C with a range of variation of ± 0.02% [3]. This isotope composition of carbon is different from that given in the IUPAC tables (1.07% with an expanded uncertainty of ± 0.08%). The results obtained in the calculations are given in Table 5 to four significant figures. The first observation is that the isotope peak distribution is quite broad with a maximum absolute abundance of 0.1863 at nominal mass 5730 (M+3 ion, exact mass 5732.6). The calculated uncertainties u(A)(+) and u(A)(-) differ now in the 4th or 5th decimal place indicating that Kragten procedure provides also a good estimation of the uncertainty for this molecule (because of the reduced uncertainty in the carbon isotope composition). The method predicts also decreasing relative uncertainties for increasing exact masses from 5729.6 (3.2% relative uncertainty) to 5733.6 (0.2% relative uncertainty). Then, the relative uncertainties increased again as the abundance decreased for higher masses. The fact that the relative uncertainties in the theoretical mass isotopomer distributions are not the same for all masses could help in the selection of the best masses for automatic chemical formula assignment procedures or for isotope dilution calculations. From Table 5 it can be also observed that the calculated uncertainties are mainly due to the natural isotope variability of carbon with an increasing contribution from sulfur particularly for the high masses.

Conclusions

The uncertainties in the theoretical mass isotopomer distribution of molecules have been traditionally ignored in the previously published computation algorithms [10-12]. From a purely metrological point of view, and for future error propagation calculations, this subject needed to be addressed. We have developed a method capable of predicting the uncertainties in the low resolution mass isotopomer distribution of small and medium sized molecules. For larger molecules, such as bovine insulin, the method provides adequate results when the most probable isotope composition of carbon in mammals is taken into account.

For most of the studied molecules the main source of uncertainty is the natural variability in the carbon isotope composition. It is well known that the isotope composition of carbon can be measured very precisely by Isotope Ratio Mass Spectrometry (IRMS) for different compounds by coupling GC or LC to the IRMS using a combustion or oxidation interface. Then, better estimates of the isotope distribution of molecules could be accomplished.

There are two other aspects of the proposed procedure which may need to be addressed in future studies. First, interelemental correlations have not been taken into account but could be also present (a given isotope enrichment on ^{15}N could be correlated with the enrichment on ^{13}C or ^{18}O). The second aspect is that the isotope composition of carbon in a molecule may not be identical for all carbon atoms. Those two aspects would need to be addressed in future studies for a better evaluation of the mass isotopomer distribution uncertainties.

However, in its present form, the procedure developed here may find different applications. For example, in the formula pre-screening of tentative molecules using isotope peak abundances [4], where the uncertainties in the theoretical isotope abundances can be used to provide weighing factors in the algorithms. In addition, the full understanding and validation of quantitative methodologies based on the measurement of isotopomer abundances in organic isotope dilution analysis requires the calculation of full uncertainty budgets [18]. The uncertainties in the isotope composition of the molecules cannot be ignored from those budgets as they may be important contributors to the total uncertainty on the final analyte concentration.

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Table 1. Input data and intermediate results for the implementation of the Kragten procedure for the determination of the mass isotopomer distribution of phenol and its uncertainty.

A. Input Data.

Isotope	**Abundance (nominal)**	**Standard uncertainty**	**^{1}H**	**^{2}H**	**^{12}C**	**^{13}C**	**^{16}O**	**^{17}O**	**^{18}O**
^{1}H	0.999885	0.000035	**0.999920**	0.999885	0.999885	0.999885	0.999885	0.999885	0.999885
^{2}H	0.000115	0.000035	0.000115	**0.000150**	0.000115	0.000115	0.000115	0.000115	0.000115
^{12}C	0.9893	0.0004	0.9893	0.9893	**0.9897**	0.9893	0.9893	0.9893	0.9893
^{13}C	0.0107	0.0004	0.0107	0.0107	0.0107	**0.0111**	0.0107	0.0107	0.0107
^{16}O	0.99757	0.00008	0.99757	0.99757	0.99757	0.99757	**0.99765**	0.99757	0.99757
^{17}O	0.000380	0.000005	0.000380	0.000380	0.000380	0.000380	0.000380	**0.000385**	0.000380
^{18}O	0.00205	0.00007	0.00205	0.00205	0.00205	0.00205	0.00205	0.00205	**0.00212**
B. Calculated Isotopomer distributions.									
	Nominal Mass	**Mass isotopomer distribution**	**^{1}H**	**^{2}H**	**^{12}C**	**^{13}C**	**^{16}O**	**^{17}O**	**^{18}O**
	94	0.934570	0.934766	0.934570	0.936839	0.934570	0.934645	0.934570	0.934570
	95	0.061649	0.061662	0.061846	0.061774	0.063916	0.061654	0.061654	0.061649
	96	0.003626	0.003627	0.003639	0.003633	0.003753	0.003626	0.003626	0.003691
	97	0.000151	0.000151	0.000152	0.000152	0.000159	0.000151	0.000151	0.000156
C. Calculated standard uncertainties.									
		Nominal Mass	**$u(y,^{1}H)$**	**$u(y,^{2}H)$**	**$u(y,^{12}C)$**	**$u(y,^{13}C)$**	**$u(y,^{16}O)$**	**$u(y,^{17}O)$**	**$u(y,^{18}O)$**
		94	0.000196	0.000000	0.002270	0.000000	0.000075	0.000000	0.000000
		95	0.000013	0.000196	0.000125	0.002267	0.000005	0.000005	0.000000
		96	0.000001	0.000013	0.000007	0.000127	0.000000	0.000000	0.000066
		97	0.000000	0.000001	0.000000	0.000008	0.000000	0.000000	0.000004

Table 2. The isotope composition of natural abundance and labelled phenols and the distribution of their uncertainty sources between the different elements (in %).

A. Natural abundance phenol

Nominal Mass	Mass isotopomer distribution	u(A)(+)	u(A)(-)	u(A) experimental (n=3) [18]	Carbon	Hydrogen	Oxygen
94	0.9346	0.0023	0.0023	0.00092	99	1	0
95	0.0616	0.0022	0.0022	0.00066	99	1	0
96	0.00363	0.00014	0.00013	0.000072	76	1	23
97	0.000151	0.000008	0.000008	0.000050	73	1	26

B. $^{13}C_1$-phenol

Nominal Mass	Mass isotopomer distribution	u(A)(+)	u(A)(-)	u(A) experimental (n=3) [18]	Carbon	Hydrogen	Oxygen	^{13}C
94	0.0094	0.0055	0.0055	0.00015	0	0	0	100
95	0.9358	0.0055	0.0055	0.00029	12	0	0	88
96	0.0516	0.0018	0.0018	0.00014	97	1	0	2
97	0.00307	0.00011	0.00010	0.000095	57	1	39	3
98	0.000118	0.000006	0.000006	0.000026	66	1	32	1

C. $^{13}C_6$-phenol

Nominal Mass	Mass isotopomer distribution	u(A)(+)	u(A)(-)	u(A) experimental (n=3) [18]	Hydrogen	Oxygen	^{13}C
98	0.0014	0.0021	0.0011	0.00023	0	0	100
99	0.057	0.031	0.031	0.00079	0	0	100
100	0.939	0.033	0.032	0.00036	0	0	100
101	0.00112	0.00020	0.00020	0.00012	98	0	2
102	0.001929	0.000095	0.000094	0.000093	0	48	52

Table 3. Isotope composition of tetrabrominated diphenyl ether ($C_{12}H_6OBr_4$) and its uncertainty sources (%).

A. Natural abundance

Nominal Mass	Abundance	u(A)(+)	u(A)(-)	u(A) experimental (n=5) [19]	Carbon	Hydrogen	Oxygen	Bromine
482	0.05785	0.00032	0.00032	0.00062	76	0	0	24
483	0.00757	0.00025	0.00025	0.00023	99	0	0	1
484	0.2257	0.0011	0.0011	0.00098	92	0	0	8
485	0.02949	0.00097	0.00097	0.00018	100	0	0	0
486	0.3307	0.0015	0.0015	0.00058	100	0	0	0
487	0.0431	0.0014	0.0014	0.00036	100	0	0	0
488	0.21626	0.00091	0.00091	0.0012	89	0	0	11
489	0.02806	0.00092	0.00092	0.00026	100	0	0	0
490	0.05392	0.00020	0.00020	0.00077	44	0	0	56
491	0.00690	0.00023	0.00023	0.00028	99	0	0	1

B. Isotope labelled ($^{81}Br_4$)

Nominal Mass	Abundance	u(A)(+)	u(A)(-)	u(A) experimental (n=5) [19]	Carbon	Hydrogen	Oxygen	^{81}Br
486	0.000115	0.000005	0.000005	0.00045	1	0	0	99
487	0.000015	0.000001	0.000001	0.00081	37	0	0	63
488	0.01624	0.00035	0.00035	0.0012	5	0	0	95
489	0.002125	0.000082	0.000083	0.0038	71	0	0	29
490	0.8600	0.0042	0.0042	0.0055	99	0	0	1
491	0.1125	0.0037	0.0037	0.0021	100	0	0	0

C. Isotope labelled ($^{13}C_{12}$)

Nominal Mass	Abundance	u(A)(+)	u(A)(-)	u(A) experimental (n=5) [19]	Hydrogen	Oxygen	Bromine	^{13}C
493	0.0071	0.0038	0,0037	0.00044	0	0	0	100
494	0.0599	0.0020	0,0028	0.00071	0	0	1	99
495	0.028	0.014	0,014	0.00052	0	0	0	100
496	0.229	0.013	0,014	0.00090	0	0	0	100
497	0.041	0.021	0,021	0.00092	0	0	0	100
498	0.333	0.022	0,021	0.0015	0	0	0	100
499	0.026	0.013	0,013	0.00029	0	0	0	100
500	0.216	0.015	0,014	0.00089	0	0	0	100
501	0.0066	0.0032	0,0033	0.00022	0	0	0	100
502	0.0527	0.0038	0,0036	0.00031	0	0	0	100

Table 4. Isotope composition of natural abundance peptide NIST 8327 RM ($C_{68}H_{107}N_{17}O_{25}$) and its uncertainty sources (%).

Exact Mass	Abundance	u(A)(+)	u(A)(-)	Carbon	Hydrogen	Oxygen	Nitrogen
1561.8	0.420	0.012	0.012	97	2	1	0
1562.8	0.3444	0.0022	0.0024	90	2	8	0
1563.8	0.1610	0.0052	0.0050	97	2	1	0
1564.8	0.0548	0.0030	0.0028	95	2	3	0
1565.8	0.0150	0.0012	0.0010	93	2	5	0
1566.8	0.0035	0.0003	0.0003	91	2	7	0
1567.8	0.0007	0.0001	0.0001	89	2	9	0
1568.8	0.0001	0.0000	0.0000	87	2	11	0

Table 5. The isotope composition of bovine insulin ($C_{254}H_{377}N_{65}O_{75}S_6$) and its uncertainty sources (%).

Exact Mass	Abundance	u(A)(+)	u(A)(-)	Relative uncertainty (%)	Carbon	Hydrogen	Oxygen	Nitrogen	Sulfur
5729.6	0.0286	0.0009	0.0009	3.2	67	18	4	4	7
5730.6	0.0902	0.0021	0.0020	2.3	60	16	7	4	13
5731.6	0.1539	0.0023	0.0022	1.5	52	14	11	4	19
5732.6	0.1863	0.0013	0.0013	0.7	37	11	20	3	30
5733.6	0.1780	0.0003	0.0003	0.2	45	11	30	3	11
5734.6	0.1421	0.0012	0.0011	0.8	63	17	3	4	13
5735.6	0.0981	0.0015	0.0015	1.6	54	15	7	4	21
5736.6	0.0600	0.0014	0.0014	2.3	48	13	10	3	25
5737.6	0.0331	0.0010	0.0010	3.1	45	12	12	3	28
5738.6	0.0166	0.0006	0.0006	3.9	41	12	13	3	31
5739.6	0.0077	0.0004	0.0003	4.7	39	11	14	3	33
5740.6	0.0033	0.0002	0.0002	5.5	36	10	15	3	35
5741.6	0.0013	0.0001	0.0001	6.4	34	10	16	2	37
5742.6	0.0005	0.0000	0.0000	7.3	32	9	17	2	38
5743.6	0.0002	0.0000	0.0000	8.2	31	9	18	2	40

Figure 1. Visual Basic macro for Excel used

```
Sub CalDistIsot()
Dim Cpatt(2000): Dim c(18) As Integer: Dim Npeak(18) As Integer: Dim
Abund(18, 10): Dim D(2000)
Rem Read data from worksheets “calculation” and “database”
Natom = 18
For i = 1 To Natom: c(i) = Worksheets("calculation").Range("B" & 6 + i).Value:
Next i
For i = 1 To Natom: Npeak(i) = Worksheets("calculation").Range("C" & 6 +
i).Value: Next i
Count = 1
For i = 1 To Natom
   For j = 1 To Npeak(i)
      Count = Count + 1
      Abund(i, j) = Worksheets("database").Range("C" & 4 + Count).Value
   Next j
Next i
Cpatt(1) = 1
Rem Calculation of the mass isotopomer distribution
P = 1
Q = 1
For j = 1 To Natom
   For i = 1 To c(j)
      For k = 1 To 2000: D(k) = 0: Next k
      For k = P To Q
         For l = 1 To Npeak(j)
         D(k + l - 1) = D(k + l - 1) + Cpatt(k) * Abund(j, l)
         Next l
      Next k
      Q = Q + Npeak(j) - 1
      For k = 1 To 2000: Cpatt(k) = 0: Next k
      For k = P To Q
         Cpatt(k) = D(k)
      Next k
   Next i
Rem Write isotopomer mass distribution into worksheet “calculation”
   For i = 1 To 20
   Worksheets("calculation").Range("E" & i + 1) = Cpatt(i)
   Next i
Next j
End Sub
```

Figure 2. Three-isotope abundance plot for silicon calculated using the mass dependent fractionation law. (○) ^{29}Si, (●) ^{28}Si.

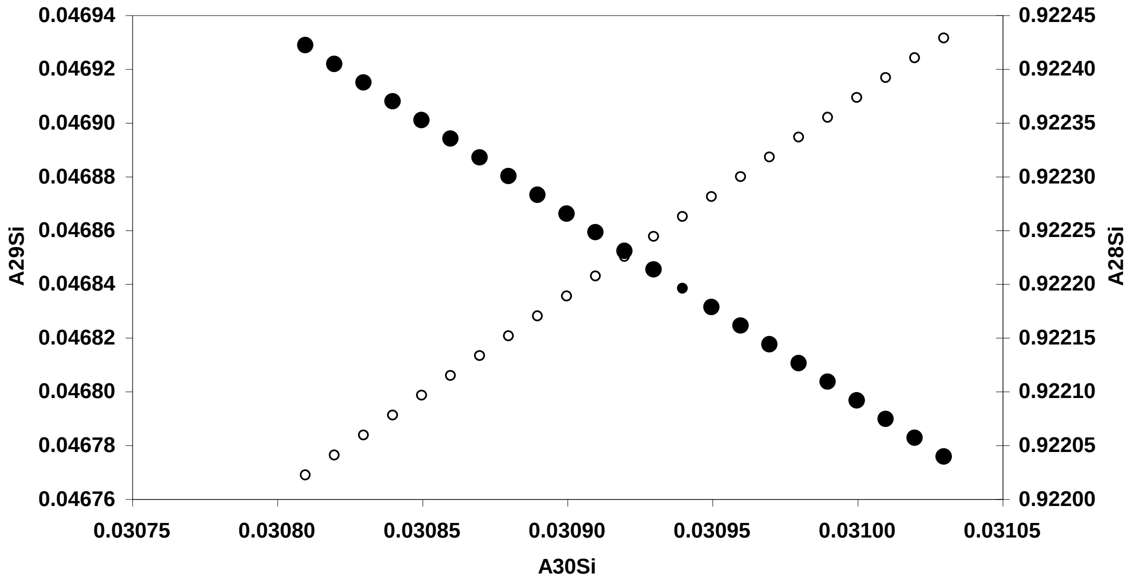

Figure 3. The isotope composition of the iron-transporting molecule in plants $(Fe_3C_{18}H_{15}O_{22})^{2-}$ and its associated uncertainty (error bars). The circular graphs represent the uncertainty sources (in %) distributed for the different elements at each selected mass.

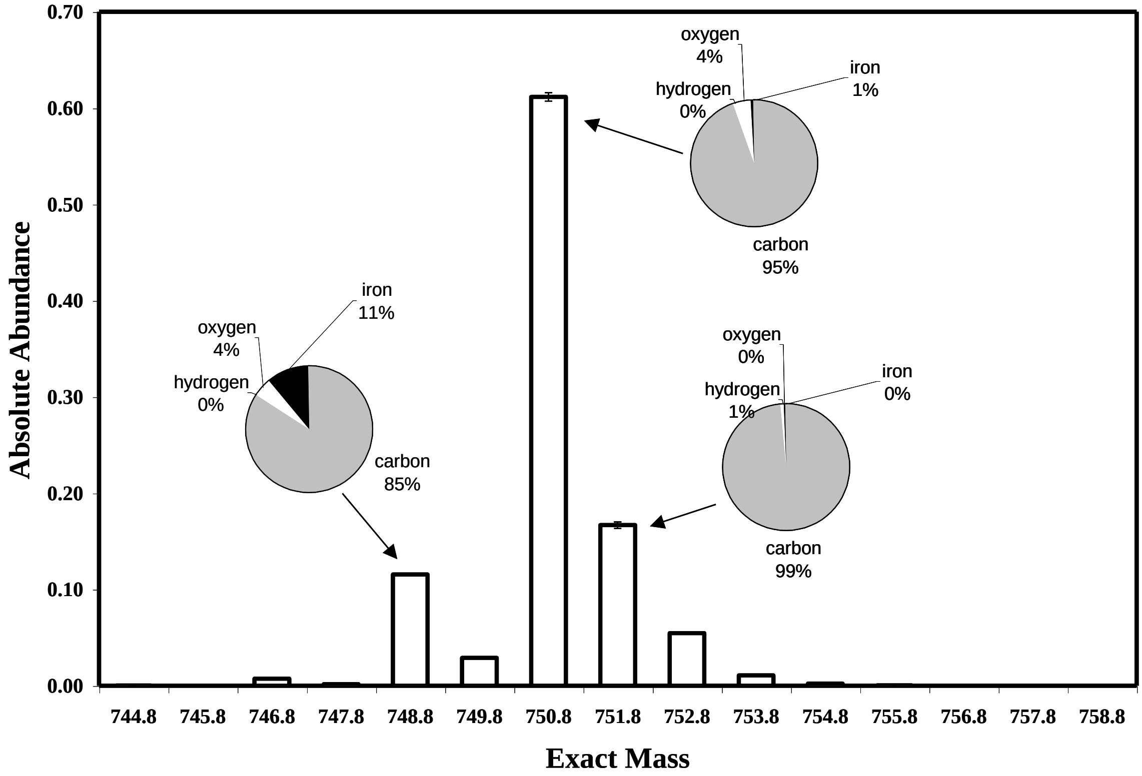

Figure 4. The calculated mass isotopomer distribution and their uncertainties for the dimethylated peptide DAEPDILELATGYR (NIST 8327) both using natural abundance (grey bars) or 99% enriched ^{13}C (white bars) formaldehyde as reagent.