Rapid determination of isotopic purity of stable isotope (D, 15N or 13C)-labeled organic compounds by electrospray ionization-high resolution mass spectrometry

Chenlong Liang1,2, Lin Ling2, Hao-Yang Wang2,\*

1School of Materials and Chemistry, University of Shanghai for Science and Technology, Shanghai, People’s Republic of China

2Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, People’s Republic of China

Correspondence: Hao-Yang Wang, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, 200032, People’s Republic of China.

Email: [haoyangwang@sioc.ac.cn](mailto:haoyangwang@sioc.ac.cn)

**Rationale:** Stable isotope-labeled organic compounds, containing D, 15Nor 13C, have widespread applications in chemistry, biology, environmental science, and agriculture. However, the isotopic purity calculations for these labeled organic compounds are usually complicated, especially in mixed isotopes-labeled scenarios. Herein, the electrospray ionization-high resolution mass spectrometry (ESI-HRMS) was applied to determinate the isotopic purity for stable isotope-labeled organic compounds, containing D, 15Nor 13C.

**Methods:** The representative isotopolog ion with its specific molecular formula was proposed to represent various labeled states. The isotopic purity was calculated with the corrected intensities of representative isotopolog ions by removing the natural isotopic contributions from preceding peaks. A unified equation has been proposed for the calculation of isotopic purity for various labeled situations including D, 15Nor 13C.

**Results:** Several case studies were presented and our calculated isotopic purities were all consistent with the isotopic purities provided in the certificate. In-source CID method was applied for the labeled compound (molecular weight＞400 u), when the maxim resolution setting was insufficient to differentiate isobaric isotopolog ions.

**Conclusion:** Finally, a workflow with a Python calculation program was summarized for determinations of the isotopic purity for mono isotope-labeled or mixed isotopes-labeled organic compounds, involving D, 15Nor 13C, by using ESI-HRMS to assigning the representative isotopolog ions with accurate mass and excluding the isobar interference.

**1. INTRODUCTION:**

In current researches on agriculture,1,2 chemistry,3 environment,4 food,5 and biochemistry,6,7 isotope-labeled compounds have emerged as powerful tools for protein interaction network analyses,8 analyzing complex biological processes.9 For radioactive isotope-labeled compounds and stable isotope-labeled compounds. There are practical disadvantages in using radioisotope labeling: first, it requires facility management and costly radioactive waste disposal (especially for 3H and 14C); second, the variety of radiolabeled reagents is limited; third, sensitivity of radiometric detection is inversely related to the half-life of radionuclides, requiring short half-life radionuclides (such as 32P) for sensitive experiments.10 Stable isotopes typically include 13C, 2H (D), 15N, 17O, 18O, 33S, 34S, 37Cl, and 81Br. The most commonly utilized isotope-labeled organic compounds typically contain a single type of stable isotope, such as deuterium (D), carbon-13 (13C), or nitrogen-15 (15N). These stable isotopes are non-radioactive, and no special precautions are required during separation, synthesis, preparation, or use, making the process safer to handle.11

With the rapid development of analytical instruments and increasing requirements under new trends, the use of isotopic purity characterization methods has substantially increased.20 For example, isotopic purity analysis methods based on mass spectrometry (MS)12-14, nuclear magnetic resonance (NMR)15-18, and Fourier-transform infrared spectroscopy (FTIR)19 have been developed. Mass spectrometry (MS) offers superior sensitivity and requires smaller sample amounts compared to NMR, IR, and other isotopic purity methods. MS is more versatile, capable of analyzing gases, liquids, and solids, and can efficiently analyze complex mixtures at faster speeds, especially with modern HR-MS. In contrast, NMR may not be suitable for all D-labeled compounds, particularly those prone to tautomerization at D-labeled positions to isomers.20

The development of mass spectrometry-based methods for isotopic purity characterization has progressed rapidly. Wei et al.21 developed a method using GC-MS data of standards to identify the signature ion from EI spectra of isotope-labeled samples, and a linear regression model deconvoluted the intensity of overlapping isotopologues. Li et al.22 proposed a Selected Ion Monitoring (SIM) method with Gas Chromatography-Mass Spectrometry (GC-MS) to enhance the signal-to-noise ratio, measurement precision and accuracy of 13C detection in cell metabolism studies. MOU et al.23 proposed a method based on the length of the carbon chains of the parent and daughter ions and whether the daughter ions contain 13C atoms, the one-to-one method, one-to-many method and SIM method were established for measuring 13C isotopic abundance. Lars et al.24 proposed a direct quantitative Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method for biological analysis, using internal standards for stable isotopic ratio calibration. Zheng et al.25 analyzed the isotopic abundance of 15N-labeled amino acids using electrospray ionization mass spectrometry centroid method. Du et al.26 developed a method combining MSTFA derivatization and GC-MS to determine amino acids, the isotopic abundance of amino acids in the 15N-labeled arginine fermentation broth was calculated based on the ion fragmentation information from the EI spectrum.

However, in certain or specialized applications, mixed-labeled organic compounds that incorporate two or even three types of isotopes are sometimes used.27,28 Most of the isotopic purity calculation methods reported in the reference for isotopic purity analysis are only applicable to a mono isotope-labeled organic compounds or cannot calculate the isotopic purity of each isotope individually in mixed-labeled organic compounds. As a continuation of previous research on the isotopic purity analyses of deuterated compounds,20 we further explored how to determinate the isotopic purity of 13C and 15N, and even for mixed-labeled compounds by electrospray ionization-high resolution mass spectrometry in this article. We want to establish a unified equation to calculate the isotopic purity of mono isotope-labeled organic compounds and the isotopic purity of each isotope in mixed isotope-labeled organic compounds. In order to simplify the calculation of isotopic purity in complex labeling situations, we have developed a Python program to facilitate the calculation of isotopic purity. This Python program is not only suitable for the calculation of isotopic purity for mono isotope-labeled organic compounds but also for the calculation of isotopic purity for mixed isotopes-labeled organic compounds.

**2. EXPERIMENTAL SECTION：**

**2.1 Reagents and Chemicals**

Methanol and dichloromethane were purchased from Merck KGaA (Darmstadt, Germany). Difenzoquat-13C6 (C1213C₆H₂oN₂O₄S, isotopic purity provided in the certificate: 98.50%), Sulfaquinoxaline-13C6 (C₆13C₈H₁₂N₄O₂S, isotopic purity provided in the certificate: 99.12%), Paclobutrazol-15N3 (C15H20Cl15N3O, isotopic purity provided in the certificate: 99.40%), Hydroxy ipronidazole-D3 (C7H8D3N3O3, isotopic purity provided in the certificate: 99.00%), Climbuterol-D9 (C13H10D9N3O, isotopic purity provided in the certificate: 98.41%), 3,5-Dinitrosalicylhydrazide-15N2 (C7H6N215N2O6, isotopic purity provided in the certificate: 96.50%), 2-NP-SCA-13C, 15N2 (C713CH8N215N2O3, isotopic purity provided in the certificate: 15N 98%;13C 99%), Ritonavir-13C, D3 (C3613CH45D3N6O5S2, isotopic purity provided in the certificate: 99.56%) were purchased from ANPEL-TRACE Standard Technical Services (Shanghai) CO. Ltd. (Shanghai, China). L-Methionine-D3 (C5H8D3NO2S, isotopic purity provided in the certificate: 99.90%) were purchased from Shanghai Yizhun Biology Co. Ltd. (Shanghai, China). Fipronil-sulfone-13C6 (C613C6H4Cl2F6N4O2S, isotopic purity provided in the certificate: 98.90%) were purchased from ANPEL Laboratory Technologies (Shanghai) Inc. (Shanghai, China). L-Glutamine-13C5 (13C5H10N2O3, isotopic purity provided in the certificate: 98.10%) were purchased from Shanghai Haoyuan ChemExpress Co. Ltd. (Shanghai, China). 2-Oxo Clopidogrel-13C, D3 Hydrochloride (C1513CH14D3Cl2NO3S, isotopic purity provided in the certificate: 98.40%) were purchased from Toronto Research Chemicals (Toronto, Canada).

**2.2 Mass spectrometric conditions**

The electrospray ionization-high resolution mass spectrometry experiments were performed on a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a standard ESI interface. Nitrogen was used as the sheath gas, auxiliary gas and collision gas. The basic ESI conditions were: spray voltage, 3500 V; capillary temperature, 275 °C; probe heater temperature, 300 °C; sheath gas flow rate, 35 arbitrary units; auxiliary gas flow rate, 10 arbitrary units; while the selected collision energy depended on the dissociation capability of the precursor ion. Data acquisition and analysis were carried out with the Xcalibur software package (version 4.2.47, Thermo Scientific).

**2.3 Sample handling**

The isotope-labeled organic compounds studied in this article did not contain active D atoms, thus there were no special requirements regarding the use of solvents. All samples examined in this article were dissolved in methanol to prepare solutions with a concentration of approximately 10 μg/mL for mass spectrometric analyses. All sample solutions were analyzed using the direct injection method. The injection volume was 0.20 μl, mass range was *m/z* 100–1000.

**3. RESULTS AND DISCUSSION：**

**3.1** **Calculations of isotopic purity based on the corrected intensity of representative isotopolog ions**

For a labeled organic compound, the general molecular formula is L(13CaDb15Ne). There would be various labeled states L(12C(a-x)13CxH(b-y)Dy14N(e-z)15Nz, where “a”, “b”, and “e” represent the maximum number of 13C, D, or 15N atoms at the labeling positions, 0 ≤ x ≤ a, 0 ≤ y ≤ b, and 0 ≤ z ≤ e, "L" refers to various natural elements at the non-labeled positions of this compound. Based on the suggestions of Attygalle,29 we proposed the representative isotopolog ions rather than the “monoisotopic ions” to represent various labeled states in the isotopic purity calculations. Each representative isotopolog ion has its specific molecular formula. In most cases for dealing small organic compounds, the mass of representative isotopolog ions could be calculated with Llowest(12C(a-x)13CxH(c-y)Dy14N(e-z)15Nz), Llowest represents the isotopes with the lowest mass for all elements within L. However, the mass peak of certain representative isotopolog ion might also include the natural isotopic contributions from preceding peaks (i.e. D, 15N, 13C). Therefore, the calculations of isotopic purity should be based on the corrected intensity of representative isotopolog ions by removing the natural isotopic contributions from preceding peaks.30,31,32 The ratios of the corrected intensities of representative isotopolog ions represent the molar ratios of various labeled states.

Following our previous studies on the isotopic purity of D-labeled organic compounds, electrospray ionization-high resolution mass spectrometry was also applied to determine the isotopic purity for mono isotope-labeled or mixed isotopes-labeled scenarios involving D, 15Nor 13C. The role of high-resolution mass spectrometry herein is the assigning the representative isotopolog ions with their specific molecular formulas by accurate mass measurements and excluding the adjacent interfering isobar ions in very close *m/z* values.33 In Figure 1, the mass spectra (in narrow band) for some extreme cases in singly positive-charged condition under infinite resolution were mimicked and showed to explain the situations we might encounter in our studies.



**FIGURE 1.** Three mimic mass spectra (in narrow band) for some extreme cases in singly positive-charged condition under infinite resolution. (A) The molar ratio of the two representative isotopolog ions is 1:1. The black portion represents the ionic species of protonated CvHw(HD0), and the gray striped portion represents the ionic species of protonated CvHw(H0D). (B) The molar ratio of the two representative isotopolog ions is 1:1. The black portion represents the ionic species of protonated CsHt(14N15N0), and the gray diagonal-striped portion represents the ionic species of protonated CsHt(14N015N). (C) The molar ratio of the three representative isotopolog ions is 1:1:1. The black portion represents the ionic species of protonated CP(12C213C0), the white portion represents the ionic species of protonated CP(12C213C0), and the gray portion represents the ionic species of protonated CP(12C213C0).

Figure 1A showed the example of protonated CvHw(D) with isotopic purity 50%, the *m/z* difference between the representative isotopolog ion of non-labeled state CvHw(HD0) and completely-labeled state CvHw(H0D) is 1.0063 u. The adjacent interfering isobar ion from 13C-isotopic peak of non-labeled state CvHw(HD0) with distance of only 0.0029u to the mass peak of the representative isotopolog ion of protonated CvHw(H0D) was showed. Since the natural relative abundance of D in nature is very low (0.0015%), we can ignore such contribution in practice. Figure 1B is the example of protonated CsNt(15N) with isotopic purity 50%, the *m/z* difference between the representative isotopolog ion of non-labeled state CsNt(14N15N0) and completely-labeled state CsNt(14N015N1) is 0.9970 u. The natural relative abundance of 15N is 0.369%, therefore the 15N-isotopic peak of non-labeled state of CsNt(14N15N0) from Nt should be removed. The distance between the mass peak of representative isotopolog ion of completely-labeled state CsNt(14N015N) and 13C-isotopic peak of non-labeled state of CsNt(14N15N0) from Cs is 0.0064 u. Such isobaric interference mostly could be separated in most situations for compounds (molecular weights ≤ 400 u) when applying 240000-resolution setting in mass analyses.

For the protonated Cp(13C2) with isotopic purity 50% containing same amount of Cp(12C13C) and Cp(12C213C0), the mimic mass spectrum was shown in Figure 1C. The mass peak of the representative isotopolog ion of protonated Cp(13C2) at [12Cp(12C013C2)+H]+ has contributions from the first 13C-isotopic ion of protonated Cp(12C13C) and the second 13C-isotopic ion of protonated Cp(12C213C0). Meanwhile, the mass peak of the representative isotopolog ion of protonated Cp(12C13C) at [12Cp(12C13C)+H]+ has contributions from the first 13C-isotopic ion of protonated Cp(12C213C0). Thus, the contributions of these preceding isotopic ions has to be removed during the calculations of isotopic purity of Cp(13C2).

Based on our previous studies20 and the discussions above, Equations (1-2) were presented to correct intensities of representative isotopolog ions by removing all natural isotopic contributions from preceding mass peaks and to calculate isotopic purity. Equation 1 describes the process of correcting the intensities of representative isotopolog ions. Because C, H, and N were all treated as di-isotopic elements, so the Equation 2 can be used to calculate the isotopic purity for all labeled scenarios involving D, 15N, and 13C. Here is our Equation 1:34,35

Note: “*f*” is the number of natural H, N or C atoms at non-labeled positions. 0≤*h*≤*j*-i and “*h*” is a positive integer. “*r*” is their relative natural abundance (as their sum being 100%) according to IUPAC36: D (0.0115%); 13C (1.07%); 15N (0.368%). “*j*” is the representative isotopolog ion being studied; “*i*” is all the preceding representative isotopolog ions (0≤*i*≤*j*-1) after corrections. *Ij(measured)* is the measured intensity of the mass peak containing certain representative isotopolog ion. *Ij(corrected)* is the intensity of certain representative isotopolog ion after removing all the natural isotopic contributions of preceding ionic species.

For a detailed example for explaining how to use Equation 1 with the mass spectrometric data in Figure 1C, please refer to the supporting information section 2. Therefore, the isotopic purity for all labeled scenarios involving D, 15N, and 13C could be calculated according toEquation 2. "*a*" represents the maximum number of isotope atoms at the labeling positions. Here is our Equation 2:

Based on the isotopic purity calculation Equation 1-2 presented above, we have developed a Python program (<https://github.com/samurai164/Isotopic-Purity-Calculation-Program>) for rapid isotopic purity calculations by inputting the compound molecular formulas and the mass spectrometry data list of the intensities of the mass peaks containing representative isotopolog ions. The detailed instructions for using the program can be found in the supporting information section 3.

**3.2** **Case studies**

**3.2.1 D-labeled organic compound**



**SCHEME 1.** The structures of stable isotope (D, 13C or 15N)-labeled organic compounds studied in this article.

The structure of Cimbuterol-D9 was showed in Scheme 1. The representative isotopolog ions with their specific molecular formulas for ten possibly labeled states and the mass peak intensities in ESI-HRMS spectrum (Figure 2A) were summarized in Table 1. Four representative isotopolog ions were detected and assigned by their accurate *m/z* values with relative error ≤5×10-6. From the Figure 2A, the 13C-isotopic ion of protonated C13H10N3O(HD8), [12C1213CH1014N316O(HD8)+H]+ at *m/z* 243.2141, could be well separated and the representative isotopolog ion of protonated C13H10N3O(H0D9), [12C13H1014N316O(H0D9)+H]+ at *m/z* 243.214, with only 0.0029 u distance when using the 240000-resolution setting. However, when the resolution is set at 15000, the two peaks cannot be well separated, which fully demonstrates the power of ultra-high mass spectrometry resolution to exclude the interference of nearby isobaric ions.37 Based on the corrected intensities of the representative isotopolog ions with removing the natural D contributions in Table 1, the isotopic purity (D) is calculated to be 98.69% with considerations of the natural D contributions and to be 98.69% without considerations of the natural D contributions. Such results showed the natural relative abundance of D in nature is too low (0.0015%), their contributions could be ignored in most cases. Both calculated isotopic purities were consistent with its isotopic purity (98.41%) provided in the certificate.

**TABLE1.** The data for the representative isotopolog ions of protonated Cimbuterol-D9 (Scheme 1) in its ESI-HRMS spectrum (Figure 2A).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Elemental compositions of representative isotopolog ions | Measured total intensity | Natural isotope correction | Natural isotope corrected intensity | |
| [12C13H1014N316O(H9D0)+H]+ | 0.0 | 0.0 | | 0.0 |
| [12C13H1014N316O(H8D1)+H]+ | 0.0 | 0.0 | | 0.0 |
| [12C13H1014N316O(H7D2)+H]+ | 0.0 | 0.0 | | 0.0 |
| [12C13H1014N316O(H6D3)+H]+ | 0.0 | 0.0 | | 0.0 |
| [12C13H1014N316O(H5D4)+H]+ | 0.0 | 0.0 | | 0.0 |
| [12C13H1014N316O(H4D5)+H]+ | 0.0 | 0.0 | | 0.0 |
| [12C13H1014N316O(H3D6)+H]+ | 70418.8 | 0.0 | | 70418.8 |
| [12C13H1014N316O(H2D7)+H]+ | 3018336.5 | 89.1 | | 3018247.4 |
| [12C13H1014N316O(HD8)+H]+ | 60049352.0 | 3818.6 | | 60045533.4 |
| [12C13H1014N316O(H0D9)+H]+ | 497517760.0 | 75968.5 | | 497441791.5 |

\* The relative error for all *m/z* values of representative isotopolog ions are within 5×10-6.



**FIGURE 2.** ESI-HRMS spectrum of the compound Cimbuterol-D9 at the 240000-resolution (A) and 15000-resolution (B) setting. In Figure 2A, the *m/z* difference between the representative isotopolog ion [12C13H1014N316O(HD8)+H]+ at *m/z* 242.2109 and representative isotopolog ion [12C13H1014N316O(H0D9)+H]+ at *m/z* 243.2170 is 1.0061 u.

**3.2.2 15N-labeled organic compounds**

The structure of Paclobutrazol-15N3 was showed in Scheme 1. The representative isotopolog ions with their specific molecular formulas for four possibly labeled states and the mass peak intensities in ESI-HRMS spectrum (Figure 3A) were summarized in Table 2. Four representative isotopolog ions were detected and assigned by their accurate *m/z* values with relative error ≤5×10-6. From the Figure 3A, the 13C-isotopic ion of protonated C15H20ClO(14N115N2), [13C12C14H2016O35Cl(14N115N2)+H]+ at *m/z* 297.1346, could be well separated and the representative isotopolog ion of protonated C15H20ClO (14N015N3), [12C15H2016O35Cl(14N015N3)+H]+ at *m/z* 297.1284, with only 0.0062 u distance when using the 240000-resolution setting. However, when the resolution is set at 15000, the two peaks cannot be well separated, this indicates that similar to the analysis of D isotopic purity the analysis of 15N isotopic purity also requires ultra-high mass spectrometry resolution to exclude the interference of nearby isobaric ions. Since there is no natural N at non-labeled positions in this compound, we can directly calculate the isotopic purity using the measured representative isotopolog ions. The isotopic purity (15N) is calculated to be 99.25%, which was consistent with its isotopic purity (99.40%) provided in the certificate.



**FIGURE 3.** ESI-HRMS spectrum of the compound Paclobutrazol-15N3 at the 240000-resolution (A) and 15000-resolution (B) setting. In Figure 3A the difference between the representative isotopolog ion [12C15H2016O35Cl(14N15N2)+H]+ at *m/z* 296.1316 and therepresentative isotopolog ion at [12C15H2016O35Cl(14N015N3)+H]+ at *m/z* 297.1284 is 0.9969u.

**TABLE 2.** The data for the representative isotopolog ions of protonated Paclobutrazol-15N3 (Scheme 1) in its ESI-HRMS spectrum (Figure 3A).

|  |  |  |  |
| --- | --- | --- | --- |
| Elemental compositions of representative isotopolog ion | Measured peak intensity | Natural isotope correction | Natural isotope corrected intensity |
| [12C15H2035Cl16O(14N315N0)+H]+ | 210201.3 | 0.0 | 210201.3 |
| [12C15H2035Cl16O (14N215N)+H]+ | 254757.2 | 0.0 | 254757.2 |
| [12C15H2035Cl16O (14N15N2)+H]+ | 11874951.0 | 0.0 | 11874951.0 |
| [12C15H2035Cl16O (14N015N3)+H]+ | 564177600.0 | 0.0 | 564177600.0 |

\* The relative error for all *m/z* values of representative isotopolog ion are within 5×10-6.

**3.2.3 13C-labeled organic compounds**

The structure of Fipronil-sulfone-13C6 was showed in Scheme 1. The representative isotopolog ions with their specific molecular formulas for seven possibly labeled states and the mass peak intensities in ESI-HRMS spectrum (Figure 4) were summarized in Table 3. Five representative isotopolog ions were detected and assigned by their accurate *m/z* values with relative error ≤5×10-6. From the Figure 4, the 37Cl-isotopic ion of sodium adduct C6H4Cl2F6N4O2S(12C313C3), [12C6H435Cl37Cl19F614N416O232S (12C313C3)+Na]+ at *m/z* 479.93321, could be well separated and the representative isotopolog ion of sodium adduct C6H4Cl2F6N4O2S(12C113C5), [12C6H435Cl219F614N416O232S(12C113C5)+Na]+ at *m/z* 479.9409, with only 0.0088 u distance when using the 240000-resolution setting. So when a 13C-labeled organic compound contains elements such as Cl, Br, or S, it is best to use ultra-high mass spectrometry resolution to exclude the interference of nearby isobaric ions. When the 13C-labeled organic compound does not contain these elements, LR-MS can also be used. Based on the corrected intensities of the representative isotopolog ions with removing the natural 13C contributions in Table 3, the isotopic purity (13C) is calculated to be 98.85%, which was consistent with its isotopic purity (98.90%) provided in the certificate.



**FIGURE 4.** ESI-HRMS spectrum of the compound Paclobutrazol-15N3 at the 240000-resolution setting.

**TABLE 3.** The data for the representative isotopolog ions of sodium adduct Fipronil-sulfone-13C6 (Scheme 1) in its ESI-HRMS spectrum (Figure 4).

|  |  |  |  |
| --- | --- | --- | --- |
| Elemental compositions of representative isotopolog ion | Measured peak intensity | Natural isotope correction | Natural isotope corrected intensity |
| [12C6H435Cl219F614N416O232S (12C613C0)+Na]+ | 170127.8 | 0.0 | 170127.8 |
| [12C6H435Cl219F614N416O232S (12C513C)+Na]+ | 47197.8 | 11040.3 | 36157.5 |
| [12C6H435Cl219F614N416O232S (12C413C2)+Na]+ | 0.0 | 0.0 | 0.0 |
| [12C6H435Cl219F614N416O232S (12C313C3)+Na]+ | 350545.1 | 67.8 | 350477.3 |
| [12C6H435Cl219F614N416O232S (12C213C4)+Na]+ | 0.0 | 0.0 | 0.0 |
| [12C6H435Cl219F614N416O232S (12C13C5)+Na]+ | 6165492.5 | 615.0 | 6164877.5 |
| [12C6H435Cl219F614N416O232S (12C013C6)+Na]+ | 115318352.0 | 400074.7 | 114918277.3 |

\* The relative error for all *m/z* values of representative isotopolog ions are within 5×10-6.

**3.3 The Cases for mixed stable isotope labeled organic compounds**

**3.3.1** **Case for mixed-labeled organic compounds with sufficient resolution**



**SCHEME 2.** The structures of the mixed-labeled organic compounds studied in this article.

A case study for the determination of isotopic purity of a compound (2-NP-SCA-13C,15N2 with one 13C atom and two 15N atoms labeling is presented herein. Its structure was shown in Scheme 2 and its ESI-HRMS spectrum showing strong sodium adduct ion signals in Figure 6. Based on our discussions above, six possible representative isotopolog ions of the sodiated 2-NP-SCA-13C, 15N2 that may appear in ESI-HRMS spectrum were proposed by showing their specific molecular formulas and their relations in Figure 5. As shown in Figure 6, the *m/z* difference between the mass peak of the representative isotopolog ion at [12C7H814N216O3(12C13C0,14N015N2)+Na]+ and the mass peak of the representative isotopolog ion at [12C7H814N216O3(12C013C,14N15N)+Na]+ is 0.0062u. At the 240000-resolution setting, such two representative isotopolog ions are well separated.

**FIGURE 5.** Due to the presence of one 13C atom and two 15N atoms labeling in this compound, six possible representative isotopolog ions of the sample 2-NP-SCA-13C,15N2 (C713CH8N215N2O3) that may appear in ESI-HRMS were proposed with their formulas and their theoretical *m/z* values were calculated. Form this Figure, the relations between six possible representative isotopolog ions of the sodiated 2-NP-SCA-13C,15N2 were also clearly presented. Four representative isotopolog ions could be assigned in Figure 6.

Since this compound contained one 13C atom and two 15N atoms labeling, the isotopic purity of each isotope could be calculated separately as: isotopic purity (13C) = 99.48%, isotopic purity (15N) = 99.79%, based on the corrected intensities data of I*j(corrected)* in Table 4. Our calculated isotopic purity (13C) and (15N) were consistent with the separated isotopic purities (13C 99%, 15N 98%, respectively) provided in the certificate. Finally, the total isotopic purity of this compound is calculated be 99.48% × 99.79% = 99.27% according the equation 3 (the abbreviation “*Ip*” is for “Isotopic purity”). Total isotopic purity represents the contribution of the labeled specie with one 13C atom and one or two 15N atoms in this compound. The contribution of the labeled species with one 13C atom and one 15N atom is 0.19%, and the contribution of the labeled species with one 13C atom and two 15N atoms is 99.07%. The sum of these two values was 99.27%, which was the total isotopic purity we calculated herein.

Then, the contribution of the specie labeled only with one 13C atom and non 15N atom in this compound is calculated to be 99.48% × (100% - 99.79%) = 0.21%. The contribution of the specie labeled only with one or two 15N atoms and non 13C atom in this compound is 99.79% × (100% - 99.48%) = 0.52%. The contribution of the specie with neither 13C nor 15N labeling in this compound is (100% - 99.79%) × (100% - 99.48%) = 0.000011%.



**FIGURE 6.** ESI-HRMS spectrum of the compound 2-NP-SCA-13C,15N2 (C713CH8N215N2O3) at the 240000-resolution setting showing strong sodium adduct ion signals.

**TABLE 4.** The data for the representative isotopolog ions of sodium adduct 2-NP-SCA-13C,15N2 (Scheme 2) in its ESI-HRMS spectrum (Figure 6).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Ion type | Elemental compositions of representative isotopolog ion | Measured peak intensity | Natural isotope correction | Natural isotope corrected intensity |
| 13C0 | [12C7H814N216O3(12C13C0, 14N015N2)+Na]+ | 606194.7 | 0.0 | 606194.7 |
| [12C7H814N216O3(12C13C0, 14N15N)+Na]+ | 0.0 | 0.0 | 0.0 |
| [12C7H814N216O3(12C13C0, 14N215N0)+Na]+ | 0.0 | 0.0 | 0.0 |
| 13C1 | [12C7H814N216O3(12C013C, 14N015N2)+Na]+ | 116530680.0 | 49252 | 116481428.0 |
| [12C7H814N216O3(12C013C, 14N15N)+Na]+ | 454551.0 | 171.3 | 454379.7 |
| [12C7H814N216O3(12C013C, 14N215N0)+Na]+ | 23186.2 | 0.0 | 23186.2 |
| 15N0 | [12C7H814N216O3(12C013C, 14N215N0)+Na]+ | 23186.2 | 0.0 | 23186.2 |
| [12C7H814N216O3(12C13C0, 14N215N0)+Na]+ | 0.0 | 0.0 | 0.0 |
| 15N1 | [12C7H814N216O3(12C013C, 14N15N)+Na]+ | 454551.0 | 171.3 | 454379.7 |
| [12C7H814N216O3(12C13C0, 14N15N)+Na]+ | 0.0 | 0.0 | 0.0 |
| 15N2 | [12C7H814N216O3(12C013C, 14N015N2)+Na]+ | 116530680.0 | 49252 | 116481428.0 |
| [12C7H814N216O3(12C13C0, 14N015N2)+Na]+ | 606194.7 | 0.0 | 606194.7 |

\*The relative error for all *m/z* values of representative isotopolog ions are within 5×10-6.

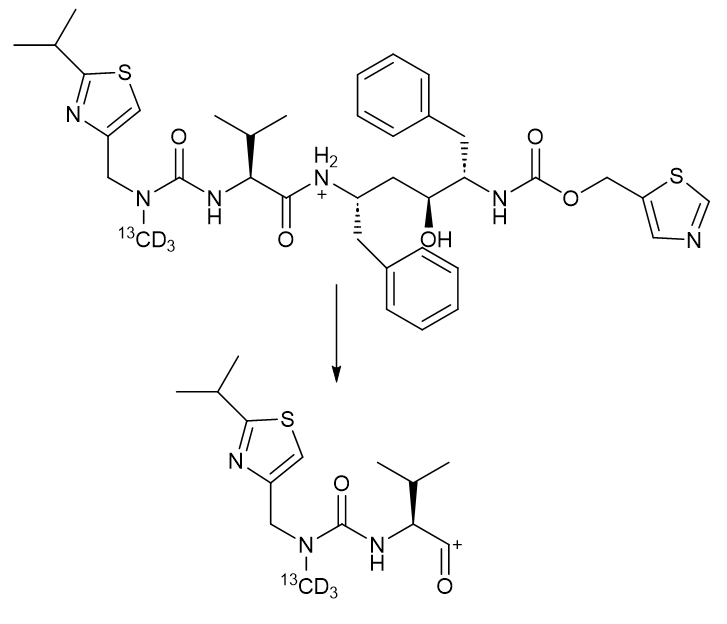
**3.3.2** **Case for mixed-labeled organic compounds with insufficient resolution**

When the molecular weight of the organic compound, such as Ritonavir-13C,D3 in Scheme 2, is above 400 u, the high resolution mass spectrometer might not be able to fully separate the mass peaks with distance Δ*m/z*(D - H) - Δ*m/z*(13C - 12C) = 0.0029 u even at 240000-resolution setting (Figure 7A). The in-source CID might solve such problem by generating the fragment ions containing all the labeled positions, because these fragment ions retained the isotopic information of the labeled elements in the compound.38 First we tested the feasibility of this strategy using a small labeled compound, Sulfaquinoxaline-13C6 (C₆13C₈H₁₂N₄O₂S), with molecular weight at about 300 u. The isotopic purity calculated directly was 99.05% and the isotopic purity calculated based on fragment ion containing all the labeled positions by in-source CID was 99.21% (seeing supporting information section 4). Both calculated isotopic purities was consistent with the isotopic purity (99.12%,) provided in the certificate. This demonstrates the feasibility of such strategy.

From the Figure 7A, we found that even at the 240000-resolution setting, we could not assigned the ion at *m/z* 724.3353 as the representative isotopolog ion [12C36H4514N616O532S2(12C13C0,H0D3)+H]+ at *m/z* 724.3389 or as the representative isotopolog ion [12C36H4514N616O532S2 (12C013C,HD2)+H]+ at *m/z* 724.3359. To address such issue, we used the in-source CID method to obtain a small fragment ion at *m/z* 300 containing all the stable isotope-labeled segments (Figure 7B). The in-source CID fragmentation process to give such fragment ion was shown in Scheme 3. From Figure 7B, we can see that two mass peaks containing the representative isotopolog ions [12C13H1914N316O232S(12C13C0,H0D3)]+ at *m/z* 299.1608 and [12C13H1914N316O232S (12C013C,HD2)]+ at *m/z* 299.1579 respectively, are well separated and assigned within accuracy ≤5×10-6. Thus, we could find three mass peaks containing representative isotopolog ions in Figure 7B. Themass spectrometric data summarized in Table 5, and the isotopic purity (13C) = 99.19%, isotopic purity (D) = 99.89%, total isotopic purity = 99.08% could be calculated, respectively. Our calculated isotopic purities were consistent with the isotopic purity (99.56%,) provided in the certificate.



**FIGURE 7.** (A) The expanded ESI-HRMS spectrum of Ritonavir-13C,D3 (C3613CH45D3N6O5S2) at massrange from *m/z* 720 to *m/z* 732 with the 240000-resolution setting. (B) The expanded ESI-HRMS spectrum of Ritonavir-13C,D3 (C3613CH45D3N6O5S2) at *m/z* range from *m/z* 295 to *m/z* 307 with the 240000-resolution setting with in-source CID, showing the fragment ion containing isotopic information.



**SCHEME 3.** The in-source CID fragmentation process of protonated Ritonavir-13C,D3 (C3613CH45D3N6O5S2) to fragment ion containing isotopic information.

**TABLE 5.** The data for the representative isotopolog ions of protonated Ritonavir-13C,D3 (Scheme 2) after in-source CID in its ESI-HRMS spectrum (Figure 7).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Ion type | Elemental compositions of representative isotopolog ion | Measured peak intensity | Natural isotope correction | | Natural isotope corrected intensity |
| 13C0 | [12C13H1914N316O232S(12C13C0, H0D3)]+ | 959950.1 | | 0.0 | 959950.1 |
| [12C13H1914N316O232S(12C13C0, HD2)]+ | 0.0 | | 0.0 | 0.0 |
| [12C13H1914N316O232S(12C13C0, H2D)]+ | 0.0 | | 0.0 | 0.0 |
| [12C13H1914N316O232S(12C13C0, H3D0)]+ | 0.0 | | 0.0 | 0.0 |
| 13C1 | [12C13H1914N316O232S(12C013C, H0D3)]+ | 118001360.0 | | 135804.6 | 117865555.4 |
| [12C13H1914N316O232S(12C013C, HD2)]+ | 380440.9 | | 0.0 | 380440.9 |
| [12C13H1914N316O232S(12C013C, H2D)]+ | 0.0 | | 0.0 | 0.0 |
| [12C13H1914N316O232S(12C013C, H3D0)]+ | 0.0 | | 0.0 | 0.0 |
| D0 | [12C13H1914N316O232S(12C013C, H3D0)]+ | 0.0 | | 0.0 | 0.0 |
| [12C13H1914N316O232S(12C13C0, H3D0)]+ | 0.0 | | 0.0 | 0.0 |
| D1 | [12C13H1914N316O232S(12C013C, H2D)]+ | 0.0 | | 0.0 | 0.0 |
| [12C13H1914N316O232S(12C13C0, H2D)]+ | 0.0 | | 0.0 | 0.0 |
| D2 | [12C13H1914N316O232S(12C013C, HD2)]+ | 380440.9 | | 0.0 | 380440.9 |
| [12C13H1914N316O232S(12C13C0, HD2)]+ | 0.0 | | 0.0 | 0.0 |
| D3 | [12C13H1914N316O232S(12C013C, H0D3)]+ | 118001360.0 | | 135804.6 | 117865555.4 |
| [12C13H1914N316O232S(12C13C0, H0D3)]+ | 959950.1 | | 0.0 | 959950.1 |

\*The relative error for all *m/z* values of representative isotopolog ions are within 5×10-6.

Based on our discussions above, we summarized a workflow for determinations of the isotopic purity for mono isotope-labeled or mixed isotopes-labeled scenarios involving D, 15Nor 13C by electrospray ionization-high resolution mass spectrometry.

1. If it has been confirmed in advance that the compound is only labeled with 13C and contains only small amount of N atoms, it is also feasible to calculate the isotopic purity using low-resolution mass spectrometry.

2. For only D-labeled compound, the resolution setting should be able to separate the preceding 13C isotopic peak and the nearby representative isotopolog ion of D-labeled specie with mass difference of Δ*m/z*(D - H) - Δ*m/z*(13C - 12C) = 0.0029 u.

3. For only 15N-labeled compound, the resolution setting should be able to separate the preceding 13C isotopic peak and the nearby representative isotopolog ion of 15N-labeled specie with mass difference of Δ*m/z*(13C - 12C) - Δ*m/z*(15N - 14N) = 0.0064 u.

4. For mixed-labeled compound, the resolution setting should be able to separate isobaric ions with mass differences of Δ*m/z*(D - H) - Δ*m/z*(13C - 12C) = 0.0029 u, Δ*m/z*(13C - 12C) - Δ*m/z*(15N - 14N) = 0.0064 u, and Δ*m/z*(D - H) - Δ*m/z(*15N - 14N) = 0.0093 u, simultaneously. Since contained more than on kind labeling isotope, the isotopic purity of each isotope could be calculated separately. Finally, the total isotopic purity of the mixed-labeled compound, could be calculated according the equation 3.

5. For the molecular weight＞400 u, the resolution setting might not be able to fully separate isobaric ions. we used the in-source CID method to obtain small fragment ions containing all the stable isotope-labeled segments.****

**FIGURE 8.** The workflow for determinations of the isotopic purity for mono isotope-labeled or mixed isotopes-labeled scenarios involving D, 15Nor 13C by electrospray ionization mass spectrometry. After the qualified mass spectrometric data were collected, the mass spectrometry data can be extracted as a spectrum list and imported into the calculation program to obtain the isotopic purity.

**4. CONCLUSIONS:**

This process of determining the isotopic purity is often complicated if labeled organic compounds contains a variety of labeled atoms, including D, 15Nor 13C, especially in mixed isotopes-labeled scenarios. The representative isotopolog ion with its specific molecular formula was proposed rather than the “monoisotopic ion” to represent various labeled states. Then, the isotopic purity was calculated with the corrected intensities of representative isotopolog ions by removing the natural isotopic contributions from preceding peaks and a unified equation has been proposed for the calculation of isotopic purity for various situations including D, 15Nor 13C. Several case studies were presented and our calculated isotopic purities were all consistent with the isotopic purities provided in the certificate. Based on our discussions above, we summarized a workflow with a Python calculation program for determinations of the isotopic purity for mono isotope-labeled or mixed isotopes-labeled scenarios involving D, 15Nor 13C by electrospray ionization-high resolution mass spectrometry to assigning the representative isotopolog ions with accurate mass and excluding the isobar interference. Therefore, such a rapid analytical method is useful in isotopic purity measurement for mono isotope-labeled or mixed isotopes-labeled scenarios.

**ACKNOWLEDGEMENTS**

The authors thank the financial support from National Natural Science Foundation of China for (Grant Nos. 21772227) and Shanghai Institute of Organic Chemistry (Grant Nos. E22ZZ61).

**CONFLICT OF INTEREST**

There are no conflicts to declare.

**AUTHOR CONTRIBUTIONS**

Chenlong Liang: Investigation; writing—original draft. Lin Ling: Methodology; writing—review and editing. Hao-Yang Wang: Conceptualization, writing—review and editing; supervision. They thank Prof. Jing Zhang for helpful discussions on isotopic purity calculations.

**DATA AVAILABILITY STATEMENT**

Data available on request from the authors.

**REFERENCES**

1. Xu Q, Liu H, Shang X, et al. 15N isotope tracing analysis of nitrogen accumulation and ammonia volatilization in upper leaves of flue-cured tobacco varieties. *Tob Sci Technol (China).* 2019;52(12):10-16. doi:10.16135/j.issn1002-0861.2019.0044.

2. Liu F, Wang Y-Q, Zhang Y, et al. Effect of Long-term Straw Returning on the Mineralization and Priming Effect of Rice Root-carbon. *Environmental Science.* 2022;43(8):4372-4378. doi:10.13227/j.hjkx.202112040.

3. Zhu SY, Zhao XE, Liu HW. Recent advances in chemical derivatization-based chromatography-mass spectrometry methods for analysis of aldehyde biomarkers. *Chin J Chromatogr.* 2021;39(8):845-854. doi:10.3724/SP.J.1123.2021.02023.

4. Yang XZ, Lu DW, Wang WC, Yang H, Liu Q, Jiang GB. Nano-Tracing: Recent Progress in Sourcing Tracing Technology of Nanoparticles. *Acta Chim Sin.* 2022;80(5):652-658. doi:10.6023/A21120612.

5. Zhang SJ, Rui C, Wang XT, Ji ZY, Li L, You JM. Preparation of Cationic Stable Isotope Labeling Probe and Its Application in Analysis of a-Dicarbonyl Compounds. *Chin J Anal Chem.* 2022;50(8):1269-1277. doi:10.19756/j.issn.0253-3820.221172.

6. Su M, Li Y, Wang H, Diao S, Wang M. Subcellular imaging and metabolic analysis of Isotopically labeled carbon compounds in biological sample: an ion microprobe (Nano SIMS) study. *J Beijing Norm Univ, Nat Sci (China).* 2016;52(2):223-227. doi:10.16360/j.cnki.jbnuns.2016.02.019.

7. Hu H, Zhao D, Liu C. Identification of arene degradation bacteria using carbon isotope labeling method. *J China Univ Pet (China).* 2015;39(6):57-62. doi:10.3969/j.issn.1673-5005.2015.06.007.

8. Huang P, Zhu J, Li H, Wang Y, Tang Y, Liu Q. Bioinformatic analysis of differentially expressed proteins in the dorsal raphe nucleus of rats after continuous treatment with olanzapine. *Journal of Southern Medical University.* 2022;42(8):1221-1229. doi:10.12122/j.issn.1673-4254.2022.08.15.

9. Guo X, Liu C, Wang G-B, Xu M-G. Quantitative proteomics and bioinformatics analyses of human coronary artery endothelial cell injury induced by Kawasaki disease. *Chinese journal of contemporary pediatrics.* 2020;22(7):796-803. doi:10.7499/j.issn.1008-8830.2001069.

10. Junoothula S, Sharma P. RECENT APPLICATION OF RADIOLABELING CHEMISTRY AND RADIO ANALYTICAL METHODS TO ASSESS THE BIOLOGICAL UPTAKE OF HAZARDOUS SUBSTANCE. 2022;20(16):140. doi:10.14704/NQ.2022.20.16.NQ88018.

11. Wang W, Hou X, Liu J, Jiang G. Application and research progress of traditional stable isotope technology in environmental science. *Environmental Chemistry.* 2021;40(12):3640-3650. doi:10.7524/j.issn.0254-6108.2021041601.

12. Li J, Zhao M, Liu Z, et al. Method for determining isotope abundance of 13C - labeled amino acids and metabolites in HepG2 cells. *Journal of Shenyang Pharmaceutical University.* 2020;37(11):990-997. doi:10.14066/j.cnki.cn21-1349/r.2020.11.005.

13. Cheng M, Wang H, Yao J, Sun J, Shi Y. Rapid determination of urea in urea 13C breath test kit by GC/MS. *Chinese Journal of Pharmaceutical Analysis.* 2020;40(2):268-272. doi:10.16155/j.0254-1793.2020.02.10.

14. Lei W, Du X, Zhang W. Isotope Abundance and Chemical Purity Determination of 13C -Fatty Acid by GC/MS. *Journal of Chinese Mass Spectrometry Society.* 2015;36(5):434-441. doi:10.7538/zpxb.youxian.2015.0024.

15. El'man AR, Davydov IE, Kononov LO, Zinin AI, Dugin SN. Synthesis of (13C-Methoxy)Methacetin for Isotopic Breath Tests. *Pharm Chem J.* 2014;48(4):279-283. doi:10.1007/s11094-014-1094-7.

16. Pironti C, Ricciardi M, Motta O, Camin F, Bontempo L, Proto A. Application of 13C Quantitative NMR Spectroscopy to Isotopic Analyses for Vanillin Authentication Source. *Foods.* 2021;10(11):11. doi:10.3390/foods10112635.

17. Akoka S, Remaud GS. NMR-based isotopic and isotopomic analysis. *Prog Nucl Magn Reson Spectrosc.* 2020;120-121:1-24. doi:10.1016/j.pnmrs.2020.07.001.

18. Xie L, Zhao Y, Sheng L, et al. Determination of isotope abundance for deuterium-labeled compounds by quantitative 1H NMR + 2H NMR. *J Labelled Comp Radiopharm.* 2022;65(9):234-243. doi:10.1002/jlcr.3990.

19. Marriott AS, Boyd AM, Quirk E, Chadwick J. A chemometric model for the quantitative determination of isotopic impurities in d3-methylamine hydrochloride by Fourier-transform infrared spectroscopy. *J Pharm Biomed Anal.* 2021;205:114337. doi:10.1016/j.jpba.2021.114337.

20. Zhang Q, Xia Y, Song W, Chen C, Wang HY. Rapid characterization of isotopic purity of deuterium-labeled organic compounds and monitoring of hydrogen-deuterium exchange reaction using electrospray ionization-high-resolution mass spectrometry. *Rapid Commun Mass Spectrom.* 2023;37 Suppl 1:e9453. doi:10.1002/rcm.9453.

21. Wei X, Shi B, Koo I, et al. Analysis of stable isotope assisted metabolomics data acquired by GC-MS. *Anal Chim Acta.* 2017;980:25-32. doi:10.1016/j.aca.2017.05.002.

22. Li M-C, Huang M-Z, Liu Y-W, Chu J, Zhuang Y-P, Zhang S-L. Accurate Determination of 13C Isotopic Abundance of Free Intracellular Amino acids with Low Concentration by GC-MS-Selective Ion Monitoring Method. *Chin J Anal Chem.* 2014;42(10):1408-1413. doi:10.11895/j.issn.0253-3820.140371.

23. Mou H, Hong M, Liu X-Y, et al. Accurate Determination of Isotopic Abundance of Intracellular Metabolites of Saccharopolysporaerythraea Based on Ultra Performance Liquid Chromatography-Triple Quadrupole Mass Spectrometry. *Chin J Anal Chem.* 2017;45(9):1264-1270. doi:10.11895/j.issn.0253-3820.170205.

24. Nilsson LB, Eklund G. Direct quantification in bioanalytical LC-MS/MS using internal calibration via analyte/stable isotope ratio. *J Pharm Biomed Anal.* 2007;43(3):1094-1099. doi:10.1016/j.jpba.2006.09.030.

25. Zheng B, Du X, Zhang W, Song MM, Cai YP. Rapid Isotope Abundance Characterization of 15N－Labeled Amino Acid by Electrospray Ionization Mass Spectrometry Centroid Method. *Shandong Chem Industry.* 2013;42:68-76. doi:10.19319/j.cnki.issn.1008-021x.2013.05.023.

26. Hou J, Lei W, Du X, Ren Z, Yue H. Determination of 15N Labeled Arginine Fermentation Broth and Isotope Abundance by GC-MS. *Journal of Instrumental Analysis.* 2016;35(9):1132-1136. doi:10.3969/j.issn.1004-4957.2016.09.010.

27. Lefebvre D, Blanco-Valle K, Feraudet-Tarisse C, et al. Quantitative Determination of Staphylococcus aureus Enterotoxins Types A to I and Variants in Dairy Food Products by Multiplex Immuno-LC-MS/MS. *J Agric Food Chem.* 2021;69(8):2603-2610. doi:10.1021/acs.jafc.0c07545.

28. Wang S, Tian RJ, Li L, Figeys D, Wang LS. An enhanced chemically defined SILAC culture system for quantitative proteomics study of human embryonic stem cells. *Proteomics.* 2011;11(20):4040-4046. doi:10.1002/pmic.201100052.

29. Attygalle AB, Pavlov J, Ruzicka J. Monoisotopic Mass? *J Am Soc Mass Spectrom.* 2022;33(1):5-10. doi:10.1021/jasms.1c00110.

30. Thompson A, Chahrour O, Malone J. Determination of Isotopic Purity by Accurate Mass LC/MS. ALMAC; 2013. doi:10.13140/2.1.4776.3208.

31. HG/T 5170-2017. Determination of abundance for stable isotope deuterium labeling compound: Gas chromatography-mass spectrometry. Chemical industry Standard of the People's Republic of China.

32. Zheng B, Du XN, Zhang WB. Isotopic abundance detection method of D, 13C or 15N labeled organic compounds. China patent CN201310147732.9. October 29, 2014.

33. Tian T, Liu SY, Li SQ, Li XQ, Zhang QH. Determination of Isotope Distribution and Abundance of Deuterium Labeled Compounds by High Resolution Mass Spectrometry. *Chin J Anal Chem.* 2021;49(4):563-570. doi:10.19756/j.issn.0253-3820.201620.

34. Gross JH. *Mass spectrometry: a textbook. 3rded.* Springer; 2017 :86-146. doi:10.1007/978-3-319-54398-7.

35. Beynon JH. Mass spectrometry and its applications to organic chemistry. 1960:62-94.

36. Rosman KJR, Taylor PDP. Isotopic compositions of the elements 1997. *J Phys Chem Ref Data.* 1998;27(6):1275-1287. doi:10.1063/1.556031.

37. Zhang Q, Song W, Wang HY. Recent Progress on Mass Spectrometry Analysis-oriented Stable Isotopic Labeling Technologies. *Huaxue Shiji*. 2022,44(1),1~9. doi:10.13822/j.cnki.hxsj.2022008517.

38. Wang HY, Guo YL, Lu L. Studies of rearrangement reactions of protonated and lithium cationized 2-pyrimidinyloxy-N-arylbenzylamine derivatives by MALDI-FT-ICR mass spectrometry. *J Am Soc Mass Spectrom.* 2004;15(12):1820-1832. doi:10.1016/j.jasms.2004.08.014.