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TECHNICAL REPORT AccuCor2: isotope natural abundance correction for dual-isotope tracer experiments

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Stable isotope labeling techniques have been widely applied in the field of metabolomics and proteomics. Before the measured mass spectral data can be used for quantitative analysis, it must be accurately corrected for isotope natural abundance and tracer isotopic impurity. Despite the increasing popularity of dual-isotope tracing strategy such as ¹³C-¹⁵N or ¹³C-²H, there are no accurate tools for correcting isotope natural abundance for such experiments in a resolution-dependent manner. Here, we present AccuCor2 as an R-based tool to perform the correction for ¹³C-¹⁵N or ¹³C-²H labeling experiments. Our method uses a newly designed algorithm to construct the correction matrices that link labeling pattern and measured mass fractions, then use non-negative least-squares to solve the labeling patterns. Our results show that the dual-isotope experiments often require a mass resolution that is high enough to resolve ¹³C and ¹⁵N or ¹³C and ²H. Otherwise, the labeling pattern is not solvable. However, this mass resolution may not be sufficiently high to resolve other non-tracer elements such as oxygen or sulfur from the tracer elements. Therefore, we design AccuCor2 to perform the correction based on the actual mass resolution of the measurements. Using both simulated and experimental data, we show that AccuCor2 performs accurate and resolution-dependent correction for dual-isotope tracer data.

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INTRODUCTION

Stable isotope tracers have been widely used to study the metabolism of carbohydrates, protein, lipids, and metabolic cofactors [1]. Tracers can be used to determine the origins of specific metabolites by quantifying the incorporation of the isotope atoms from the labeled substrates [2-4]. Tracers can also be infused in vivo in a non-perturbative way to measure the turnover rates of circulating metabolites [5, 6]. Another application of stable isotope tracing is metabolic flux analysis, which provides a quantitative evaluation of the rates of biochemical reactions in the entire metabolic network [7–9]. The most commonly used tracing isotopes are 13 C, 2 H, and 15 N. 13 C tracers are frequently used to study the biosynthesis of metabolites and quantify the carbon flow in the metabolic network [10]. However, there are also many metabolic reactions such as transamination, (de)hydration, and redox reactions that do not involve the formation or cleavage of carbon-carbon bonds [11]. In these cases, the ¹³C tracers are not informative and tracers that include ²H or ¹⁵N should be used instead. ²H tracers are commonly used to study isomerase reactions and dehydrogenase reactions that involve NADH or NADPH as co-factors [12, 13]. Meanwhile, ¹⁵N tracers are widely used to investigate the metabolism of amino acids and nucleotides [14, 15]. Modern mass spectrometry provides a high mass resolution that enables unambiguous detection of various stable isotopes simultaneously. As a result, tracers containing dual isotopes (e.g., ¹³C-¹⁵N and ¹³C-²H) have been increasingly deployed in biological experiments. For example, L-[${}^{13}C_{6}$, ${}^{15}N_{4}$] arginine was used to investigate autophagy and melanoma growth [16]. L-[1- ${}^{13}C$, ${}^{15}N$] leucine was used to study the whole-body amino acid metabolism [17]. Methyl[${}^{2}H_{3}$]- ${}^{13}C$ methionine was used to study protein methylation and breakdown [18]. In these applications, the biological interpretation of the results depends on the accurate determination of the isotopic tracer incorporation in the metabolites.

It should be noted that the measured mass isotopologue distribution does not directly reflect the isotope incorporation from tracers. This is because of the natural abundance (NA) of isotopes. For example, the unlabeled metabolite NAD⁺ ($C_{21}H_{27}N_7O_{14}P_2$) would have 19% being M+1 because of the NA of ¹³C. Furthermore, 2.8% of unlabeled NAD⁺ could be M+2 because of the NA of ¹⁸O. These NAs must be accounted for to reveal the actual tracer isotope incorporation, which is done through a process called isotope natural abundance correction (INAC).

Many efforts have been made to advance the methodology of INAC. Biemann [19] proposed the first INAC algorithm, which subtracts the isotopologue fractions in a stepwise manner (i.e., NA contributions from m + 0 were corrected first, followed by contributions from m + 1, m + 2, etc.). Although the stepwise approach is mathematically correct, this method is prone to experimental errors since the measurement error in one mass fraction may be propagated across all other mass fractions and lead to a biased result. This issue was solved by Brauman [20], Wittmann and Heinzle [21], and Van Winden et al. [22] using a least-squares approach where all measurement errors were weighted equally. This method first uses probability theory to generate a correction matrix that links the isotope labeling pattern and the measured mass fractions affected by NA. Then, a least-squares approach is applied

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to solve the isotope labeling pattern. Millard et al. [23] further improved this method by applying a non-negative least-squares method to solve the labeling pattern and developed a wellestablished software IsoCor. IsoCor is an efficient tool that can perform both tracer impurity correction and INAC for many different tracer elements on large datasets. While IsoCor is accurate for lowresolution mass spectrometry data, it may have an over-correction problem when handling high-resolution data. This is because under high resolution, some isotopologues may be resolved from the tracer isotopologues and therefore, the corresponding fraction should not be subtracted. To address this issue, Su et al. [24] developed the first resolution-dependent INAC tool called AccuCor. AccuCor uses resolution information to evaluate the impact of all non-tracer isotopes and only corrects the fractions that affect the tracer isotopologue channels. Millard et al. [25] and Du et al. [26] further refined this method by calculating the correction limits for non-tracer isotope combinations and developed IsoCor2 and ElemCor, respectively. As the use of dual-isotope tracers becomes increasingly popular, several tools have been developed to perform INAC on multi-isotope experiments [27-29]. However, these tools assume that all the non-tracer isotopologues can be resolved from tracer isotopologues with an ultra-high-resolution instrument, which is not necessarily true and would be discussed later.

In this study, we developed AccuCor2 as the first tool to perform a resolution-dependent INAC in data from dual-isotope tracers. We found that such correction problems require the two tracer isotopes to be resolved under sufficiently high mass resolution. The non-tracer isotopes are often not fully resolved, and AccuCor2 can account for them in a resolution-dependent manner. We also compared the performance of AccuCor2 with two other available tools IsoCorrectoR [28] and PICor [29], using both simulated and experimental data. The result shows both IsoCorrectoR and PICor under-corrected isotopologues caused by non-tracer isotope NA, whereas AccuCor2 correctly solved this problem.

MATERIALS AND METHODS Theory of INAC

INAC is the process whereby isotope labeling patterns are solved from the measured mass fractions. The isotope labeling pattern (denoted by **L**) and the measured mass fractions (denoted by **M**) can be expressed as column vectors. These two vectors are related by the correction matrix (denoted by **CM**): $CM \times L = M$ [21, 22]. The ith row and jth column element of the correction matrix **CM**_{ij} represents the probability of the jth labeled fraction contributing to the ith measured mass fraction due to the isotope NA and the isotopic impurity of the tracer [21, 22]. The INAC algorithm that is suitable for high-resolution mass spectrometry data will construct the correction matrix based on the chemical formula of the ion, the mass resolution of the measurements, and the isotopic purity of the tracer [24–26]. Once the correction matrix is built, the labeling pattern can be solved by non-negative least-squares algorithms such as Hanson–Lawson or L-BFGS-B [23, 24]. Here, we will demonstrate the construction of the correction matrix for dual-isotope data.

The form of the correction matrix is determined by the forms of the isotope labeling pattern vector L and measured mass fractions vector M. Here, we use serine ([M-H]⁻ C₃H₆NO₃) as an example. If serine is only labeled with ¹³C isotope in the experiment, the labeling pattern vector L = [¹³C₀, ¹³C₁, ¹³C₂, ¹³C₃]^T and the measured mass fractions vectors would be a 4 × 4 matrix. In contrast, if serine is labeled with both ¹³C and ¹⁵N, L and M vectors and their relationship can be expressed as Eq. (1).

	¹³ C ₀ ¹⁵ N ₀	1	M + C0N0
	¹³ C ₁ ¹⁵ N ₀	$ \begin{vmatrix} \mathbf{N}_0 \\ \mathbf{N}_0 \\ \mathbf{N}_0 \\ \mathbf{N}_1 \end{vmatrix} = $	M + C 1 N 0
	¹³ C ₂ ¹⁵ N ₀		$\mathbf{M} + \mathbf{C} 2 \mathbf{N} 0$
[Correction]	¹³ C ₃ ¹⁵ N ₀		M + C 3 N 0
_ matrix] ×	¹³ C ₀ ¹⁵ N ₁		$\mathbf{M} + \mathbf{C}0\mathbf{N}1$
	¹³ C ₁ ¹⁵ N ₁		M + C 1 N 1
	¹³ C ₂ ¹⁵ N ₁		M + C 2 N 1
	¹³ C ₃ ¹⁵ N ₁		M + C 3 N 1

(1)

In Eq. (1), the element $^{13}C_m$ $^{15}N_n$ in the L vector denotes the fraction with m- ^{13}C and n- ^{15}N labeling. The element M+CiNj in the M vector denotes the observed mass fraction that corresponds to the incorporation of i ^{13}C and j ^{15}N atoms. As we shall see in "Results", in the dual-isotope labeling experiment, it is necessary to be able to resolve mass fractions of the same nominal mass such as M+C1N0 and M+CON1. If the measurements are done under unit mass resolution, and the M + 1 (m/z 105 for serine) fraction is obtained instead of the resolved M+C1N0 (m/z 105.03867 for serine) and M+CON1 (m/z 105.03235 for serine) fractions, the correction may fail to generate the accurate labeling pattern. For serine, which has three C and one N atoms, the correction matrix CM is an 8×8 matrix.

Construction of the correction matrix

CM can be factorized into three matrices: the tracer isotopic purity matrix **IPM**, the tracer elements matrix **TM**, and the non-tracer elements matrix **NTM**: **CM** = **NTM** × **TM** × **IPM**. We begin the demonstration with the construction of **TM** because this matrix usually has the greatest impact on **CM**. For convenience, we always use the following convention to arrange the terms in the L and M vectors: ascending numbers of ¹³C followed by ascending numbers of ²H or ¹⁵N (Eq. (1)). Therefore, the **TM** for ¹³C-¹⁵N labeling of serine can be constructed (Eq. (2)).

	$\Gamma_{0}^{0}P_{C} \times {}^{0}P_{N}$	0	0	0	0	0	0	0 Т	
TM =	${}^{1}_{3}P_{C} \times {}^{0}_{1}P_{N}$	${}^{0}_{0}P_{C} \times {}^{0}_{1}P_{N}$	0	0	0	0	0	0	
	$^{2}_{3}P_{C} \times ^{0}_{1}P_{N}$	${}^{1}_{2}P_{C} \times {}^{0}_{1}P_{N}$	${}^{0}_{1}P_{C} \times {}^{0}_{1}P_{N}$	0	0	0	0	0	
	${}^{3}_{3}P_{C} \times {}^{0}_{1}P_{N}$	${}^{2}_{2}P_{C} \times {}^{0}_{1}P_{N}$	${}^{1}_{1}P_{C} \times {}^{0}_{1}P_{N}$	${}^{0}_{0}P_{C} \times {}^{0}_{1}P_{N}$	0	0	0	0	
	${}^{0}_{3}P_{C} \times {}^{0}_{1}P_{N}$	0	0	0	${}^{0}_{3}P_{C} \times {}^{0}_{0}P_{N}$	0	0	0	
	${}^{1}_{3}P_{C} \times {}^{1}_{1}P_{N}$	${}^{0}_{2}P_{C} \times {}^{1}_{1}P_{N}$	0	0	${}^{1}_{3}P_{C} \times {}^{0}_{0}P_{N}$	${}^{0}_{2}P_{C} \times {}^{0}_{0}P_{N}$	0	0	
	${}_{3}^{2}P_{C} \times {}_{1}^{1}P_{N}$	${}_{2}^{1}P_{C} \times {}_{1}^{1}P_{N}$	${}^{0}_{1}P_{C} \times {}^{1}_{1}P_{N}$	0	${}^{2}_{3}P_{C} \times {}^{0}_{0}P_{N}$	${}^{1}_{2}P_{C} \times {}^{0}_{0}P_{N}$	${}^{0}_{1}P_{C} \times {}^{0}_{0}P_{N}$	0	
	$^{3}P_{C} \times ^{1}P_{N}$	${}^{2}_{2}P_{C} \times {}^{1}_{1}P_{N}$	$^{1}_{1}P_{C} \times ~^{1}_{1}P_{N}$	${}^{0}_{0}P_{C} \times {}^{1}_{1}P_{N}$	${}^{3}_{3}P_{C} \times {}^{0}_{0}P_{N}$	${}^{2}_{2}P_{C} \times {}^{0}_{0}P_{N}$	${}^{1}_{1}P_{C} \times {}^{0}_{0}P_{N}$	${}^{0}_{0}P_{C} \times {}^{0}_{0}P_{N}$	
								(2	2)

As shown in Eq. (2), for a metabolite with **m** carbon atoms and **n** nitrogen atoms, **TM** is a square matrix with $(m + 1) \times (n + 1)$ row and columns. In fact, **TM** is composed of $(n + 1) \times (n + 1)$ blocks where each block is an $(m + 1) \times (m + 1)$ submatrix. For example, the element in row 7 column 6 (element [7, 6]) is within block [2, 2] and position [3, 2] in the submatrix. This element represents the probability of the ${}^{13}C_1 {}^{15}N_1$ -labeled fraction that is appearing as $\mathbf{M} + \mathbf{C} 2\mathbf{N} 1$ due to the carbon and nitrogen NA. Since the ${}^{13}C_1 {}^{15}N_1$ -labeled fraction of serine already has one ${}^{13}C$ from the tracer, only two carbon atoms are natural carbon. Similarly, since the $^{13}C_1$ $^{15}N_1$ -labeled fraction of serine has one ^{15}N from the tracer, no nitrogen atom is natural. We use the term ${}_{2}^{1}P_{C}$ to represent the probability of having on ${}^{13}C$ out of two natural carbon atoms: ${}_{2}^{1}P_{C} = {}_{(1)}^{2} \times p_{C}^{1} \times (1 - p_{C})^{2-1} = 2 \times (0.011)^{1} \times (0.989)^{1}$, in which $p_{C} = 0.011$ represents the NA of ${}^{13}C$. Therefore, the element [7, 6] is ${}_{2}^{1}P_{C} \times {}_{0}^{0}P_{N}$. More generally, for the carbon–nitrogen **TM**, the element $[(i - 1) \times (m + 1)]$ $+f_{i}(j-1)\times(m+1)+q$, which is in the ith row and ith column of the blocks and the fth row and gth column of the submatrix has the value of for $f_{m+1-g}^{-g} P_C \times i_{n+1-j}^{i-j} P_N$. The element [7,6] has the block indices of i = 2, j = 2and submatrix indices f = 3, g = 2, and this element has the value of 3-2, j = 2 and $1-2 P_C \times i_{n+1-2}^{2-2} P_N = \frac{1}{2} P_C \times 0 P_N$. Because $f_{m+1-g}^{-g} P_C \times i_{n+1-j}^{i-j} P_N = 0$ when f < g or i < j, **TM** is a lower-triangular matrix, and each nonzero submatrix is also a lower-triangular matrix. Because of this triangularity, TM is always well-conditioned.

Similar to **TM**, we can also construct the isotopic purity matrix **IPM** for $^{13}C^{-15}N$ -labeled serine (Eq. (3)).

IPM =	C 0 PIPC × 0 PIPN 0 0 0 0 0 0 0 0 0	${}^{1}_{1}P_{IPC} \times {}^{0}_{0}P_{IPN}$ ${}^{0}_{1}P_{IPC} \times {}^{0}_{0}P_{IPN}$ 0 0 0 0 0 0	$\begin{array}{c} \frac{2}{2} P_{IPC} \times \ _{0}^{0} P_{IPN} \\ \frac{1}{2} P_{IPC} \times \ _{0}^{0} P_{IPN} \\ \frac{3}{2} P_{IPC} \times \ _{0}^{0} P_{IPN} \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ $	${}^{3}_{3}P_{IPC} \times {}^{0}_{0}P_{IPN}$ ${}^{2}_{3}P_{IPC} \times {}^{0}_{0}P_{IPN}$ ${}^{3}_{3}P_{IPC} \times {}^{0}_{0}P_{IPN}$ ${}^{0}_{3}P_{IPC} \times {}^{0}_{0}P_{IPN}$ 0 0 0	$ \begin{array}{c} {}^{0}_{0} P_{IPC} \times \ {}^{1}_{1} P_{IPN} \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ P_{IPC} \times \ {}^{0}_{1} P_{IPN} \\ 0 \\ 0 \\ 0 \\ 0 \end{array} $	$ \begin{array}{c} {}^{1}_{1}P_{IPC} \times ~{}^{1}_{1}P_{IPN} \\ {}^{0}_{1}P_{IPC} \times ~{}^{1}_{1}P_{IPN} \\ 0 \\ 0 \\ {}^{0}_{0}P_{IPC} \times ~{}^{0}_{1}P_{IPN} \\ {}^{0}_{1}P_{IPC} \times ~{}^{0}_{1}P_{IPN} \\ 0 \\ \end{array} $	$\begin{array}{c} \frac{2}{2} P_{IPC} \times \ \frac{1}{1} P_{IPN} \\ \frac{1}{2} P_{IPC} \times \ \frac{1}{1} P_{IPN} \\ \frac{0}{2} P_{IPC} \times \ \frac{1}{1} P_{IPN} \\ 0 \\ \frac{2}{2} P_{IPC} \times \ \frac{0}{1} P_{IPN} \\ \frac{1}{2} P_{IPC} + \ \frac{0}{1} P_{IPC}$	$\label{eq:2} \begin{array}{l} & \frac{3}{3} P_{IPC} \times \ 1 P_{IPN} \\ & \frac{3}{3} P_{IPC} \times \ 1 P_{IPN} \\ & \frac{1}{3} P_{IPC} \times \ 1 P_{IPN} \\ & \frac{3}{3} P_{IPC} \times $
	Lo	0	0	0	0	0	0	${}^{0}_{3}P_{IPC} \times {}^{0}_{1}P_{IPN}$
								(3)

For a metabolite with **m** carbon atoms and **n** nitrogen atoms, **IPM** is a square matrix composed of $(n + 1) \times (n + 1)$ blocks where each block is an $(m + 1) \times (m + 1)$ submatrix. The element in row 5 column 7 (element [5, 7]) is within block [2, 2] and position [1, 3] in the submatrix. This element represents the probability of the ¹³C₂ ¹⁵N₁-labeled fraction that is appearing as **M** + **CoN1** due to the tracer isotopic impurities. We use the term ${}_2^2P_{IPC}$ to represent the probability of having two natural carbon atoms out of two tracer carbon atoms: ${}_2^2P_{IPC} = ({}_2^2) \times (1 - p_{IPC})^2 \times p_{IPC}^{2-2} = 1 \times (1 - 0.99)^2 \times (0.99)^0$, in which $p_{IPC} = 0.99$ represents the isotopic purity of ¹³C in the tracer. Therefore, the



Fig. 1 NTM construction algorithm for ¹³C-¹⁵**N labeling experiments.** In this example, carbon and nitrogen are the tracer elements, and we assume, for simplicity, that the non-tracer elements only include hydrogen and oxygen. The algorithm takes the chemical formula of the ion, the charge and the mass resolution as the input information. The output is the resolution dependent NTM. Algorithm for ¹³C-²H labeling experiments follows the same structure.

element [5, 7] is ${}_{2}^{2}P_{|PC} \times {}_{0}^{0}P_{|PN}$. More generally, for the carbon-nitrogen **IPM**, the element $[(i-1) \times (m+1) + f, (j-1) \times (m+1) + g]$, which is in the ith row and jth column of the blocks and the fth row and gth column of the submatrix has the value of ${}_{g-1}^{\mathbf{g}}P_{|PC} \times {}_{j-1}^{\mathbf{j}}P_{|PN}$. The element [5, 7] has the block indices of i=2, j=2 and submatrix indices f=1, g=3, and this element has the value of ${}_{3-1}^{3-1}P_{|PC} \times {}_{2-1}^{2-1}P_{|PN} \times {}_{2}^{2-1}P_{|PN} \times {}_{$

Unlike the construction of **TM** and **IPM** described above, the construction of **NTM** is affected by the mass resolution of the measurement [24]. For serine that is ${}^{13}C_{-}{}^{15}N_{-}$ labeled, it takes a minimum nominal mass resolution of 50,800 (defined at m/z 200 on Orbitrap analyzer) [24] to resolve ${}^{18}O_{1}$ and ${}^{13}C_{2}$ peaks but a minimum nominal mass resolution of 140,000 to resolve ${}^{17}O_{1}$ and ${}^{13}C_{1}$ peaks on the mass spectrum. Therefore, if data were measured at a mass resolution of 100,000, **NTM** should include the term of ${}^{17}O_{1}$ but exclude the term of ${}^{18}O_{1}$ to avoid overcorrection. In our previous work, **NTM** was further factorized. The contributions from each non-tracer element such as oxygen or sulfur

were described by separate matrices. These matrices were multiplied together to obtain the full NTM. Later, Millard et al. [25] pointed out that the combination of certain non-tracer elements may be harder to resolve than individual elements. For example, a minimum resolution of 50,800 and 42,500 is required to resolve ${}^{18}O_1$ versus ${}^{13}C_2$ and ${}^{2}H_1$ versus ${}^{13}C_1$ of serine, respectively. However, it takes a minimal resolution of 260,000 to resolve ${}^{18}O_1 + {}^{2}H_1$ versus ${}^{13}C_3$ of serine. As a result, a single **NTM** should be constructed instead of a product of matrices for individual elements. The latest version of AccuCor has adopted such an algorithm for improved accuracy. For AccuCor2, we also construct a single NTM to account for the NA of the non-tracer elements. The algorithm for constructing NTM is shown in Fig. 1. First, the algorithm uses the input data to calculate the mass limit (ML) of resolvable peaks. Second, the algorithm generates a list of atom combinations that satisfy the following two criteria: (1) for each element, the atom number in the list does not exceed the atom number in the chemical formula; (2) the difference between the mass shift caused by the tracer atoms and the non-tracer atoms is within the ML. Last, the probability of each atom combination in the list is calculated and added to the corresponding position of NTM. The construction completes when all entries in the list are accounted



As shown in Eq. (4), for a metabolite with **m** carbon and **n** nitrogen atoms, **NTM** is a square matrix composed of $(n + 1) \times (n + 1)$ blocks where each block is an $(m + 1) \times (m + 1)$ submatrix. The element in row 4 column 1 (element [4, 1]) represents the probability of the ¹³C₀ ¹⁵N₀-labeled fraction contributing to the M + C3N0 m/z channel due to the non-tracer elements. Under 100,000 resolution, the only non-tracer element combination that makes such mass shift is ²H₁ + ¹⁸O₁, which has the probability of ${}_{6}^{1}P_{H} \times {}_{0.1}^{0.1}P_{O}$. The term ${}_{6}^{1}P_{H}$ represents the probability of having one ²H atom out of six natural hydrogen atoms. The term ${}_{3}^{1}P_{O}$ represents the probability of having zero ¹⁷O and one ¹⁸O atom out of three natural oxygen atoms. Note that **NTM** is not strictly lower-triangular since element [4, 5] is nonzero. This represents the probability of ${}^{13}C_0$ is nonzero. This represents the probability of ${}^{13}C_1$ is nonzero. This represents the probability of ${}^{13}C_0$ is nonzero. This represents the probability of ${}^{13}C_0$ is nonzero. This represents the probability of ${}^{13}C_0$ of the fact that ${}^{15}N_1$ + ${}^{2}H_2$ and ${}^{13}C_3$ are not resolved for serine under 100,000 resolution.

Materials

LCMS-grade methanol (A456), acetonitrile (A955), acetic acid (A35), and water (ACROS 61515) were purchased from Fisher Chemicals (Pittsburgh, PA). $^{13}C_{3-}^{15}N_{1}$ L-serine (99% isotopic purity on both ^{13}C and ^{15}N , CNLM-474-H-PK) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). L-serine (S4500) was purchased from MilliporeSigma (Burlington, MA).

Sample preparation

One hundred micrometer of L-serine, ${}^{13}C_{3}$ - ${}^{15}N_{1}$ L-serine, or 1:1 mixture were dissolved in 40:40:20 (acetonitrile:methanol:water) and centrifuged for 10 min at 4 °C. The supernatant was collected for LC–MS analysis.

Liquid chromatography-mass spectrometry

The LC–MS method is the same as previously published [30]. Conditions were optimized on an HPLC–ESI–MS system fitted with a Vanquish Horizon UHPLC and a Thermo Q Exactive Plus MS. The MS scans were obtained in negative ionization mode with a nominal mass resolution of 70,000 (defined at m/z 200), in addition to an automatic gain control target of 3×10^6 and m/z scan range of 72–1000. Metabolite data were obtained using the MAVEN software package [31] with each labeled isotope fraction (mass accuracy window: 16 ppm).

Simulation of mass fractions

Simulations of NAD⁺ mass fractions were done using Thermo Xcalibur Qual Browser software with 750,000 nominal mass resolution (defined at m/z 200).

Isotope natural abundance correction (INAC)

AccuCor2 works for data acquired on Obitrap mass analyzers. AccuCor2 calculates the ML for correction based on the nominal mass resolution for data acquisition and the m/z values of the metabolites [24].

$$\text{Resolution} = \frac{\text{Nominal resolution} \times \sqrt{200}}{\sqrt{m}}$$
(5)

AccuCor2 supports the isotopes of ¹³C, ²H, ¹⁵N, ¹⁷O, ¹⁸O, ³³S, ³⁴S, ²⁹Si, ³⁰Si, ³³Cl, and ⁸¹Br. The isotope abundance data are taken from Isotopic Compositions of the Elements 1997 [32], and the users are allowed to change the isotope abundance values. The natural isotope abundance correction code was written in R [33]. The labeling pattern solving was done using the Hanson–Lawson non-negative least-squares method, which was implemented in the NNLS package [34], to avoid negative fractions. We used PICor [29] and IsoCorrectoR [28] to compare the performance of INAC. For the correction of NAD labeling, the root-mean-square deviation (RMSD) is calculated using the sum of squared residue between the corrected values and the theoretical values of the labeling pattern. For serine and the sodium acetate adduct of serine, we only calculated the RMSD for Sample 3 which is the 100% ¹³C₂, ¹⁵N₁. The AccuCor2 code is freely available for both ¹³C-²H, and ¹³C-¹⁵N labeling studies (https://github.com/wangvujue23/AccuCor2).

RESULTS

Requirement on the mass resolution for dual-isotope tracer experiment

Early isotope tracer experiments that use a single isotope were typically done on unit resolution mass spectrometers. The dualisotope tracer experiments, however, often have a minimum requirement on the mass resolution of the instrument. Take ${}^{13}C^{-15}N$ serine as an example, there are eight possible labeled fractions of serine: ${}^{13}C_{0-3}$ - ${}^{15}N_{0-1}$. The labeling pattern of serine can be correctly solved if the sample is measured on a high-resolution instrument (see "Materials and methods: Theory of INAC"). Under low mass resolution, different labeled fractions with the same nominal mass are combined (e.g., M + C1N0 and M + C0N1 are combined as M + 1), resulting in fewer mass fraction measurements than the labeled fractions. Therefore, when measured on a unit resolution mass spectrometer, serine has only five mass fractions M + 0-M + 4. It is obviously impossible to solve eight labeling fractions based on the measurements of only five mass fractions. Therefore, the labeling fractions of the metabolite cannot be determined.

Even if the choice of the tracer limits the number of possible labeling fractions to be no more than the number of measured mass fractions, the correction may still fail. Suppose serine can be labeled in only three forms: ${}^{13}C_{1}{}^{-15}N_{0}$, ${}^{13}C_{0}{}^{-15}N_{1}$, and ${}^{13}C_{1}{}^{-15}N_{1}$. Under unit mass resolution, the ${}^{13}C_{1}{}^{-15}N_{0}$ and ${}^{13}C_{0}{}^{-15}N_{1}$ serine are both measured as M + 1 and the ${}^{13}C_{1}{}^{-15}N_{0}$ and ${}^{13}C_{0}{}^{-15}N_{1}$ serine also contribute to the M + 2 and M + 3 fractions; the ${}^{13}C_{1}{}^{-15}N_{1}$ serine also contributes to the M + 3 fraction. Therefore, it would appear to be possible to determine three labeling fractions ${}^{13}C_{1}$ - ${}^{15}N_{0}$, ${}^{13}C_{0}$ - ${}^{15}N_{1}$, and ${}^{13}C_1 - {}^{15}N_1$ from the measurement of three mass fractions M + 1, M+2, and M+3. However, Fig. 2 provides a set of examples to demonstrate that such correction cannot be done. We calculated six combinations of labeling fractions (Fig. 2A, Samples 1–6), which have the ${}^{13}C_1$ - ${}^{15}N_0$ fraction ranging from 0 to 95%. These samples have exactly the same M + 1, M + 2, and M + 3 fractions when measured under unit mass resolution (Fig. 2B, Supplementary Table 1). Therefore, the labeling fractions cannot be calculated from the measured mass fractions. In fact, there are infinitely many combinations that could fit these mass measurements. The mathematical reason behind this phenomenon is that low resolution causes certain columns of the correction matrix, such as those corresponding to M + C1N0 and M + C0N1, to be combined as M + 1. These combinations make the correction matrix singular. With a singular correction matrix, the mass fraction vector M becomes insensitive to the changes in L. Therefore, given a measurement of **M**, the labeling pattern vector **L** cannot be uniquely determined.

In contrast, when these samples were measured under a mass resolution that is high enough to resolve ¹³C and ¹⁵N, the labeling fraction of ¹³C₁-¹⁵N₀, ¹³C₀-¹⁵N₁, and ¹³C₁-¹⁵N₁ would be measured as M + C1N0, M + C0N1, and M + C1N1, respectively (Fig. 2C). ¹³C₁-¹⁵N₀ would also contribute to M + C2N0 due to NA, which can be resolved from M + C1N1 under high mass resolution. In this case, all six samples give different mass fraction measurements so that their respective labeling patterns can be solved accurately. For serine ([M-H]⁻, m/z 104.03532), the nominal mass



Fig. 2 Dual-isotope tracer experiment requires high mass resolution. A Serine isotopologue mixtures $({}^{13}C_{1}-{}^{15}N_{0}, {}^{13}C_{0}-{}^{15}N_{1}$ and ${}^{13}C_{1}-{}^{15}N_{1})$ were measured under low and high resolution MS. **B** Low resolution do not distinguish ${}^{13}C$ and ${}^{15}N$, leading to the same observation among all samples. **C** Consequently, the labeling pattern is unsolvable under low resolution. In contrast, high resolution can distinguish ${}^{13}C$ and ${}^{15}N$ isotopologues, leading to unique solution of labeling pattern.



Fig. 3 Effect of resolution on isotopologue separation. The minimum nominal mass resolution (defined at m/z 200 on Orbitrap analyzer) for separating isotopologues according to the mass of ion and mass difference (Δm) for experiments using ${}^{13}C{}^{-15}N$ (A) and ${}^{13}C{}^{-2}H$ (B) tracer experiments. The most commonly observed isotopologue pairs are shown in different colors. All isotopologues shown were measured with -1 charge.

resolution (for Orbitrap instrument, defined at m/z 200) [24, 35] should be >19,700 to resolve ¹³C and ¹⁵N. In general, the mass resolution should be high enough to resolve all the labeling fractions of interest. This requirement should be taken into consideration when designing a dual-isotope tracer experiment.

Resolution-dependent correction for the non-tracer elements

Dual-isotope tracer experiments require a minimum mass resolution under which the tracer isotopes can be resolved. However, this resolution may not be high enough to resolve other nontracer elements, which should also be corrected. As shown by Su et al. [24], the minimum resolution for isotopologue separation depends on both the absolute mass difference (Δm) and m/z(represented by *m* in the formula).

Nominal resolution
$$\geq \frac{1.66 \times m^{3/2}}{\Delta m \times \sqrt{200}}$$
 (5)

Equation (5) suggests that as the mass difference Δm goes lower, a higher mass resolution is required to resolve the corresponding isotopologues. Figure 3 provides a visual illustration of the relationship among the resolution requirement, the mass difference, and the mass of the ion. For serine ([M-H]⁻, m/z 104.03532) that is ¹³C- and ¹⁵N-labeled, it only takes a minimum resolution of 19,700 to resolve the ¹³C₁-¹⁵N₀ and ¹³C₀-¹⁵N₁ isotopologues. However, it takes a minimum resolution of 50,800 to resolve $^{13}C_2$ and ¹⁸O₁ ($\Delta m = 0.00245$). Similarly, for serine that is ¹³C- and ²H-labeled, it takes a minimum resolution of 42,500 to resolve the ¹³C₁-²H₀ and ¹³C₀-²H₁ isotopologues. However, it takes a minimum resolution of 42,600 to resolve the ¹³C₁-²H₀ and ¹³C₀-²H₁ asotopologues. However, it takes a minimum resolution of $^{13}C_{0}$ -2H₁ and $^{13}C_{0}$ -2H₁ and $^{13}C_{1}$ -1⁸N₁ and $^{13}C_{1}$ -($\Delta m = 0.00247$).

Moreover, as the mass of the ion goes up, the required resolution also goes up (Fig. 3). For acetyl-CoA ([M-H]⁻, $C_{23}H_{37}N_7O_{17}P_3S$) that is ¹³C-¹⁵N-labeled, it takes a minimum resolution of 427,000 to resolve the ¹³C₁-¹⁵N₀ and ¹³C₀-¹⁵N₁ isotopologues, which is achievable on modern Orbitrap instruments. However, it takes a minimum resolution of 697,000 to resolve ¹³C₁-¹⁵N₁ and ¹⁸O₁ ($\Delta m = 0.00387$); 1,100,000 to resolve ¹³C₂ and ¹⁸O₁ ($\Delta m = 0.00245$); and 1,550,000 to resolve ³⁴S₁ and ¹⁵N₂ ($\Delta m = 0.00174$). The mass resolution for resolving these isotopologues is not attainable on many current mainstream instruments. Fortunately, as long as the tracer isotopes are resolved, the non-tracer correction matrix is always well-conditioned so that the correction can be done even when the non-tracer isotopes are not resolved.

To accurately account for the contribution from the NA of the non-tracer elements, **NTM** should be constructed based on the actual mass resolution of the experiment (see "Materials and methods: Theory of INAC"). AccuCor2 will calculate whether a specific isotopologue is resolved from any of the tracer isotopologues based on the mass resolution of the measurement. If the resolution is not high enough so that the non-tracer NA contributes to a mass channel of a labeling fraction, AccuCor2 will add associated probability to **NTM** and correct for it. If the resolution is high enough to resolve the non-tracer isotopologue from the labeling fraction, AccuCor2 will leave this fraction off from **NTM** to avoid over-correction.

AccuCor2 performs accurate NA correction from dual-isotope tracer data

To test the performance of AccuCor2, we first used a simulated dataset of ${}^{13}C{}^{-2}H{}^{-1}abeled NAD^+$ ([M-H]⁻, $C_{21}H_{26}N_7O_{14}P_2$) (Fig. 4). The simulation assumed a mass resolution of 750,000, which is



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Fig. 4 Performance of PICor, IsoCorrectoR, and AccuCor2 in simulated data. Uncorrected data are generated using NAD⁺ MS spectra simulated by Thermo Xcalibur Qual Browser at 750,000 resolution. The original labeling patterns are sample 1: 100% non-labeled; sample 2: 50% nonlabeled + 10% $^{13}C_6^{-2}H_2 + 40\% \, ^{13}C_6^{-2}H_3$; sample 3: 36% nonlabeled + 14% $^{13}C_6^{-2}H_2 + 50\% \, ^{13}C_6^{-2}H_3$; sample 4: 100% $^{13}C_6^{-2}H_3$.

sufficient to resolve ¹³C and ²H. The simulation also assumed the following labeling patterns: sample 1: 100% non-labeled; sample 2: 50% non-labeled + 10% ${}^{13}C_{6}{}^{-2}H_{2} + 40\% {}^{13}C_{6}{}^{-2}H_{3}$; sample 3: 36% non-labeled + 14% ${}^{13}C_{6}{}^{-2}H_{2} + 50\% {}^{13}C_{6}{}^{-2}H_{3}$; sample 4: 100% $^{13}C_{6}$ - $^{2}H_{3}$. These isotopologues were chosen because they are possible products when ${}^{13}\breve{C}_{6}{}^{-2}H_{4}$ nicotinamide tracer is used [36]. The simulated dataset shows that besides the expected labeled fractions such as M + C0H0, M + C6H2, and M + C6H3, there are many unexpected isotopologues in the uncorrected data. For example, in sample 4, M + C8H3 is 3.4% of the total intensity and M + C9H1 is 2.1% of the total intensity (Fig. 4). Carbon NA alone does not explain the intensity of these signals. The observed signals are in fact mainly contributed by ¹³C₆-²H₃-¹⁸O₁ and $^{13}C_6{}^{-2}H_3{}^{-15}N_1$, respectively. The mass difference between $^{13}C_6{}^{-2}H_3{}^{-18}O_1$ and $^{13}C_8{}^{-2}H_3$ NAD⁺ is 0.00245, so that they are not resolved when the resolution is below 816,000. The mass difference between ${}^{13}C_{6}-{}^{2}H_{3}-{}^{15}N_{1}$ and ${}^{13}C_{9}-{}^{2}H_{1}$ NAD⁺ is 0.00048, so that they are not resolved when the resolution is below 4,200,000. AccuCor2 recognized these fractions that arose due to NA of the non-tracer elements and corrected them successfully. To quantitatively evaluate the performance of different tools, we also calculated the RMSD to show how far the corrected results deviate from the expected labeling pattern. The corrected results from AccuCor2 are mostly identical to the expected labeling pattern of the samples (RMSD = $7.7 \times 10^{-7} \pm 8.9 \times 10^{-7}$). PICor and IsoCorrectoR, on the other hand, failed to correct these fractions and the corrected results are heavily deviated from the expected data $(\text{RMSD} = 1.1 \times 10^{-2} \pm 5.9 \times 10^{-3})$ $1.2 \times 10^{-2} \pm 6.0 \times 10^{-2}$ and respectively). We believe that this is because these tools essentially assume infinite mass resolution and ignore non-tracer elements and therefore lead to under-correction of the data (Fig. 4).

In addition to the simulated data, we have also tested the performance of AccuCor2 using experimental data (Fig. 5). In



Fig. 5 Performance of PICor, IsoCorrectoR, and AccuCor2 in experimental data. Raw data are serine (A) and sodium acetate adduct of serine (B) $(C_5H_{10}NO_5Na)$ measured under 70,000 nominal mass resolution (defined at m/z 200 on Orbitrap analyzer). The original labeling patterns are sample 1: 100% non-labeled; sample 2: 50% non-labeled + 50% $^{13}C_3^{-15}N_1$; sample 3: 100% $^{13}C_3^{-15}N_1$. Each sample on the plot is the average of four replicates.

this experiment, we used [¹³C₃-¹⁵N₁] serine and unlabeled serine to make the following samples: sample 1: 100% nonlabeled; sample 2: 50% non-labeled + 50% $^{13}C_3$ - $^{15}N_1$; sample 3: 100% $^{13}C_3$ - $^{15}N_1$. The measurement was performed at 70,000 resolution. For serine (Fig. 5A), this resolution is sufficient to resolve all the non-tracer elements. The only unanticipated measured mass fractions are M + 2C1N and M + 3C0N due to the isotopic impurity of the tracer (99% isotopic purity on both ¹³C and ¹⁵N). AccuCor2 and IsoCorrectoR both return the expected results (RMSD = $9.5 \times 10^{-5} \pm 4.2 \times 10^{-5}$ and 9.5×10^{-5} $\pm 4.2 \times 10^{-5}$, respectively for Sample 3), whereas PICor left the ${}^{13}C_2{}^{-15}N_1$ and ${}^{13}C_3{}^{-15}N_0$ fractions uncorrected (RMSD = $1.6 \times 10^{-2} \pm 5.6 \times 10^{-5}$ for Sample 3) because PICor does not handle tracer isotopic impurity (Fig. 5A). In this experiment, serine is also observed as a sodium acetate adduct ([M+NaAc-H]⁻, $C_5H_9NO_5Na$) [30]. We observed signals that correspond to M +C5N1 serine + NaAc (Fig. 5B). Since the NaAc is from the LC mobile phase, which is unlabeled, this fraction should have been generated because of isotope NA. In fact, serine + NaAc has the m/z of 186.04, which means a minimum resolution of 120,000 is needed to resolve $^{13}C_2$ and $^{18}O_1$. The observed $^{13}C_5\text{-}^{15}N_1$ fraction is in fact $^{13}C_3\text{-}^{15}N_1\text{-}^{18}O_1$ fraction. The correction results show that AccuCor2 recognized this fraction and corrected it (RMSD = 0.0 ± 0.0 for Sample 3). In contrast, PICor and IsoCorrectoR failed to correct this fraction (RMSD = $1.1 \times 10^{-2} \pm 5.6 \times 10^{-4}$ and $3.2 \times 10^{-3} \pm 2.8 \times 10^{-4}$, respectively, for Sample 3). Our tests using simulated and experimental data demonstrate that the resolution-dependent correction, as

implemented in AccuCor2, is important for the accuracy of INAC for dual-isotope tracer experiments.

DISCUSSION

AccuCor2 is the first resolution-dependent method to perform INAC on dual-isotope tracer experiments. As shown in "Results", the reason AccuCor2 performs better INAC than other tools is because AccuCor2 does not assume all the non-tracer isotopologues are resolvable from the tracer isotopologues. Instead, AccuCor2 first use the resolution information to determine whether a specific non-tracer isotopologue is resolvable from the tracer isotopologues and then only subtract the fractions of non-resolvable isotopologues from the measured mass fraction. Previous tools for dual-INAC take the assumption that the nontracer elements are fully resolved due to the use of ultra-highresolution instrument. However, as we calculated in "Results", in order to resolve ${}^{2}H_{2}$ - ${}^{15}N_{1}$ and ${}^{13}C_{3}$ in NAD⁺, a minimum nominal mass resolution of 4,200,000 is required. This requirement is far beyond the capability of current ultra-high-resolution LC-MS instruments. Therefore, the resolution-dependent feature of AccuCor2 is critical for accurate INAC.

It is important to note that accurate input data are crucial for INAC. The input data should contain all the detectable labeled isotopologues and the isotopologues that are not resolved from the labeled ones. When picking and integrating the peaks for metabolites, the mass tolerance window should be made narrow enough to exclude the resolved mass peaks. In reality, this is rarely an issue because most people use narrow mass tolerance window of 5 or 10 ppm for peak picking, which is smaller than the resolvable ML. For serine ([M-H]⁻, m/z 104.03532) measured under the nominal mass resolution of 70,000, the resolvable ML is 0.00178, which is 17 ppm. On instruments with mass resolution >200,000, the mass tolerance window need to be carefully adjusted to avoid resolved mass peaks. On the other hand, if two mass peaks are not fully resolved, the m/z of the centroid peak is the intensity-weighted average m/z of the two peaks. This may cause some labeled isotopologue peaks to shift from the calculated m/z. We suggest the users to carefully inspect the raw data to ensure the accuracy of peak picking.

Several limitations of this study are noted. First, although sharing major features with AccuCor, AccuCor2 is specifically designed to handle data generated from dual-isotope experiments. For single-isotope experiments, it is not efficient to use AccuCor2 since additional input parameters are required. In the future, we plan to combine the functions of AccuCor and AccuCor2 into a single R package and make it available in CRAN. Second, AccuCor2 only handle data generated from ¹³C-²H and ¹³C-¹⁵N tracers, which suits the most needs of dual-isotope experiments. In the future, however, the use of other combinations of isotopes such as ²H-¹⁵N and ¹³C-¹⁸O might become popular. To perform INAC on these combinations of isotopes, similar algorithms as shown in Fig. 1 can be designed. Last, the current version of AccuCor2 only works for Orbitrap mass analyzers. We plan to add support for TOF analyzers in the updated versions.

In summary, we developed AccuCor2 as a new tool to perform INAC of data from dual-isotope tracer experiments in a resolutiondependent manner. Our results show that the dual-isotope tracer experiment should use a mass resolution that is high enough to resolve all the labeling fractions of interest. Otherwise, the labeling pattern may not be solvable. The non-tracer elements may require an even higher mass resolution to fully resolve, but this can be handled by the resolution-dependent correction in AccuCor2. AccuCor2 is freely available in open-source format and would enable more accurate calculation of metabolite labeling patterns from MS.

DATA AVAILABILITY

The datasets are available from GitHub repository, https://github.com/wangyujue23/ AccuCor2.

CODE AVAILABILITY

The codes are available from GitHub repository, https://github.com/wangyujue23/AccuCor2.

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AUTHOR CONTRIBUTIONS

YW and XS wrote the main draft. YW, LRP, and XS wrote the code. All authors have reviewed the manuscript. All authors have given approval to the final version of the manuscript.

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The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study do not require ethical approval.

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