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REVIEW

Incorporation and Dilution Values—Their Calculation in Mass Spectrally Assayed Stable Isotope Labeling Experiments

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The significance and validity of incorporation and dilution values in radioisotope tracer work are reviewed. The parallels between specific activity and enrichment factor are drawn. Methods of obtaining enrichment factors from mass spectral data are examined, and examples are given. Natural-abundance correction procedures are documented.

INTRODUCTION

Incorporation[1] ($I\%$) and dilution (D) values have been used extensively over the last two decades in tracer studies of biosynthetic pathways. One of the criteria that A is an intermediate in the biosynthesis of B is that when isotopically labeled A is fed to the system making B, the $I_{A\to B}\%$ value should be high, the $D_{A\to B}$ value, low (*2–5*). The vast majority of extant work uses radioisotopes for which the two parameters are defined as:

$$I_{A\to B}\% = \frac{\text{total dpm isolated in } B \text{ at end of experiment } (Q_B(t)) \times 100}{\text{total dpm fed in } A \text{ at beginning of experiment } (Q_A(t_0))} \tag{1}$$

$$= \frac{\text{sp act}_B(t) \times M_B(t) \times 100}{\text{sp act}_A(t_0) \times M_A(t_0)} \tag{2}$$

$$D_{A\to B} = \frac{\text{sp act}_A(t_0)}{\text{sp act}_B(t)} = \frac{100}{I_{A\to B}\%} \times \frac{M_B(t)}{M_A(t_0)}, \tag{3), (4}$$

where t_0 signifies the beginning, and t the end of the experiment, M_A and M_B are the molar intracellular pool sizes of A and B, respectively, and sp act = specific activity (e.g., in dpm/μmole). Frequently the approximation is made that sp act$_A(t_0)$ and $M_A(t_0)$ are the specific activity and mole fraction, respectively, of the fed material prior to introduction to the biosynthetic system, i.e., no account is taken of the endogenous pool of A. Such inaccuracies have no influence on $I_{A\to B}\%$ but can perturb $D_{A\to B}$.

Recently, stable isotopes have regained their former favor with biosynthetic chemists (see *6, 7* for early stable isotope history). Isotope assays are performed either mass spectrally or by nuclear magnetic resonance (*5, 8, 9*). Particularly in the former case, problems can occur in computing incorporation and dilution values. This article indicates how these problems can be avoided. It assumes the reader has some experience with radioisotopes.

[1] This is the "absolute incorporation rate" of Luckner (*1*)

A BASIC DIFFERENCE BETWEEN RADIO AND STABLE ISOTOPE MEASUREMENTS

In radioisotope work the basic measurement is of the disintegration rate (dpm). Implicitly this is the number of isotopic atoms in the sample under examination since by the Rutherford–Soddy equation,

$$\frac{-dN}{dt} = N\lambda, \tag{5}$$

where

$$\frac{dN}{dt} = \text{disintegration rate},$$

N = number of isotopic atoms in the sample, and

λ = decay constant

$$= \frac{0.639}{\text{half-life of isotope involved}}. \tag{6}$$

The specific activity is derived from the disintegration rate by dividing it by the mole fraction of sample giving rise to it. Since the number of atoms of a particular sort in a compound is proportional to the mole fraction considered, this derived sp act is simply an enrichment factor. Example 1 in the Appendix makes this point clearly. Hence, in radioactive work the number of isotopic atoms is determined directly, the enrichment factor (*EF*) indirectly.

With stable isotopes the procedure is reversed. As will be shown later, *EF* values are obtained directly from mass spectral data. The number of stable isotopic atoms in the particular sample, should this figure be needed, is obtained indirectly by calculation from the *EF* (see example 2 in the Appendix). This reversal with its subtle change in emphasis is a principal cause of trouble with $I\%$ and D values in stable isotope work. Ironically, it arises from a misconception in the radioisotope area. Stable isotope *EF* values (recorded as atom %) are always understood for what they are; atomic, not molecular, parameters. A specific atom in a molecule is enriched to a certain extent. Specific activities, although there are bona fide enrichment factors, are almost always considered to be molecular parameters. A molecule, as opposed to an atom in a molecule, has a certain specific activity. Within the radioactive area this misconception causes only minor problems; it does, however, inhibit direct projection of radioisotope knowledge into the stable isotope field.

$I\%$ AND D VALUES DEFINED FOR STABLE ISOTOPES

Since *EF* and specific activity are parameters of an ilk, and since $I\%$ and D can be defined in terms of specific activity, it should follow that, for stable isotopes (*I*)

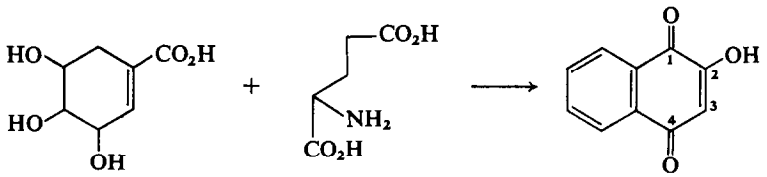
$$I_{A\to B}\% = \frac{EF_B(t) \times M_B(t) \times 100}{EF_A(t_0) \times M_A(t_0)} \tag{7}$$

$$D_{A\to B} = \frac{EF_A(t_0)}{EF_B(t)}. \tag{8}$$

Such is indeed the case, if it is understood that $I\%$ and D values, like specific activity and EF, have significance only at the atomic level. Biosynthetic workers, the present author included, have become so used to considering $I\%$ and D values in molecular terms, calculating them indiscriminately for all precursor/product relationships, that awareness of the meaning of the $I\%$ and D functions has dulled. In the next section examples of valid and invalid use of $I\%$ and D in the radioisotope field are first reviewed.

VALID (AND INVALID) USES OF $I\%$ AND D VALUES IN RADIOISOTOPE WORK

Case I. Singly labeled precursor: singly labeled product. Lawsone (I) is biosynthesized in *Impatiens balsamina* from shikimate (II) and glutamate (III) (*10*, and references therein). When [2-^{14}C]glutamate (130 μCi*/μmole, 1.0 μmole) was fed, lawsone (10^5 dpm/μmole, 20 μmoles) was isolated. Chemical degradation showed that all the label



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was located at C-4 of lawsone. The $I\%$ and D values from Eqs. 2 and 3, are 0.69% and 1:2886, respectively.[2] This is a valid use and represents the conversion of glutamate C-2 to lawsone C-4. Thought may be on the molecular scale but since only a single isotope is involved, its specific activity is equal in magnitude to that of the molecule in which it is found. From the $I\%$ and D point of view, this is the most meaningful type of experiment. A situation where two or more labels in the procursor are transferred intact to the product can be considered a simple extension of this case, e.g., [3,4-^{14}C]glutamate to [2,3-^{14}C]lawsone. The activity is treated as if it were centered at only one site.

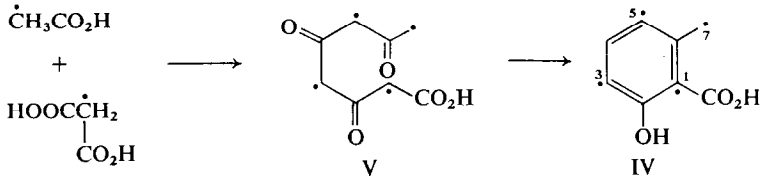
Case II. Singly labeled precursor: multiply labeled product. If the sample of lawsone, biosynthesized above, were dimerized in a subsequent intracellular step, the dimer (for instance, 1.0 μmole) might also have a specific activity of 10^5 dpm/μmole. To claim, however, that the glutamate/lawsone dimer conversion had proceeded with an $I\%$ of 0.035% and D of 1:2886 (direct use of Eqs. 2 and 3) would be invalid. The "molecular" specific activity of 10^5 dpm/μmole in the dimer is actually the sum of two "atomic" specific activities of 5×10^4 dpm/μmole. The valid conclusion from the feeding experiment is that C-2 of glutamate is incorporated to the extent of 0.017%, with a dilution of 1:5772 into each of the C-4 atoms of lawsone dimers. Only the fact that the specific activities of atoms in a molecule are additive permits us to think loosely, and calculate "molecular" specific activities. To get the valid "atomic values" suitable chemical degradations have to be performed to isolate individual carbon centers.

Case III. Several singly labeled precursors: multiply labeled product. 6-Methylsalicylate

* Ci = curie = 2.22×10^{12} dpm.

[2] For convenience, pre-existing intracellular precursor pools are neglected in $I\%$ and D calculations in this article.

(IV) is produced in fungi by condensation of one molecule of acetate with three of malonate (*11*). Malonate itself derives from acetate. If, on feeding [2-^{14}C]acetate (100 μCi/



μmole, 1.0 μmoles), methylsalicylate (10^6 dpm/μmole, 10 μmoles) was obtained, it would be invalid to report $I\%$ and D values of 4.5% and 1:222. Separate values for C-1, C-3, C-5, and C-7 have to be obtained through chemical degradation. In practice, we err and no great harm is seen to be done; valid $I\%$ and D values are implied in the degradation results of the paper.

Several other cases can be envisioned, e.g., triply labeled precursor to doubly labeled products, but enough has been said to establish the need for care in $I\%$ and D value calculation from radioisotope data. If this habit is set, the switch to stable isotopes will be uneventful. With stable isotopes under mass spectral assay conditions, one cannot fail to factorize overall molecular enrichment into its atomic components—that is the way the data come.

CALCULATION OF EF FROM MASS SPECTRAL DATA—SINGLE ISOTOPE INVOLVEMENT

In this section and the next the influence of stable isotope labeling on the parent molecular ion (PMI) of a substance will be examined. Fragment ions can also be used but care must be taken that the natural abundance satellite is not adulterated by other nonrelated fragment ions of higher mass. In this section and the next we also simplify the scene by neglecting the contribution of natural abundance; these effects are covered in the examples in the Appendix.

Returning to Case I of the last section, suppose the glutamate had been obtained with a ^{13}C at C-2 of $EF = 50$ ^{13}C atom %. This means that for every glutamate molecule with a ^{13}C at C-2 there is another one with a ^{12}C. The mass spectrum of this sample would be that of a binary mixture of [2-^{12}C]glutamate and [2-^{13}C]glutamate, with the PMI of the former at 147 amu, of the latter at 148 amu.[3] Since isotopic enrichment does not influence significantly ionization efficiency, the PMI and PMI + 1 would be of equal intensity and the equations below give the EF of glutamate C-2

$$EF = \frac{\text{intensity of (PMI + 1)} \times 100}{\text{sum of intensities of PMI and (PMI + 1)}} \tag{9}$$

or

$$= \frac{R \times 100}{(R+1)}, \tag{10}$$

[3] Traditionally, the term "PMI" in this situation is reserved for the parent ion of the unenriched species; that of the enriched is referred to as the "PMI + n", where n is difference between its m/e value and that of the unenriched.

where

$$R = \frac{\text{intensity of (PMI + 1)}}{\text{intensity of (PMI)}}, \tag{11}$$

The PMI cluster of the synthesized lawsone would also have a (PMI + 1) contribution from which the *EF* of C-4 could be calculated. Equations 7 and 8 would then give valid $I\%$ and D values.

Although not actually a single-isotope incorporation situation, cases where multiple labels go in en bloc can be treated as such. Thus, if lawsone were methylated by an enzyme system using *S*-adenosylmethionine, a CD_3-methionine feeding could be followed by measuring PMI and (PMI + 3) intensities in substrate and product. There are two absolute requirements for this approach to succeed: (a) in the starting CD_3 unit the *EF* of each deuterium must be 100%, and (b) none of the deuterium atoms of the CD_3 unit must be equilibrated with hydrogen in the course of the study.

CALCULATION OF *EF* FROM MASS SPECTRAL DATA—MULTIPLE ISOTOPE INVOLVEMENT

Consider Case II in the stable isotope context. The glutamate assay is straightforward but complexities occur with the lawsone dimer. Two labels are present therein, but their effect is not simply additive as in the ^{14}C-labeled case. There will be three distinct molecular species formed: one with no ^{13}C, one with one ^{13}C, and one with two ^{13}C. Their relative proportions, and hence the intensity ratios of the PMI cluster, are governed by the enrichment factor of each individual carbon. Let the EF of both carbons (we assume them equal as before) by m ^{13}C atom %. At any of the labeled loci in the lawsone dimer the ratio $^{12}C:^{13}C$ will be:

$$\frac{100-m}{100} : \frac{m}{100}.$$

Simple probability theory states that if two such loci are combined, the distribution of $2\ ^{12}C:(1\ ^{12}C + 1\ ^{13}C):2\ ^{13}C$ is:

$$\left(\frac{100-m}{100}\right)^2 : \frac{2m}{100} \times \left(\frac{100-m}{100}\right) : \frac{m}{100}^2,$$

i.e., in the ratio of the terms of the expansion of the binomial:

$$\left(\left(\frac{100-m}{100}\right) + \frac{m}{100}\right)^2.$$

If the ion due to the unenriched species is called the PMI and is set to unit intensity, the PMI cluster intensities will be:

$$\begin{aligned}\text{PMI}:(\text{PMI}+1):(\text{PMI}+2) &= \left(\frac{100-m}{100}\right)^2 : \frac{2m}{100}\left(\frac{100-m}{100}\right) : \frac{m}{100}^2 \\ &= 1 : \left(\frac{2m}{100-m}\right) : \left(\frac{m}{100-m}\right)^2 \left[\text{dividing by} \left(\frac{100-m}{100}\right)^2\right].\end{aligned}$$

Whence m can be obtained by setting $2m/(100-m)$ and $m^2/(100-m)^2$ equal to the experimentally determined relative intensity of PMI + 1 and PMI + 2, respectively (see example 4 in Appendix). This value of m can be used, in conjunction with the EF of glutamate, in Eqs. 7 and 8 to get valid $I\%$ and D data for the transfer of glutamate C-2 to each of the labeled portions in the dimer.

In general, where multiple labeling occurs with n atoms of the same $EF = m$ the PMI cluster consists of $n + 1$ peaks whose relative intensity is given by the ratio of the terms of the expansion of:

$$\left(\left(\frac{100-m}{100}\right)+\frac{m}{100}\right)^n.$$

The value of m can be obtained by counting the peaks in the cluster (to get value of n) and then solving the multiple algebraic equations (see example 5 in the Appendix).

Stable isotope assay by mass spectrometry assumes a further level of complexity when isotopes are present in a molecule at various levels of enrichment, e.g., Case III. If [2-^{13}C]acetate were used in the biosynthesis, methylsalicylate with up to four ^{13}C atoms could be formed. The PMI cluster would contain 5 ions whose intensity ratio would be given by the expansion of:

$$(A_1+B_1)(A_2+B_2)(A_3+B_3)(A_4+B_4), \tag{12}$$

where

$$A_i=\frac{100-m_i}{100}; \qquad B_i=\frac{m_i}{100}$$

and m_i $(i = 1,4) = EF$ of salicylate carbons 1,3,5,7, respectively. This could be simplified to:

$$(A_1+B_1)(A_2+B_2)^3 \tag{13}$$

if it could be assumed that all the malonate EF values were the same, i.e., there was no serial dilution of the malonate pool as the polyketide (V) was being formed. For the simplified case (Eq. 13) the PMI cluster intensity ratio would be:

$$\begin{aligned}&\text{PMI}:(\text{PMI}+1):(\text{PMI}+2):(\text{PMI}+3):(\text{PMI}+4)\\&=A_1A_2^3:(3A_2^2B_2A_1+B_1A_2^3):(3A_2B_2^2A_1+3A_2^2B_2B_1):(B_2^3A_1+3A_2B_2^2B_1):B_1B_2^3\\&=1:(3R_2+R_1):(3R_2^2+3R_1R_2):(R^3{}_2+3R_1R_2^2):R_1R_2^3,\end{aligned}$$

where

$$R_1=\frac{B_1}{A_1} \quad \text{and} \quad R_2=\frac{B_2}{A_2}.$$

Values of R_1 and R_2, and, therefore, of the two enrichment factors, can be obtained by simple algebra (see example 4 in Appendix). Since there are essentially four equations in two unknowns in the above, a statistically significant answer should be available. In the more complex case (Eq. 12) with four unknowns, statistical significance may be low, especially if one or more of the intensities is small. In such situations and in more heavily labeled products, it may be advisable to degrade the biosynthetic product to more simple species prior to analysis.

Before attempting a binomial expansion analysis of a complex PMI cluster, it is important to check that it was formed from homogeneously labeled precursors. The reverse

sometimes occurs when deuterium labeling is used in the methylene and methyl groups of precursor, and one or more isotopic atoms are partly "washed out." The biosynthetic starting material is, therefore, a mixture of, for instance, mono-, di-, and trideuterated forms. Products made from such a "precursor" would give a complex PMI cluster whose intensities would resist attempts to relate them to binomial expansion terms. The best that can be done in such cases is the mole % analysis of Biemann (*12*). The biosynthetic significance in terms of $I\%$ and D values of this type of experiment is suspect.

NATURAL ABUNDANCE—CORRECTIONS FOR ITS EXISTENCE

Carbon, hydrogen, oxygen, and nitrogen all exist naturally, enriched to a small extent with heavy isotopes. The precise composition of each element varies slightly with radiochemical history, but average values are:

Carbon	1.069 ^{13}C atom %
hydrogen	0.016 ^{2}H atom %
nitrogen	0.380 ^{14}N atom %
oxygen	0.039 ^{17}O atom % and 0.200 ^{18}O atom %.

Because of this, any calculation of EF made and planned for use in $I\%$ and D computations has significance only to the extent that it is in excess of the natural abundance. Such corrected EFs, expressed as atom % excess, are obtained traditionally by subtracting the natural abundance from the directly observed enrichment factor.

The significance of the value so computed, however, is of doubt for the following reason. Consider a sample of graphite containing m ^{13}C atom and n ^{12}C atoms. The EF is $m/(m+n)$. Let the natural abundance of ^{13}C in graphite be $p/(o+p)$ i.e., p ^{13}C for every o ^{12}C atoms. The "corrected" EF is by the above definition:

$$\begin{aligned}\text{corr. } EF &= \frac{m}{m+n} - \frac{p}{p+o} && {}^{13}\text{C atom \% excess} \\ &= \frac{m(p+o)-p(m+n)}{(m+n)(p+o)} && {}^{13}\text{C atom \% excess} \\ &= \frac{mo-pn}{(m+n)(p+o)} && {}^{13}\text{C atom \% excess.} \end{aligned} \tag{14}$$

Another way to approach the question is to remove from the m ^{13}C atoms in the sample that number $(n \times p/o)$ naturally associated with n ^{12}C and then calculate the EF from the ratio:

$$\frac{\text{number of } {}^{13}\text{C in sample, excluding those occurring naturally}}{\text{number of total carbon atoms, excluding those } {}^{13}\text{C occurring naturally}}$$

$$\begin{aligned} &= \frac{m - \frac{np}{o}}{n + m - \frac{np}{o}} \\ &= \frac{mo-np}{no+mo-np}. \end{aligned} \tag{15}$$

Note that the expressions 14 and 15 will be equal only if

$$no + mo - np = mp + np + om + on,$$

i.e.,
$$mp + 2np = 0$$

i.e.
$$p(m + 2n) = 0$$

$$p = 0 \quad \text{or} \quad m = n = o$$

both of which are impossible in reality.[4] The latter method is the one used in what follows.

Because of the natural abundance phenomenon, the PMI of all naturally formed compounds is a cluster. The intensity ratio of the ions in that cluster is obtained by the expansion method used previously. For a compound of composition $C_wH_xN_yO_z$ the appropriate expression is

$$\left(\frac{100-c}{100}+\frac{c}{100}\right)^w\left(\frac{100-h}{100}+\frac{h}{100}\right)^x\left(\frac{100-n}{100}+\frac{n}{100}\right)^y\left(\frac{100-o_1-o_2}{100}+\frac{o_1}{100}+\frac{o_2}{100}\right)^z,$$

where c, h, n, o_1 and o_2 are the natural abundance of ^{13}C, ^{2}H, ^{14}N, ^{17}O, and ^{18}O, respectively. Luckily a table of precomputed values is available (*13*). As is shown in the examples that follow, these natural intensities are subtracted from the experimental intensities prior to calculating the *EF*. Such values are, therefore, corrected and should be stated as atom % excess.

APPENDIX

Example 1. A sample of $^{14}CO_2$ has sp act $= 7.50 \times 10^6$ dpm/μmole. The number of ^{14}C atoms per μmole is, therefore, N, where

$$N = \frac{1}{\lambda}\left|\frac{dN}{dt}\right|$$

$$= \frac{7.50 \times 10^6}{2.12 \times 10^{-10}} = 3.54 \times 10^{16}$$

(for ^{14}C, $t_{1/2} = 5.72 \times 10^3$ yr $= 3.01 \times 10^9$ min; by Eq. 6, $\lambda = 2.12 \times 10^{-10}$/min).

In 1 μmole, there are Avogadro's number $\times 10^{-6}$ molecules of CO_2. This is also the number of atoms of carbon. The enrichment factor of the carbon, therefore, is

$$\frac{\text{number of } ^{14}C \text{ atoms}}{\text{number of } (^{14}C + ^{12}C) \text{ atom}} = \frac{3.54 \times 10^{16}}{6.02 \times 10^{23} \times 10^{-6}} = 5.88 \times 10^{-2}$$

or 5.88 ^{14}C atoms % total carbon.

[4] A simple and convincing way to demonstrate the error in the "classical" calculation is to consider a compound containing a pure ^{13}C atom at one site. The *EF* of that carbon will be 100 ^{13}C atom %. The atom % excess value will also be 100% since no ^{12}C is present at that site to give rise to a natural abundance. Notwithstanding, the classical calculation would by definition set atom % excess = 100 − natural ^{13}C abundance = 98.931.

Example 2. Consider 1.000 g of graphite with an enrichment factor of 30.00 ^{13}C atom %. An atom of ^{13}C weighs (13.00/Avogadro's number) g, that of ^{12}C (12.00/Avogadro's number) g. One hundred atoms of the sample will weigh

$$\left(\frac{30 \times 13.00}{A} + \frac{70 \times 12.00}{A}\right) g = \frac{12.3 \times 10^2}{6.02 \times 10^{23}} g \qquad \text{(footnote 5)}$$

(A = Avogadro's number = 6.02×10^{23}).

1.000 g the sample will, therefore, contain

$$\frac{30 \times 6.02 \times 10^{23}}{12.3 \times 10^2} \text{ atoms of } ^{13}C$$

$$= 1.47 \times 10^{22} \text{ atoms of } ^{13}C.$$

Example 3. Figure 1a shows the PMI cluster of a sample of phenacyl acetate ($C_{10}H_{10}O_3$) made from [2-^{13}C]sodium acetate (approximate *EF* 60–65 ^{13}C atom %).

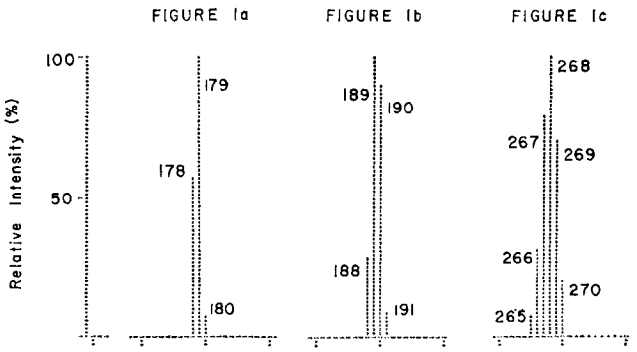


FIG. 1. PMI cluster (LKB 9000; ionization voltage, 17 eV; acceleration voltage, 3.5 kV; ion current, 60 μA; source temp. 270°C) of: (a) phenacyl-[^{13}C]-acetate; (b) [2,7-di-^{13}C]methoxynaphthalene; (c) [hexa-^{13}C]methylinositol.

The PMI:(PMI + 1):(PMI + 2) ratio is 1.00:1.75:0.13. The PMI cluster ratio due to natural abundance is PMI:(PMI + 1):(PMI + 2) = 1.0:0.11:0.01. Hence, by subtraction, the corrected ratio of PMI:(PMI + 1) is 1.00:1.64, viz:

m/e	178	179	180
	1.00	:1.75	:0.13
	(1.00)	:0.11	:0.01
	1.00	:1.64	:0.12

Applying Eq. 10, $R = 1.64$, gives an enrichment factor of 62.1 ^{13}C atom % excess. (Note—the intensity remaining at *m/e* 180 after removing the PMI + 2 contribution of the *m/e* 178 ion, represents the "PMI + 1" natural abundance contribution of the *m/e* 179 ion.)

Example 4. Shown in Fig. 1b is the PMI cluster of 2,7-dimethoxynaphthalene ($C_{12}H_{12}O_2$) prepared from methyl iodide ($^{13}CH_3$ = 61.5 ^{13}C atom %). The ratio

[5] The figure 12.3 represents the "atomic weight" of the graphite sample obtainable by standard gravimetric analysis.

PMI:(PMI + 1):(PMI + 2):(PMI + 3) is observed to be 1.0:3.34:3.01:0.31; the natural abundance ratio of the unenriched material is 1:00:0.13:0.01. Firstly correct for the natural abundance associated with the 188-amu parent ion. This is done analogously to example 3, thus:

m/e	188	189	190	191
	1.00	:3.34	:3.01	:0.31
	(1.00)	:0.13	:0.01	
	1.00	:3.21	:3.00	:0.31

Next the natural abundance of the 189-amu ion is removed. This is done by subtraction, after the natural abundance ratio has been normalized to an intensity value of m/e 189 = 3.21, thus:

m/e	188	189	190	191
	1.00:	3.21	:3.00	:0.31
		(3.21)	:0.42	:0.03
	1.00:	3.21	:2.58	:0.28

This ratio is related to the expansion of the equation:

$$(A_1 + B_1)(A_2 + B_2) \qquad \text{(footnote 6).}$$

Clearly $A_1A_2:(A_1B_2 + A_2B_1):B_1B_2 = 1.00:3.21:2.58$. Dividing the left-hand side by A_1A_2, the expression converts to

$$1:\frac{(A_1B_2 + A_2B_1)}{A_1A_2}:\frac{B_1B_2}{A_1A_2} = 1.00:3.21:2.58$$

whence

$$\frac{A_1B_2 + A_2B_1}{A_1A_2} = 3.21, \quad \text{and} \quad \frac{B_1B_2}{A_1A_2} = 2.58.$$

Let

$$R_1 = \frac{B_1}{A_1} \quad \text{and} \quad R_2 = \frac{B_2}{A_2},$$

then from the above, $R_1 + R_2 = 3.21$ and $R_1R_2 = 2.58$ whence by simple algebra $R_1 = R_2 = 1.6$. Now

$$R_i = \frac{B_i}{A_i} = \frac{EF_i}{100} \times \frac{100}{100 - EF_i} \qquad \text{[from Eq. (12)],}$$

hence

$$EF_1 = EF_2 = 61.5\ ^{13}\text{C atom \% excess.}$$

Example 5. Shown in Fig. 1c is the PMI cluster of the hexamethyl ether of inositol (264 amu, $C_{12}H_{24}O_6$), natural abundance ratio PMI:(PMI + 1):(PMI + 2) = 1.00: 0.14:0.02. There is no discernible intensity at 264 amu—the normal parent ion. The remaining six ions have intensities in the ratio (ascending) 0.11:0.46:1.15:1.46:1.04: 0.31. This ratio can be corrected for natural abundance as was done in the last example.

[6] We have chosen to use this expression, rather than the simpler $(A + B)^2$ to illustrate how to proceed if it is not known that both methyl groups were enriched to the same extent. The reader should repeat the calculation using $(A + B)^2$.

Thus:

m/e	265	266	267	268	269	270
	0.11 :	0.46 :	1.15 :	1.46 :	1.04 :	0.31
	(0.11):	0.02 :	0.00			
	0.11 :	0.44 :	1.15 :	1.46		
		(0.44):	0.06 :	0.00		
	0.11 :	0.44 :	1.09 :	1.46 :	1.04	
			(1.09):	0.15 :	0.02	
	0.11 :	0.44 :	1.09 :	1.31 :	1.02 :	0.31
				(1.31):	0.18 :	0.03
				1.31 :	0.84 :	0.28
					(0.84):	0.12
					0.84 :	0.16

i.e., 0.11:0.44:1.09:1.31:0.84:0.16. This ratio can now be related to the expansion of $(A + B)^6$ with the exclusion of the term A^6 (the PMI) thus:

$$6A^5B : 15A^4B^2 : 20A^3B^3 : 15A^2B^4 : 6AB^5 : B^6 = 0.11 : 0.44 : 1.09 : 1.31 : 0.84 : 0.16.$$

These values can then be associated in pairs by division to solve for $R = B/A$ thus:

$$\frac{\text{PMI} + 4}{\text{PMI} + 3} = \frac{15A^2B^4}{20A^3B^3} = \frac{1.31}{1.09}; \quad \text{i.e.,} \quad \frac{3}{4}R = 1.20; \quad \text{i.e.,} \quad R = 1.60.$$

$$\frac{\text{PMI} + 5}{\text{PMI} + 4} = \frac{6AB^5}{15A^2B^4} = \frac{0.84}{1.31}; \quad \text{i.e.,} \quad \frac{2}{5}R = 0.64; \quad \text{i.e.,} \quad R = 1.60,$$

etc. with other pairs. Whence

$$ER = 61.5\ ^{13}\text{C atom \% excess.}$$

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