Supporting Information

A New Concept Linking Observable Stable Isotope Fractionation to Transformation Pathways of Organic Pollutants

Martin Elsner, Luc Zwank‡, Daniel Hunkeler†, René P. Schwarzenbach.*

Swiss Federal Institute for Environmental Science and Technology (EAWAG) and Swiss Federal Institute of Technology Zurich (ETHZ), Ueberlandstrasse 133, CH-8600 Duebendorf

elsner@geology.utoronto.ca

‡ present address: s&e consult, B.P.2453 L-1024 Luxembourg

† Univ Neuchatel, Ctr Hydrogeol, Rue Emile Argand 11, CH-2007 Neuchatel, Switzerland

* Corresponding author present address: Stable Isotope Laboratory, Department of Geology, University of Toronto; 22 Russell Street, Toronto, Ontario M5S 3B1; phone: ++1 416 978 0825; Fax: ++1 416 978 3938; e-mail: elsner@geology.utoronto.ca

1. Experimental Determination of Isotope Ratios

With Compound Specific Isotope Analysis (CSIA) the average relative abundance of the heavy (^{h}E) and light (^{l}E) isotopes of a given element E is determined in molecules of a given compound (expressed by the ratio $R = {}^{h}E/{}^{l}E$). Owing to the high precision required for measurement of such isotopic ratios at natural abundance, special isotope ratio mass spectrometers (IRMS) are required *(1)*. Compared to conventional high resolution organic mass spectrometers such instruments achieve their higher precision by simultaneously recording the signals of the different isotopomers on separate collectors. For the analysis, the analytes have to be separated completely by chromatography and converted quantitatively on-line into simple gases such as CO_2, H_2, N_2 and CO for carbon, hydrogen, nitrogen and oxygen isotopic analyses, respectively. The combustion to CO_2 is usually carried out in presence of a catalyst (CuO, NiO) at temperatures > 900 °C *(2)*, whereas pyrolysis to H_2 takes place at temperatures of > 1200 °C in the absence of a catalyst *(3)*. Extensive reviews on gas chromatography isotope ratio mass spectrometry (GC-IRMS) have been published by Brenna *(1)* and Meier-Augenstein *(4)*. Recently, an online oxidation interface has been introduced that allows also the coupling of HPLC separation to an IRMS for the determination of $^{13}C/^{12}C$ ratios of compounds that can not be analyzed by gas chromatography *(5)* [ThermoFinnigan Prospect für LC ISOLINK^{TM}] Independent of the type of chromatographic separation or oxidation / pyrolysis procedure, CSIA can, of course, only yield a bulk isotopic composition of the molecule, as opposed to a position specific isotopic composition that would be obtainable from SNIF-NMR (Site-specific Natural Isotope Fractionation by Nuclear Magnetic Resonance Spectroscopy). SNIF-NMR analysis is a new, alternative method for isotope analysis in organic compounds *(6, 7)*. It has the advantage that isotope ratios can be measured specifically at each molecular position *(8)*, but the method requires large amounts (typically several milliliters) of pure substance and is, hence, not suited for analyzing mixtures of compound at low concentrations. To date, SNIF-NMR has therefore mostly been applied in the certification of food components, while for the analysis of environmental samples GC-IRMS is the method of choice.

The bulk isotopic signature of a given compound determined by gas chromatography – isotope ratio mass spectrometry (GC-IRMS) is commonly reported as difference in per mil with respect to an international standard, and is denoted as $\delta^{h}E$ *(9, 10)*:

$$\delta^{h}E = \left(\frac{R - R_{ref}}{R_{ref}} \right) \cdot 1000‰ = \left(\frac{({}^{h}E/{}^{l}E) - ({}^{h}E/{}^{l}E)_{ref}}{({}^{h}E/{}^{l}E)_{ref}} \right) \cdot 1000‰ \quad (1)$$

Note that precise results can only be obtained by measuring isotope ratios relative to a reference gas of known isotopic composition, rather than by trying to measure absolute isotope ratios. To this end different external standards as well as reference gases are available *(11)*. Hence, all analytical procedures have to be evaluated thoroughly with analytes of known isotopic compositions, and the referencing procedures have to be used correctly to yield accurate results that are reproducible also in interlaboratory comparisons *(12)*.

The moderate sensitivity of CSIA is a major difficulty for its use in environmental applications. However, using appropriate enrichment techniques (e.g. solid-phase microextraction, purge and trap), method detection limits in the low µg/L-range can be achieved *(13)*. A comprehensive overview of detection limits of CSIA for different environmentally relevant organic compounds is given in a recent review by Schmidt et al. *(14)*. Sensitivity and technical requirements (e.g., online transformation) mentioned above are the main reasons why studies in contaminant hydrology, so far, have primarily focused on measurements of $\delta^{2}H$ and, particularly, $\delta^{13}C$. The examples discussed in this review, therefore, mostly deal with these two elements.

2. Kinetic Isotope Effects in the Literature (Detailed Discussion)

2.1 Background Information Related to the Rules of Thumb

The rules of thumb presented in the main text are based on the so-called zero-point energy approximation *(15)* that can be derived within the theory of activated complexes (transition state theory *(16)*). The following discussion is a short summary of textbook knowledge such as from "Physical Chemistry" by Atkins *(17)* or, for treatment of isotopes, from "Reaction Rates of Isotopic Molecules" by Melander and Saunders *(15)*). As illustrated in Figure S1, kinetic isotope effects KIE (= the ratio of rate constants of chemical bonds containing light vs. heavy isotopes, see Equation 2 in the main article) are caused by the difference in activation energies of bonds with heavy (white arrow) vs. light (black arrow) isotopes. These activation energies depend, in turn, on the energy levels of the heavy (dashed line) vs. light (solid line) isotopic bonds in the ground and transition state.

Clearly, such activation energies are determined by the changes that a molecule experiences during reaction. The main contribution comes from the energy of electrons, which are located between the atomic nuclei and act like "glue" that keeps the atoms together in chemical bonds. However, according to the Born-Oppenheimer approximation (= electrons move so rapidly that they are unaffected by the much slower, mass-dependent motion of the atomic nuclei *(16)*) these electronic energies do not contribute to the kinetic isotope effect. Hence, the next important contribution stems from the energy caused by changes in molecular vibrations during a reaction (= periodic oscillations of the atomic nuclei within molecular bonds). In a first approximation, molecular vibrations can be described by a classical oscillator with its corresponding potential curve and energy levels corresponding to discrete quantum states *(17)*. Because the excitation of molecular vibrations is generally negligible at ambient temperatures, the vibrational energies may be characterized by their lowest ("zero point") energy levels *(15)*. This zero point energy is given by $E = h \cdot \nu/2$, where h is the Planck constant and ν the respective frequency of molecular vibrations. This frequency is in turn dependent on the force constants, f, and the

reduced masses, μ, of the respective vibration according to the formula for harmonic oscillators $\nu = 1/2\pi \sqrt{f/\mu}$ *(17)*.

The reduced mass is $\mu = (m_{Isotope} \cdot m_{Binding\ Partner}) / (m_{Isotope} + m_{Binding\ Partner})$, where $m_{Isotope}$ and $m_{Binding\ Partner}$ are the atomic masses of the isotope and the binding partner in the covalent bond. Because the reduced mass is greater for heavy than for light isotopes, zero-point energy levels are always of lower energy for bonds with heavy isotopes (dashed line in Figure S1) than with light isotopes (solid line). Moreover, this difference in reduced masses (and, hence, the spacing of the energy levels) increases with the relative difference between the mass of the isotopes (Rule of Thumb No. 1). In addition, the mass difference between the isotopes is more strongly reflected in the difference of the overall reduced masses if the mass of the binding partner is greater (Rule of Thumb No. 2). Finally, the spacing between the isotopic zero-point energy levels depends also on the force constant f of the vibration, being greater in steep (narrow) vibrational potentials with larger f and higher ν (Rule of Thumb No. 3, first part).

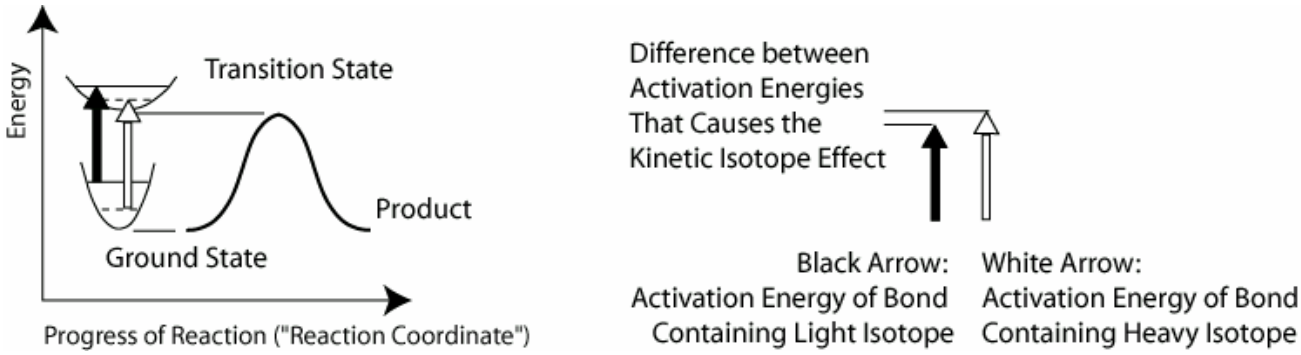

Figure S1. Activation energies (arrows) as given by the differences in zero-point energy levels (lines inside the respective vibration potentials) between ground and transition state. The activation energy and energy levels of the light isotopic bond are given by a black arrow and solid lines, respectively, and those of the heavy isotopic bond by a white arrow and dashed lines. As shown in the right part, the kinetic isotope effect is determined by the *difference* between the isotopic activation energies.

As shown in Figure S1, the difference between the isotopic activation energies does not only depend on the spacing between energy levels in the ground state, but also on the corresponding spacing in the transition state of a reaction. Hence, the more a bond is weakened in the transition state (corresponding to a smaller force constant *f* and a more shallow potential with smaller spacing) the greater the isotope effect will be (Rule of Thumb No.3, second part). On the other hand, if bonds are formed or become stronger in the transition state (corresponding to a steeper potential and greater spacing), kinetic isotope effects may even become inverse. Finally, as vibrations can only be observed in the ground, but not in the transition state, it is intrinsically difficult to obtain kinetic isotope effects from theoretical calculations. For certain reactions it has been possible to determine the geometry of the transition state structures by computational approaches, to obtain the frequency of the corresponding vibration and to subsequently calculate theoretical KIE values (see, e.g., *(18)* and references cited therein). Alternatively, as discussed in the main text, rough estimates of the maximum values (Streitwieser Limits) may be obtained by assuming the bond is completely broken (= zero spacing) in the transition state. The upper limit of the kinetic isotope effect can then simply be estimated from the spacing of the energy levels in the ground state.

2.2 Hydrogen KIE Values and Typical Carbon KIE Values in different Reactions

Many oxidation reactions involve the initial cleavage of a C-H bond. The corresponding hydrogen isotope effects KIE_H (for definition see Eq. 2 in the main article) are reported to be very large, that is, generally greater than 2, typically in the range of 3-8 and in the presence of tunnel effects as high as 40 or even 50 *(19-22)*. These large isotope effects are characteristic of all kinds of hydrogen transfer reactions, no matter whether they are oxidations (H radical transfer), reductions (hydride transfer) or acid-base reactions (proton transfer). Conversely, many hydrolysis reactions of contaminants involve a nucleophilic substitution of either the S_N2- or the S_N1-type. Typical carbon isotope effects for these reactions are between KIE_C = 1.00 and 1.03 in nucleophilic substitutions of the S_N1-type *(23)* and significantly larger, 1.03 to 1.08 (section 8.3.4 in *(15)*) or even 1.09 *(23)*, in nucleophilic substitutions of

the S_N2-type. (Note that $^{14}k/^{12}k$-effects in ref. *(23)* were converted into $^{13}k/^{12}k$-effects using the relationship in section 2.3.3 of *(15)*.)

Less data is reported on carbon isotope effects for oxidative cleavage of C-H bonds (such as in degradation of petroleum hydrocarbons) or reductive cleavage of C-Cl bonds (such as in dehalogenation of chlorinated solvents). Nonetheless it is possible to estimate approximate *maximum* kinetic isotope effects ("semiclassical Streitwieser limits") for such reactions *(24)*. As discussed in the main article, such numbers are derived from greatly simplifying assumptions. Under the caveat that these values have therefore only semi-quantitative character, the calculated Streitwieser limit for cleavage of a C-H bond would be $KIE_C = 1.02$ and for cleavage of a C-Cl bond $KIE_C = 1.06$ (see Table 1). As these are estimates for the case of complete bond cleavage in an infinitely late transition state, realistic values with transition states at about 50% bond cleavage can be expected to be half as pronounced, $KIE_C = 1.01$ (C-H bond) and $KIE_C = 1.03$ (C-Cl bond).

2.3 Isotope effects of some concerted reactions

Isotope effects are not easily predicted in concerted reactions where several bonds may be weakened and formed simultaneously. Values for such reactions must either be measured directly, or the transition state structure may be computed with *ab initio* methods from which expectation values for isotope effects may be calculated. Studies of this kind have been conducted for typical cases of oxidation at a double bond that may, for example, play a role in the oxidative degradation of chlorinated ethenes. Houk, Singleton, Strasser and coworkers found evidence that both the epoxidation and the oxidation of olefins with permanganate are concerted, although the reactions may be somewhat asynchronous depending on how unsymmetrical substituents are distributed at the double bond *(18, 25, 26)*. The average value KIE_C at each olefinic carbon atoms was 1.011 in epoxidations with m-Cl perbenzoic acid, whereas a much larger value of 1.024 was calculated in the oxidation with permanganate.

2.4 Secondary hydrogen isotope effects

Compared with primary isotope effects, secondary isotope effects are generally much smaller, and for heavier elements such as C, N, O or Cl these effects are usually neglected. Owing to the general high isotope effects associated with hydrogen, however, in the case of this element secondary isotope effects are still detectable, provided that there is no C-H breakage in which case overall fractionation would be dominated by the simultaneous primary effect. Such secondary hydrogen isotope effects may not only be characteristic of different reactions, they also depend on whether the isotopic substitution is in α-position (directly next to reacting bond), β-position (one position away from reacting bond) or γ-position (two positions apart). No effects are generally observed for isotopic substitutions further away. The following typical secondary hydrogen effects have been reported per deuterium atom for nucleophilic substitution reactions:

– S_N1-type, α-position: KIE_H = 1.1-1.2 (see section 6.1.1 in *(15)*, p. 304 in *(27)*)

– S_N1-type, β-position: KIE_H = 1.05-1.15 (see section 6.1.2 in *(15)*, p. 284 in *(27)*.

– S_N2-type, α-position: KIE_H = 0.95-1.04 (hydrolysis), KIE_H = 0.88-1.07 in general (see section 6.1.1 in *(15)*, p. 297 in *(27)*)

– S_N2-type, β-position: KIE_H = 1.01-1.04 (hydrolysis, see p. 297 in *(27)*)

– S_N2-type γ-position: KIE_H = 0.97-0.98 (hydroloysis, see p. 297 in *(27)*)

3. **Mathematical Derivation of the Novel Evaluation Procedure**

3.1 Assumption: Heavy Isotopes are of low natural abundance

In the following, it is assumed that the natural isotopic ratio $R = {}^{heavy}E/{}^{light}E$ of a given element E is very small (such as in the case of H, C, N and O) where ${}^{light}E$ and ${}^{heavy}E$ are the abundance of the lighter and heavier isotope, respectively. (Note that this is not the case, however, for elements such as S or Cl.) Molecules with more than one heavy isotope can then be neglected so that only compounds where all isotopes of E are light, $[{}^{light}E_n]$, and molecules with just one heavy isotope present, $[{}^{heavy}E{}^{light}E_{n-1}]$, need to be considered in the following derivations.

3.2 Ratios of isotopes and isotopic molecules

Because the Rayleigh-equation describes whole reacting molecules rather than single isotopes inside a compound, the ratio $R = {}^{heavy}E/{}^{light}E$ of total isotopes measured by CSIA must, strictly speaking, first be converted into the ratio of isotopic molecules, R, before the derivation of a Rayleigh-type expression can be attempted:

$$R = {}^{heavy}E/{}^{light}E \qquad \text{(e.g.: } R = [D]/[H]\text{)}$$

$$R = [{}^{heavy}E\,{}^{light}E_{n-1}]/[{}^{light}E_n] \qquad \text{(e.g.: } R = [\text{benzene-d}]/[\text{benzene}]\text{)} \qquad \text{(S1)}$$

where $[{}^{light}E_n]$ and $[{}^{heavy}E{}^{light}E_{n-1}]$ are concentrations of molecules with no and one heavy isotope, respectively ([benzene-d] is singly deuterated benzene) . The following equations can be stated:

$${}^{heavy}E = [{}^{heavy}E\,{}^{light}E_{n-1}] \qquad \text{(e.g.: } [D] = [\text{benzene-d}]\text{)} \qquad \text{(S2)}$$

$${}^{light}E = n\,[{}^{light}E_n] + (n-1)\,[{}^{heavy}E\,{}^{light}E_{n-1}]$$

$$\text{(e.g.: } [H] = 6[\text{benzene}] + 5[\text{benzene-d}]\text{)} \qquad \text{(S3)}$$

Therefore,

$$R = \frac{{}^{heavy}E}{{}^{light}E} = \frac{[{}^{heavy}E{}^{light}E_{n-1}]}{n\cdot[{}^{light}E_n] + (n-1)\cdot[{}^{heavy}E{}^{light}E_{n-1}]} = \frac{R}{n+(n-1)\cdot R} \qquad \text{(S4)}$$

and

$$R = \frac{n\cdot R}{1-(n-1)\cdot R} \qquad \text{(e.g., in the case of benzene, } R = 6R/(1-5R)\text{)} \qquad \text{(S5)}$$

However, if values of *R* are small, (see initial assumption), equation (S5) becomes approximately

$$R \approx n * R \qquad \text{(S6)}$$

Let us assume that a "typical" Rayleigh-equation can be derived for isotopic molecules (e.g., benzene-d vs. benzene):

$$\frac{R}{R_0} = \left[f \cdot \frac{(1+R_0)}{(1+R)} \right]^{(\alpha\text{-}1)} \approx f^{(\alpha\text{-}1)} \approx f^{\,\varepsilon/1000} \qquad (5)$$

If this equation is written for isotopes rather than isotopic molecules, it becomes

$$\frac{R}{R_0} = \left[f \cdot \frac{(1+n \cdot R_0)}{(1+n \cdot R)} \right]^{(\alpha\text{-}1)} \approx f^{(\alpha\text{-}1)} \approx f^{\,\varepsilon/1000} \qquad \text{(S7)}$$

Again, if *R* is very small (see first assumption) this expression can be approximated by the commonly known Rayleigh-expression (for comparison and definition of α and ε see Eqs. 6,7 in the main article)

$$\ln \frac{R}{R_0} \approx (\alpha - 1) \cdot \ln f \approx \frac{\varepsilon}{1000} \cdot \ln f \qquad (5)$$

In the following, all equations will be derived for isotopic molecules $R = [^{heavy}E\ ^{light}E_{n-1}]/\ [^{light}E_n]$ rather than isotope ratios that can be measured by CSIA after complete combustion, $R = {}^{heavy}E/{}^{light}E$. In cases of low natural abundance of the heavier isotopes the outcome will be the same in both cases. If however, isotopic abundance is large, if fractionation becomes very strong and / or even if only *n becomes sufficiently large (!)*, the number of equations (S1) to (S7) provide the framework that must be used to first convert the ratios of *R* into values of R, before the evaluations can be made that are introduced in the main article and that will be derived in the following section.

3.3 General derivation of the proposed correction for non-reacting positions

Considered is the arbitrary case of a molecule with n atoms of the element E, of which x are equivalent and located at reactive positions. In the following, the reactive positions will be indicated by bold letters and carry the subscript x, so that the molecule is denoted $[E_{(n-x)}\ \mathbf{E}_x]$. Heavy isotopes of E shall be of low natural abundance, and their presence will from now on be indicated with an asterisk

rather than applying the notation “heavy” and “light”. Heavy isotopomers are therefore written in general as $([E_{(n-x)}\mathbf{E}_x])^*$, those with heavy isotopes in the non-reacting position are denoted as $[^*E_{(n-x)}\mathbf{E}_x]$ and those with the heavy isotope in the reactive position as $[E_{(n-x)}\ \mathbf{E}_x^*]$. Molecules that do not contain heavy isotopes are written as $[E_{(n-x)}\mathbf{E}_x]$.

The following equations apply:

$$R = \frac{([E_{(n-x)}\mathbf{E}_x])^*}{[E_{(n-x)}\mathbf{E}_x]} = \frac{[^*E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]} + \frac{[E_{(n-x)}\mathbf{E}_x^*]}{[E_{(n-x)}\mathbf{E}_x]} \quad \text{(S8)}$$

$$\frac{d([E_{(n-x)}\mathbf{E}_x])^*}{d[E_{(n-x)}\mathbf{E}_x]} = \frac{d[^*E_{(n-x)}\mathbf{E}_x]}{d[E_{(n-x)}\mathbf{E}_x]} + \frac{d[E_{(n-x)}\mathbf{E}_x^*]}{d[E_{(n-x)}\mathbf{E}_x]} \quad \text{(S9)}$$

In other words, both types of isotopic molecules react essentially *independent* of each other, and the observed isotopic enrichment is the *average* of the two reactions. This is important, because they can now be considered in two separate mathematical treatments, and then the effect on the average value can be calculated. In the case of $[E_{(n-x)}\ \mathbf{E}_x^*]$ isotopic discrimination can be expected, according to the fractionation factor of the reactive position $\mathbf{E}_x$ that is denoted by α_{rp} (note that in the paper section α_{rp} is written as $\alpha_{\text{reactive position}}$):

$$\frac{d[E_{(n-x)}\mathbf{E}_x^*]}{d[E_{(n-x)}\mathbf{E}_x]} = \alpha_{rp} \cdot \frac{[E_{(n-x)}\mathbf{E}_x^*]}{[E_{(n-x)}\mathbf{E}_x]} \quad \text{(S10)}$$

In the case of $[^*E_{(n-x)}\ E_x]$ there will be no isotope effect, because all molecules have light isotopes in their reactive position so that their reaction rate is the same as of molecules that are composed of light isotopes only, $[E_{(n-x)}\ E_x]$:

$$\frac{d[^*E_{(n-x)}\mathbf{E}_x]}{d[E_{(n-x)}\mathbf{E}_x]} = 1 \cdot \frac{[^*E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]} \quad \text{(S11)}$$

Introduction of (S10) and (S11) in (S9) gives

$$\frac{d([E_{(n-x)}\mathbf{E}_x])^*}{d[E_{(n-x)}\mathbf{E}_x]} = \alpha_{rp} \cdot \frac{[E_{(n-x)}\mathbf{E}_x^*]}{[E_{(n-x)}\mathbf{E}_x]} + \frac{[^*E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]} \quad \text{(S12)}$$

Integration of equation (S11), finally, gives

$$\left(\frac{[{}^{*}E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]}\right)_t = \left(\frac{[{}^{*}E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]}\right)_0 \quad \text{(S13)}$$

which means that molecules with the isotope in a non-reacting position $[{}^{*}E_{(n-x)}\mathbf{E}_x]$ are over the whole reaction in a constant ratio to non-labelled molecules $[E_{(n-x)}\mathbf{E}_x]$ in the substrate fraction. Specifically, this is ratio is at any time identical to the ratio at time 0. Knowledge about the *initial* isotope distribution in the substrate fraction is therefore very helpful. In the case of hydrogen, where natural isotopic variation is most pronounced (see discussion in the Supporting Information Part 5), it can, in principle, be obtained from SNIF-NMR measurements on pure substrates. The outcome could be, for example, that 80% of all deuterium isotopes are located in non-reactive positions. Equation (S8) could then be written for time 0

$$R_0 = \left(\frac{[{}^{*}E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]}\right)_0 + \left(\frac{[E_{(n-x)}\mathbf{E}_x{}^{*}]}{[E_{(n-x)}\mathbf{E}_x]}\right)_0 = 0.8 \cdot R_0 + 0.2 \cdot R_0 \quad \text{(S14)}$$

If such information is not available, one may assume a random (i.e., even) distribution of isotopes inside the molecules giving

$$R_0 = \left(\frac{[{}^{*}E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]}\right)_0 + \left(\frac{[E_{(n-x)}\mathbf{E}_x{}^{*}]}{[E_{(n-x)}\mathbf{E}_x]}\right)_0 = \frac{(n-x)}{n} \cdot R_0 + \frac{x}{n} \cdot R_0 \quad \text{(S16)}$$

and for any given time of conversion:

$$R = \left(\frac{[{}^{*}E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]}\right) + \left(\frac{[E_{(n-x)}\mathbf{E}_x{}^{*}]}{[E_{(n-x)}\mathbf{E}_x]}\right) = \frac{(n-x)}{n} \cdot R_0 + \left(\frac{[E_{(n-x)}\mathbf{E}_x{}^{*}]}{[E_{(n-x)}\mathbf{E}_x]}\right) \quad \text{(S17)}$$

Possible errors associated with this assumption are discussed in greater detail in Part 5 of the Supporting Information. However, it should be pointed out once more that this assumption is not a prerequisite for the evaluation scheme. The factor (n-x)/n can easily be substituted by the appropriate percentage if initial isotope ratios are determined, for example in SNIF-NMR measurements.

Using equations (S13), (S16) and (S17), $\left(\frac{[{}^{*}E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]}\right)$ and $\left(\frac{[E_{(n-x)}\mathbf{E}_x{}^{*}]}{[E_{(n-x)}\mathbf{E}_x]}\right)$ can be written as

$$\left(\frac{[{}^{*}E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]}\right) = \frac{(n-x)}{n}\cdot R_0 \qquad \text{(S18)}$$

and $$\left(\frac{[E_{(n-x)}\mathbf{E}_x{}^{*}]}{[E_{(n-x)}\mathbf{E}_x]}\right) = R - \frac{(n-x)}{n}\cdot R_0 \qquad \text{(S19)}$$

If both expressions are introduced in equation (S12), the result is

$$\frac{d([E_{(n-x)}\mathbf{E}_x])^{*}}{d[E_{(n-x)}\mathbf{E}_x]} = \alpha_{rp}\cdot\left(R - \frac{(n-x)}{n}\cdot R_0\right) + \frac{(n-x)}{n}\cdot R_0 \qquad \text{(S20)}$$

Introducing

$$R = \frac{([E_{(n-x)}\mathbf{E}_x])^{*}}{[E_{(n-x)}\mathbf{E}_x]} \qquad \text{(S8)}$$

and separating out R_0, finally, gives

$$\frac{d([E_{(n-x)}\mathbf{E}_x])^{*}}{d[E_{(n-x)}\mathbf{E}_x]} - \alpha_{rp}\cdot\frac{([E_{(n-x)}\mathbf{E}_x])^{*}}{[E_{(n-x)}\mathbf{E}_x]} = \frac{(n-x)}{n}\cdot R_0\cdot\left(1-\alpha_{rp}\right) \qquad \text{(S21)}$$

The general solution of this first-order inhomogeneous differential equation is

$$([E_{(n-x)}\mathbf{E}_x])^{*} = \kappa\cdot[E_{(n-x)}\mathbf{E}_x]^{\alpha_{rp}} + \frac{(n-x)}{n}\cdot R_0\cdot[E_{(n-x)}\mathbf{E}_x] \qquad \text{(S22)}$$

where κ is an arbitrary constant introduced by integration. Division by $[E_{(n-x)}\mathbf{E}_x]$ gives

$$\frac{([E_{(n-x)}\mathbf{E}_x])^{*}}{[E_{(n-x)}\mathbf{E}_x]} = R = \kappa\cdot[E_{(n-x)}\mathbf{E}_x]^{(\alpha_{rp}-1)} + \frac{(n-x)}{n}\cdot R_0 \qquad \text{(S23)}$$

or, after substraction of (n-x)/n * R_0,

$$R - \frac{(n-x)}{n}\cdot R_0 = \kappa\cdot[E_{(n-x)}\mathbf{E}_x]^{(\alpha_{rp}-1)} \qquad \text{(S24)}$$

This can be expressed for t=0

$$R_0 - \frac{(n-x)}{n}\cdot R_0 = \frac{x}{n}\cdot R_0 = \kappa\cdot[E_{(n-x)}\mathbf{E}_x]_0^{(\alpha_{rp}-1)} \qquad \text{(S25)}$$

Division of the two equations gives

$$\frac{R - \frac{(n-x)}{n}\cdot R_0}{\frac{x}{n}\cdot R_0} = \left(\frac{[E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]_0}\right)^{(\alpha_{rp}-1)} \quad \text{(S26)}$$

or

$$\frac{\frac{n}{x}\cdot R - \frac{(n-x)}{x}\cdot R_0}{R_0} = \frac{R_0 + \frac{n}{x}\cdot(R - R_0)}{R_0} = \frac{R_0 + \frac{n}{x}\cdot\Delta R}{R_0} = \left(\frac{[E_{(n-x)}\mathbf{E}_x]}{[E_{(n-x)}\mathbf{E}_x]_0}\right)^{(\alpha_{rp}-1)} \quad \text{(S27)}$$

Finally, because the concentration of *total* substrate $c([E_{(n-x)}\mathbf{E}_x])_{tot}$ including labelled as well as unlabelled molecules, is

$$c([E_{(n-x)}\mathbf{E}_x])_{tot} = [E_{(n-x)}\mathbf{E}_x] + ([E_{(n-x)}\mathbf{E}_x])^* = [E_{(n-x)}\mathbf{E}_x]\cdot(1+R) \quad \text{(S28)}$$

equation (S27) can be written in the form

$$\frac{R_0 + \frac{n}{x}\cdot\Delta R}{R_0} = \left(f\cdot\frac{(1+R_0)}{(1+R)}\right)^{(\alpha_{rp}-1)} \quad \text{(S29)}$$

where *f* is the fraction of remaining *total* substrate

$$f = \frac{c([E_{(n-x)}\mathbf{E}_x])_{tot}}{\{c([E_{(n-x)}\mathbf{E}_x])_{tot}\}_0} = \frac{[E_{(n-x)}\mathbf{E}_x] + ([E_{(n-x)}\mathbf{E}_x])^*}{[E_{(n-x)}\mathbf{E}_x]_0 + ([E_{(n-x)}\mathbf{E}_x])_0^*} \quad \text{(S30)}$$

The proposed correction for non-reacting positions that was introduced in the paper section in an illustrative way is thus now derived in equation (S29) as outcome of a stringent mathematical treatment. The term n/x that was introduced by the assumption that isotopes are initially distributed evenly in the molecule, can anytime be replaced by an experimentally determined distribution. (If, for example, SNIF-NMR measurements give the result that 20% of the deuterium is present in reactive positions, n/x can be replaced by the factor 1 / 0.2 = 5).

Note, finally, that if all isotopes are located in reactive positions such as in the case of carbon in CCl_4 or also benzene, then x = n so that equation (S29) simplifies to the well-known Rayleigh-expression.

$$\frac{R_0 + \Delta R}{R_0} = \frac{R_0 + 1 \cdot (R - R_0)}{R_0} = \frac{R}{R_0} = \left(f \cdot \frac{(1 + R_0)}{(1 + R)} \right)^{(\alpha_{rp} - 1)} \quad \text{(S31)}$$

In cases where n is not equal to x, such as with toluene, MTBE and many other organic molecules, equation (S29) is the appropriate expression.

3.4 Conversion of α_{rp} into an apparent kinetic isotope effect AKIE

To convert the fractionation factor at the reactive site, α_{rp}, into the corresponding apparent kinetic isotope effect AKIE, finally, a correction for intramolecular competition must be made. Three cases can be distinguished:

1) *No intramolecular competition*

Intramolecular isotopic competition does not occur

a) in the case of primary isotope effects if there is only one atom present in the reactive position (e.g., carbon in the methyl group of MTBE) so that x = 1.

b) in the case of secondary isotope effects, where equivalent positions act in a kind of "concerted action".

c) in concerted reactions where several positions are engaged simultaneously in the reaction (see Rule of Thumb 6).

In both cases the fractionation factor at the reactive site, α_{rp}, can directly be converted into the apparent kinetic isotope effect according to

$$\alpha_{rp} = \varepsilon_{rp} / 1000 + 1 = \text{AKIE}^{-1} \quad \text{(S32)}$$

2) *Intramolecular competition, but no slow preceding steps or commitment to catalysis*

We consider a case where intramolecular competition occurs between z equivalent reactive positions (z > 1) and where at the same time the measured fractionation is representative of the actual bond

conversion, without the influence of preceding slow processes. Equation (S10) then has the following meaning:

$$\frac{d[E_{(n-x)}\mathbf{E}_x^*]}{d[E_{(n-x)}\mathbf{E}_x]} = \frac{^{heavy}k_{primary\ effect} + (z-1)\cdot {}^{heavy}k_{secondary\ effect}}{z\cdot {}^{light}k} \cdot \frac{[E_{(n-x)}\mathbf{E}_x^*]}{[E_{(n-x)}\mathbf{E}_x]} \qquad (S33)$$

The fact that the observable fractionation factor $\alpha_{rp}^{\not<}$ is intrinsic and not diminished by commitment to catalysis is indicated by the superscript "$\not<$", otherwise this factor corresponds to $\alpha_{reactive\ position}$ introduced in the main text. This factor is:

$$\alpha_{rp}^{\not<} = \frac{^{heavy}k_{primary\ effect} + (z-1)\cdot {}^{heavy}k_{secondary\ effect}}{z\cdot {}^{light}k} \qquad (S34)$$

If secondary isotope effects are neglected meaning that $^{heavy}k_{secondary\ effect} \approx {}^{light}k$, and if $^{heavy}k_{primary\ effect}$ is simply written as $^{heavy}k$, equation (S34) becomes

$$\alpha_{rp}^{\not<} = \frac{1}{z}\cdot\frac{^{heavy}k}{^{light}k} + \frac{z-1}{z} = \frac{1}{z}\cdot KIE^{-1} + \frac{z-1}{z} \qquad (S35)$$

The corresponding enrichment factor $\varepsilon_{rp}^{\not<}$ is then

$$\frac{\varepsilon_{rp}^{\not<}}{1000} = \alpha_{rp}^{\not<} - 1 = \frac{1}{z}\cdot\left(\frac{^{heavy}k}{^{light}k} - 1\right) = \frac{1}{z}\cdot\left(KIE^{-1} - 1\right) \qquad (S36)$$

and, conversely, the intrinsic kinetic isotope effect is

$$\frac{^{ligtht}k}{^{heavy}k} = KIE = \frac{1}{z\cdot\varepsilon_{rp}^{\not<}/1000 + 1} \qquad (S37)$$

2) Intramolecular competition in reactions involving slow preceding steps /commitment to catalysis

We now consider a case where both, intramolecular competition and slow preceding steps must be taken into account. As discussed in the paper section, in enzyme reactions (e.g., in biotransformations) slow preceding steps lead to observable *apparent* kinetic isotope effects AKIE that are much smaller than the corresponding *intrinsic* isotope effects KIE in the actual bond conversion. To describe this

influence of commitment to catalysis C on values of AKIE and KIE, Northrop *(28)* has derived the following equation that is valid for studies with labelled substrate:

$$\text{AKIE} = \frac{C + KIE}{C + 1} \tag{3}$$

Although expressed in terms of kinetic isotope effects, this equation describes quite generally the way how intrinsic fractionation between molecules with heavy and light isotopes at the reactive site is affected depending on the magnitude of C. Considering the fact that kinetic isotope effects are inversely related to values of α, the following general relationship can therefore be deduced from equation (3):

$$\left(\alpha_{rp}^{C}\right)^{-1} = \frac{C + \left(\alpha_{rp}^{\not C}\right)^{-1}}{C + 1} \tag{S38}$$

where the superscript "C" in α_{rp}^{C} indicates the presence of commitment to catalysis.

For reactions with no intramolecular competition (z=1), equation S38 is identical to equation (3) and thus α_{rp}^{C}, which is obtained from equation S27, can be directly transformed to AKIE by

$$\text{AKIE} = \left(\alpha_{rp}^{C}\right)^{-1} \tag{S39}$$

(see equation S32). If C is known, AKIE can further be transformed to KIE using equation 3.

For reactions with intramolecular competition, a more complicated situation arises. In this case $\left(\alpha_{rp}^{\not C}\right)^{-1}$ is given by

$$\left(\alpha_{rp}^{\not C}\right)^{-1} = \left(\frac{1}{z} \cdot KIE^{-1} + \frac{z-1}{z}\right)^{-1} \tag{S40}$$

so that, with equation (S37),

$$\left(\alpha_{rp}^{C}\right)^{-1} = \frac{C + \left(\alpha_{rp}^{\not C}\right)^{-1}}{C + 1} = \frac{C + \left(\frac{1}{z} \cdot KIE^{-1} + \frac{z-1}{z}\right)^{-1}}{C + 1} \tag{S41}$$

As can be easily seen, a correction for commitment to catalysis is in such cases no longer straightforward. Starting with the value of α_{rp}^{C} that can be obtained from the Rayleigh equation according to (S29), in a correct treatment it would be necessary to take first into account the influence

of commitment to catalysis C according to equation (S37). A value of $\alpha_{rp}^{\lessdot}$ would then be obtained that could eventually be inserted into equation (S40) in order to correct for intramolecular competition. Such an evaluation would give directly the intrinsic kinetic isotope effect KIE rather than the apparent value AKIE.

Obviously, however, such a treatment is not possible, because values of C are generally neither known (except for very few cases of well understood enzyme reactions, see, e.g., *(29)*), nor easily obtainable. Therefore, because the adequate treatment (first correcting for C, then correcting for intramolecular competition to calculate KIE) is not possible, it may be considered what values would be obtained for AKIE if the correction for intramolecular competition is directly applied. The mathematical equations of this procedure are analogous to those in the absence of commitment to catalysis, (S37), with the only difference that estimates of AKIE are now calculated instead of KIE:

$$\mathrm{AKIE} = \frac{1}{z \cdot \varepsilon_{rp}^{\subset}/1000 + 1} = \frac{1}{z \cdot (\alpha_{rp}^{\subset} - 1) + 1} \qquad \text{(S42)}$$

While such values of AKIE have now experienced some sort of correction for intramolecular competition, they are not yet corrected for commitment to catalysis. To understand the meaning of such AKIE-numbers, a comparison is therefore instructive with values of $\mathrm{AKIE_{LS}}$ that would be obtained for the same compound with labelled substrate (subscript LS). Such values of $\mathrm{AKIE_{LS}}$ are also subject to the effect of commitment to catalysis, but in their case intramolecular competition is by definition avoided, because all reactive positions are occupied exclusively by heavy isotopes (see equation 3):

$$\mathrm{AKIE_{LS}} = \left(\alpha_{rp}^{\subset}\right)_{LS}^{-1} = \frac{C + \left(\alpha_{rp}^{\lessdot}\right)_{LS}^{-1}}{C + 1} = \frac{C + \mathrm{KIE}}{C + 1} \qquad \text{(S43)}$$

Assuming different values of KIE and C, it is now possible to calculate the ratio of AKIE and $\mathrm{AKIE_{LS}}$ for different theoretical scenarios. Equation (S41) in its reciprocal form gives

$$\left(\alpha_{rp}^{\subset}\right) = \frac{C + 1}{C + \left(\alpha_{rp}^{\lessdot}\right)^{-1}} = \frac{C + 1}{C + \left(\frac{1}{z} \cdot \mathrm{KIE}^{-1} + \frac{z - 1}{z}\right)^{-1}} \qquad \text{(S44)}$$

or

$$\frac{\left(\varepsilon_{rp}^{\subset}\right)}{1000} = \left(\alpha_{rp}^{\subset} - 1\right) = \frac{C+1}{C + \left(\frac{1}{z}\cdot KIE^{-1} + \frac{z-1}{z}\right)^{-1}} - 1 \qquad \text{(S45)}$$

and if the corresponding operational correction for intramolecular competition

$$\frac{z\cdot\varepsilon_{rp}^{\subset}}{1000} = z\cdot(\alpha_{rp}^{\subset} - 1) = z\cdot\left[\frac{C+1}{C + \left(\frac{1}{z}\cdot KIE^{-1} + \frac{z-1}{z}\right)^{-1}} - 1\right] \qquad \text{(S46)}$$

is inserted into equation (S42), it gives AKIE in dependence on intrinsic KIE and commitment to catalysis, C:

$$AKIE = \left[z\cdot\left(\frac{C+1}{C + \left(\frac{1}{z}\cdot KIE^{-1} + \frac{z-1}{z}\right)^{-1}} - 1\right) + 1\right]^{-1} \qquad \text{(S47)}$$

The corresponding value $AKIE_{LS}$ is given by equation (S43)

$$AKIE_{LS} = \left(\alpha_{rp}^{\subset}\right)_{LS}^{-1} = \frac{C + \left(\alpha_{rp}^{\subset}\right)_{LS}^{-1}}{C+1} = \frac{C + KIE}{C+1} \qquad \text{(S43)}$$

(or is alternatively obtained from equation (S47) with z = 1.)

Figure S2 demonstrates that if the observable values of $\varepsilon_{rp}^{\subset}$ on the x-axis of the diagram are corrected for intramolecular competition, AKIE-values are obtained that are consistently smaller than $AKIE_{LS}$ – values obtained with labelled substrate, as shown by the ratio of AKIE / $AKIE_{LS}$ in the y-axis of the diagram. This complicates the identification of reaction mechanisms because the calculated AKIE may fall below a certain threshold value indicative of a reaction (see Table 2 in the main article) even though the reaction occurs. On the other hand, AKIE values never overestimate "true" intrinsic KIEs and thus an AKIE above a certain threshold value is a strong indication for a certain reaction.

An alternative approach to identify reactions and to rationalize isotope effects despite commitment to catalysis and intramolecular competition is to compare relative effects for different elements rather than

absolute values. Such a comparison can be carried out using the following equation given for the example of C and H isotopes

$$\frac{(\alpha^{\subset}_{rpH})^{-1}-1}{(\alpha^{\subset}_{rpC})^{-1}-1}=\frac{KIE_H-1}{KIE_C-1}\cdot\frac{1+KIE_C\cdot(z_C-1)}{1+KIE_H\cdot(z_H-1)} \qquad \text{(S48)}$$

Equation S48 can be obtained by subtracting one on both sites of equation S41 and by dividing the resulting equation for one element by an analogous equation for another element. To apply equation S48, the KIEs for the two elements have to be estimated. The expected value of the right hand side of equation S48 can then be calculated and compared with the value obtained from the experimentally determined $\alpha^{\subset}_{rp}$ for the two elements. The application of equation S48 to identify a reaction is illustrated in the paper section using biodegradation of MTBE as an example.

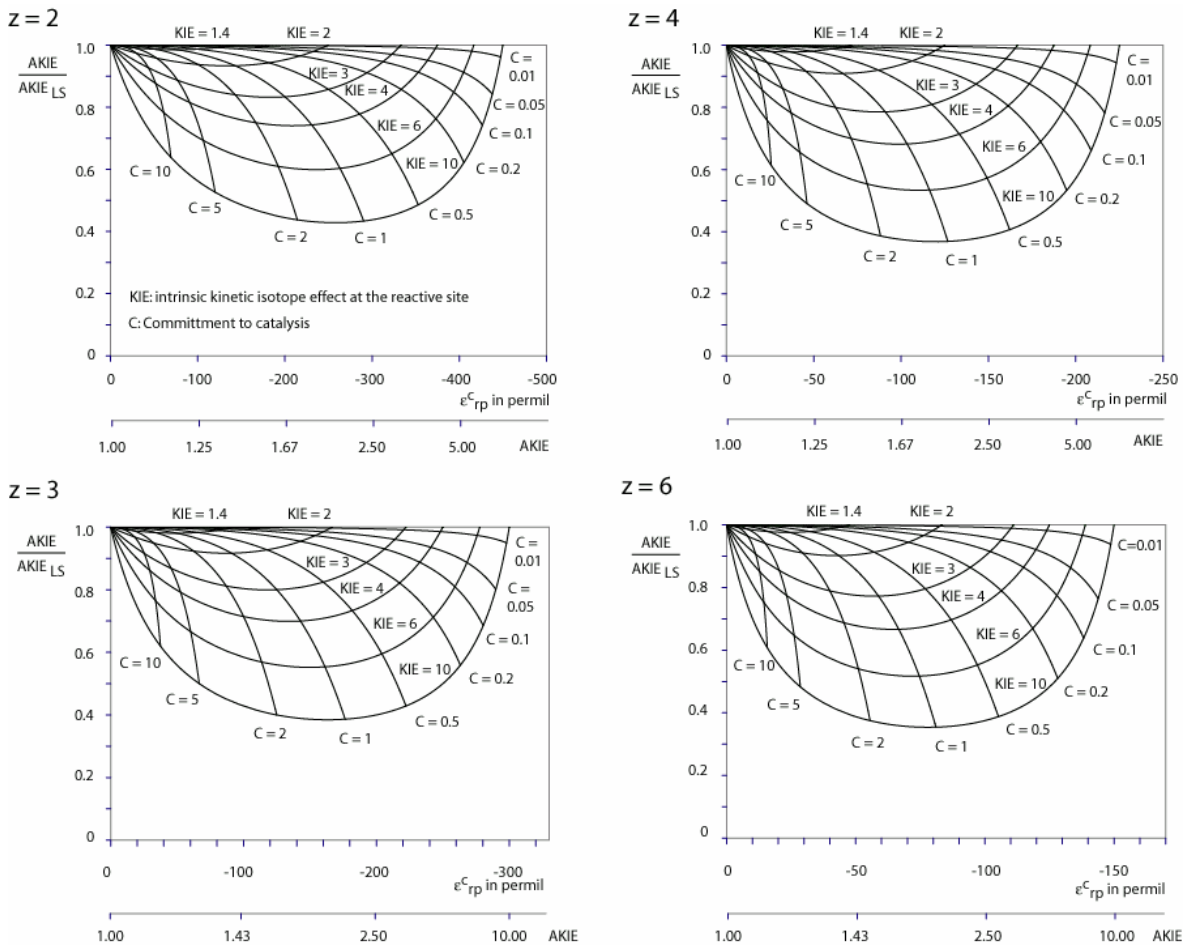

Figure S2. Differences between AKIE and $AKIE_{LS}$ for combinations of intrinsic KIE and values of C (commitment to catalysis)

Explanations to Figure S2

Figure S2 illustrates the following trends:

- Commitment to catalysis acts stronger on values of AKIE that are obtained with substrate of natural isotopic abundance than on values of $AKIE_{LS}$ that are obtained with labelled substrate.
- This difference between AKIE and $AKIE_{LS}$ is zero at C = 0, increases to maximum values at C = 0.2 to C = 5 and becomes negligible again at C = ∞.
- The difference between AKIE and $AKIE_{LS}$ becomes stronger the higher the intrinsic kinetic isotope effect and the stronger commitment to catalysis C is; it is practically only important for primary hydrogen isotope effects.

Illustrative Example:

The following example illustrates how large intrinsic isotope effects are occasionally reflected in much smaller measurable fractionation if experiments are conducted with unlabelled substrate and both, intramolecular competition and commitment to catalysis are important. For example, we consider a case in which hydrogen isotope fractionation is measured and an enrichment factor of

$$\varepsilon_{rp}^{C} = (\alpha_{rp}^{C} - 1)*1000\ \% = -160\%$$

has been determined according to equation (S29), where the reacting group is a methyl group such as in MTBE or toluene (z = 3).

If the operational correction for intramolecular competition is applied according to equation (S42)

$$AKIE = \frac{1}{z \cdot \varepsilon_{rp}^{C}/1000 + 1} = \frac{1}{z \cdot (\alpha_{rp}^{C} - 1) + 1} \qquad \text{(S42)}$$

an apparent kinetic isotope effect of

$$AKIE = 1/[\ 3*(-0.16)+1] = 1.9$$

can be calculated, which is just about large enough to be still indicative of a primary hydrogen isotope effect.

In the diagram of z = 3 a vertical line can now be drawn through ε_{rp}^{C} = -160%, which shows that this observable value can arise through many different combinations of C and KIE, for example,

C = 0.5, KIE = 3 and

C = 1, KIE ≈ 6 (exactly 5.8).

In the first case (C = 0.5, KIE = 3) the AKIE estimated from measurement with unlabelled substrate is, therefore, 1.9/3 = 63% of the intrinsic KIE, in the second case (C = 1, KIE ≈ 6) it is even only 1.9/5.8 = 33%.

We now consider what values would have been obtained if the same measurements had been conducted with labelled substrate where only commitment to catalysis plays a role, but no intramolecular competition takes place. If we consider the case (C = 0.5, KIE = 3), a horizontal line can be drawn from the first point to the y-axis, giving a value of AKIE / $AKIE_{LS}$ = 0.82. This means that the value of AKIE = 1.9 that was calculated above is in this case only about 82% of the value $AKIE_{LS}$ that would have been obtained with labelled substrate:

$AKIE_{LS}$ = 1.9 / 0.82 = 2.3

In the case of (C = 1, KIE ≈ 6) a value of AKIE / $AKIE_{LS}$ = 0.57 is obtained meaning that the value of AKIE = 1.9 is now even only 57% of the value $AKIE_{LS}$ that would have been obtained with labelled substrate:

$AKIE_{LS}$ = 1.9 / 0.57 = 3.4

Hence, the higher the intrinsic KIE is and the stronger the effect of commitment to catalysis (= slow steps preceding the catalytic conversion), the larger will be the difference between AKIE and $AKIE_{LS}$ and the less adequately can equation (S42) correct for intramolecular competition.

4. Mathematical Derivation of the Approximate Equations 16 and 22 and Illustration of Associated Errors

The correction for non-reactive locations (here for the case of carbon isotopes)

$$\ln\frac{R}{R_0} = \ln\frac{(1000+\delta^{13}C_0+\frac{n}{x}\cdot\Delta\delta^{13}C_{bulk})}{(1000+\delta^{13}C_0)} = \frac{\varepsilon^{C}_{reactive\,position}}{1000}\cdot\ln f \qquad \text{(S49)}$$

may be rewritten as

$$\ln\left(\frac{1000+\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000+\delta^{13}C_0}\right) = \ln\left(\frac{1+\frac{\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000}}{1+\frac{\delta^{13}C_0}{1000}}\right) \qquad \text{(S50)}$$

$$= \ln\left(1+\frac{\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000}\right) - \ln\left(1+\frac{\delta^{13}C_0}{1000}\right) = \frac{\varepsilon_{reactive\,postion}}{1000}\cdot\ln f$$

and the expressions $\ln\left(1+\frac{\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000}\right)$ and $\ln\left(1+\frac{\delta^{13}C_0}{1000}\right)$ can be developed in a Taylor series around 1.

If changes in isotope ratios are small so that $\frac{\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000} << 1$ and $\frac{\delta^{13}C_0}{1000} << 1$, this leads to the approximations

$$\ln\left(1+\frac{\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000}\right) \approx \frac{\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000} \quad \text{and} \quad \ln\left(1+\frac{\delta^{13}C_0}{1000}\right) \approx \frac{\delta^{13}C_0}{1000} \quad \text{so that}$$

$$\ln\left(1+\frac{\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000}\right) - \ln\left(1+\frac{\delta^{13}C_0}{1000}\right) \approx \frac{\delta^{13}C_0+\frac{n}{x}\Delta\delta^{13}C}{1000} - \frac{\delta^{13}C_0}{1000} \qquad \text{(S51)}$$

$$\approx \frac{\frac{n}{x}\Delta\delta^{13}C}{1000}$$

If the result is inserted into equation (S50), it becomes clear that the multiplication of $\Delta\delta^{13}C$ by n/x (to obtain the isotopic signature in the reactive position) is equivalent to multiplying ε by n/x (to obtain

$\varepsilon_{\text{reactive position}}$). The approximate equation (16) and, hence, also (22) is thereby derived, which may frequently be used to estimate AKIE if only ε_{bulk} values are available or in reverse to estimate ε_{bulk} from KIE. A prerequisite, however, is that values of n/x $\Delta\delta^{13}C$ do not become too large, as only then the Taylor expression can be applied. Therefore, it is important to know the systematic error associated with these equations. To quantify this error, data sets of R/R_0 where generated for given ε_{rp} (=true ε_{rp}) and n/x using the following equation:

$$\frac{R}{R_0} = \frac{(n - x)}{n} \cdot + \frac{x}{n} \cdot f^{(\alpha_{rp} - 1)} \qquad \text{(S52)}$$

Using linear regression for $\ln R/R_0$ versus ln f without forcing the regression through the origin, ε_{bulk} was determined and the approximate ε_{rp} calculated based on equation 16. The error of ε_{rp} was characterized by the ratio between approximated and true ε_{rp}. The error increases with increasing ε_{rp}, and it increases when the regression is carried out over larger intervals of ln f (Figure S3). The latter is due to an upward curvature of the Rayleigh plot for average bulk isotope ratios that increases at lower values of f. As discussed in the main text, this curvature originates from an increased proportion of molecules with heavy isotopes at reactive position as the reaction proceeds, which accelerates the fractionation trend (see discussion about non-reacting locations). For further characterization of the error as a function of ε_{bulk} and n/x, a regression interval of up to ln f = -4 was used. The error increases with increasing ε_{bulk} and increasing n/x (Figure S4). The ratio between approximated and true ε_{rp} is always >1, i.e. the magnitude of isotope fractionation is overestimated when the approximate equation is used. For carbon isotopes, the maximal error can be estimated using the maximal reported AKIE (1.09, Table 2), which corresponds to an ε_{rp} = -82 ‰ and setting x = 1. For this value, ε_{rp} is overestimated at the most by 15% (i.e., 12.3 ‰) for n/x up to 8. For hydrogen isotopes, ε_{rp} can be considerable larger and hence the approximate equations lead to substantial error (Figure 2). The error of $n \cdot \varepsilon_{bulk}$ (equation 22) is equal to that of ε_{rp} (equation 16).

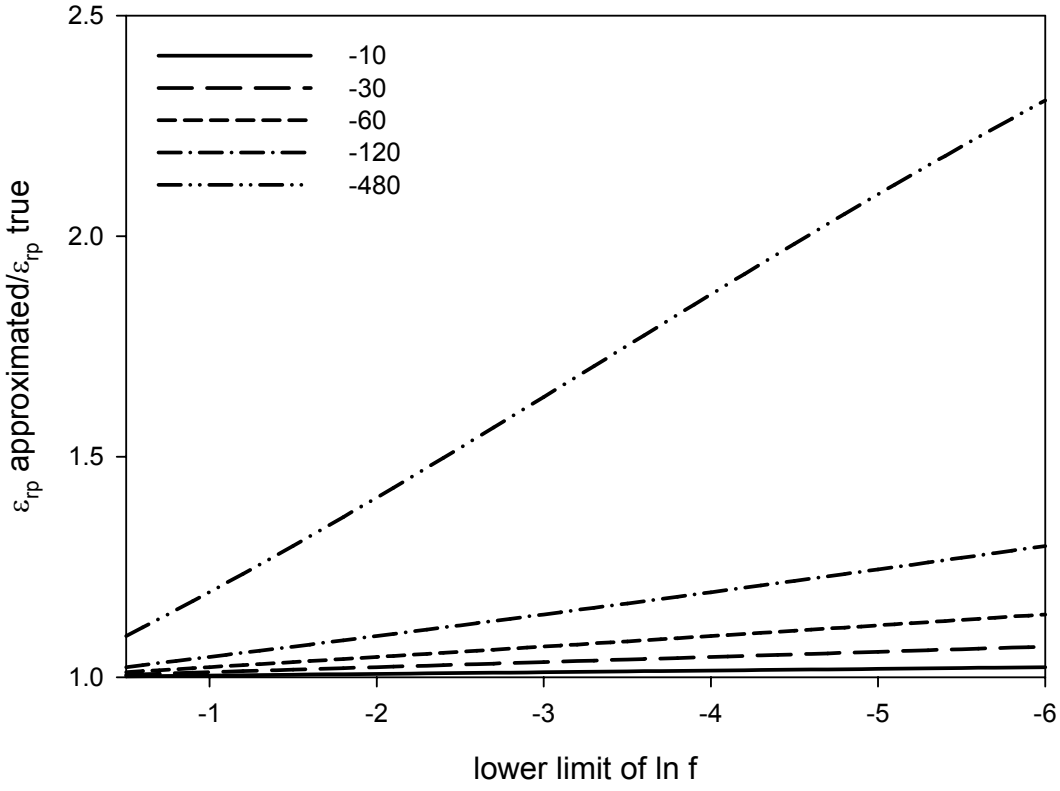

Figure S3: Ratio between approximated ε_{rp} calculated based on ε_{bulk} (eq 16) and true ε_{rp} as a function of the range of ln*f* used to determine ε_{bulk}. The relationship is illustrated for n/x=4 and different values of ε_{rp}.

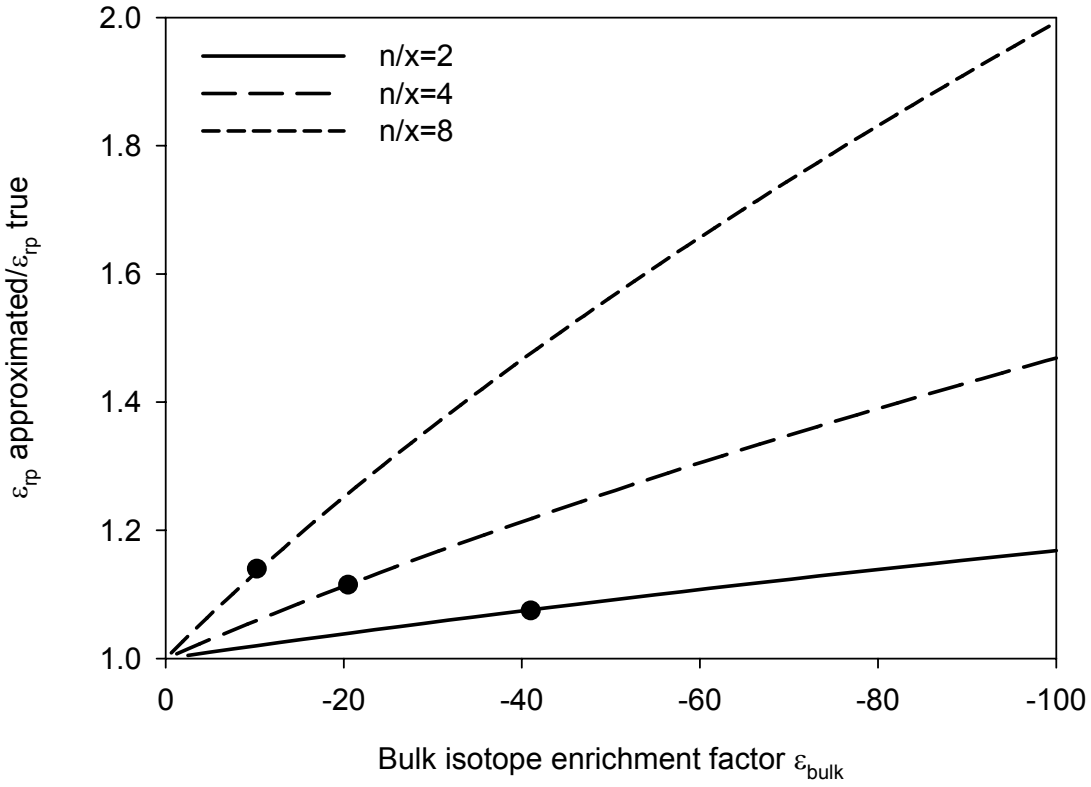

Figure S4: Ratio between approximate ε_{rp} (eq 16) and true ε_{rp} as a function of ε_{bulk} for different n/x. The point on the curves illustrates the maximal uncertainty for carbon isotopes assuming AKIE = 1.09 (see text).

5. Influence of an Uneven Isotope Distribution inside Organic Compounds

To illustrate the effect of a non-random isotope distribution inside organic molecules, anaerobic degradation of the petroleum hydrocarbon toluene may be considered. It is well established that, under anoxic conditions, this compound is degraded by reaction of the methyl group rather than at the aromatic ring *(30, 31)*. Consequently, isotope fractionation can only arise from isotopes in the methyl position, whereas no fractionation may be expected from isotopes at the aromatic ring. Because heavy isotopes of H and C are of such low abundance that only one of them at most will be present per molecule, two extreme cases may be imagined: (1) Heavy isotopes (i.e., ^{2}H, ^{13}C) are located

predominantly at non-reacting aromatic positions. Measurable isotope fractionation will then be negligible. (2) Heavy isotopes are located predominantly in the reacting methyl group. Measurable isotope fractionation will then be very strong. (Note that in both cases the *intrinsic* fractionation at the methyl group is the same, despite the fact that it is differently represented in the *measurable* fractionation, owing to the different proportion of heavy isotopes at the reacting site.) Consequently, "traditional" bulk enrichment factors ε_{bulk} for exactly the same degradation reaction may be different if they are measured with substrate that has a different intramolecular isotope distribution. Although it may be not universally realized, the current practice to compare ε_{bulk} values determined in different laboratories relies, therefore, implicitly on the silent assumption that isotopes are always distributed in the same way – probably randomly and, thus, evenly – inside organic contaminants. Our evaluation procedure makes use of this common assumption in the correction for non-reacting positions, where a factor of n/x is introduced (n = number of atoms of an element of which x are located at the reactive site). However, as pointed out in the mathematical derivation, the factor of n/x, can be easily replaced using the true percentage of reactive sites if the intramolecular isotope distribution inside a compound can be measured. In the following we summarize insight from studies where the intramolecular isotope distribution inside molecules has been measured experimentally. We discuss the systematic error that the assumption of an even isotope distribution introduces to both, ε_{bulk} values commonly reported in the literature as well as the correction applied in our evaluation.

Evidence for a non-random isotope distribution inside organic molecules was already given by a study of Abelson and Hoering *(32)* which showed that ^{13}C in natural amino acids is consistently by up to 20‰ enriched in the carboxyl group as compared to the rest of the molecule. In following decades intramolecular ^{13}C isotope ratios were measured for many more natural compounds, with maximum reported differences between different molecular positions inside the compounds of 18‰ in natural fatty acids *(33)*, 6‰ in glucose *(34)*, 15‰ in low molecular weight organic acids *(35)*, 19‰ in glycerol from corn oil *(36)* and even 46‰ in natural vanilla *(37)*. Evidence suggests that differences are generally higher in industrially manufactured compounds, 8‰ in commercial MTBE *(38)*, up to 45‰ in

synthetic amino acid analogues *(39)* and up to 66‰ in artificial vanillin *(37)*. Being industrial products, contaminants will likely show differences at the upper end of the range, and one may assume that these values are largely determined by the variation in the precursors of their synthesis, plus a possible enrichment / depletion caused by the industrial synthesis. Approximating this variability by the *total* variation of ^{13}C isotope ratios observed in nature, deviations would be between +10‰ and -60‰ relative to VPDB *(4, 40)*. Hence, applying a conservative estimate, expected total differences will be 100‰ at the highest meaning that position-specific intramolecular ^{13}C isotope ratios will not deviate by more than ± 50‰ from the average compound isotope ratio that is determined by GC-IRMS.

Clearly, if the reactive position is depleted / enriched compared to the rest of the molecule, this will result in a "dampening" / enhancement of measured isotope fractionation, with all consequences for determinations of ε_{bulk} values and subsequent uncertainties in the quantification of *in situ* degradation in the field. Therefore, if we assume that such a maximum relative error of 5% is introduced into the correction factor n/x and propagate this error according to equation (S50) and the subsequent Taylor series, it become evident that also the value of $\varepsilon_{reactiveposition}$ will be associated with a relative error of only 5%. In other words, if $\varepsilon_{reactiveposition}$ is -20‰, the absolute error will be 1‰ at the highest, if $\varepsilon_{reactiveposition}$ is -100‰, it will not be larger than 5‰, etc. In an analogous error propagation, the same conclusions can be derived for expected variations in bulk enrichment factors ε_{bulk}. Hence, in the case of carbon, variations in position-specific isotope ratios are so relatively small that they can not be expected to cause considerable bias in determined (bulk) carbon enrichment factors or calculated $AKIE_C$ values.

In contrast to carbon isotope ratios, intramolecular variations reported for hydrogen isotopes are, however, generally much larger. For example, the methyl position in ethanol from different wines was found to be consistently by about 200‰ more depleted in deuterium than the methylene position *(41)*.

(Comment: Note that such hydrogen isotopes are normally reported as total abundances of deuterium vs. hydrogen in ppm, where the abundance of the methyl position is, e.g., 104ppm and that of the methylene position 130ppm. (For comparison, the corresponding natural abundance of the

international standard VSMOW is 156ppm.) Hence, a *total* depletion of 130ppm -104ppm = 26ppm corresponds to a *relative* depletion of 26ppm / 130ppm = 20% or 200‰ in the permil notation.)

In squalene extracted from olive oil maximum intramolecular hydrogen isotope variations between different positions were up to about ± 150‰ *(42)* and in glycerol from cane sugar up to 409‰ *(43)*. Olefinic positions in fatty acids from olive oil, finally, were reported to be on average depleted by 242‰ as compared to most aliphatic positions *(44)* and the aldehyde position in artificial vanillin was even enriched by about 1000‰ compared to VSMOW *(37)*. (Note again the trend that variations become more pronounced in industrial compounds!)

It should be considered that these values represent *maximum* deviations, and that most position-specific isotope ratios reported in the cited publications are indeed much smaller, generally not larger than 100‰. Therefore, if an error of 10% is introduced into reported bulk and calculated position-specific ε-values, such variations are still not very pronounced so that interlaboratory comparisons of reported ε_{bulk} values are still valid and calculated AKIE correct. In extreme cases of 100% enrichment (or 50% depletion), however, it may happen that different ε_{bulk} values can (wrongly) be taken as evidence of different intrinsic fractionation. Application of our evaluation may then actually be helpful, because in the case of excessive enrichment of reactive positions it would lead to highly overestimated $\varepsilon_{reactive\ position}$, and application of equation 21would then lead to negative AKIE values, which are by definition physically impossible. Hence, such an analysis can immediately indicate a probable effect of intramolecular isotope distribution. As a general way to remove such bias, however, we strongly suggest that the substrate utilized in laboratory experiments should be analyzed by SNIF-NMR before the experiment, as only normalization to the initial position-specific isotope ratios will provide the sound basis for inter laboratory comparisons of measured ε values.

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