

## Measurement of (carbon) kinetic isotope effect by Rayleigh fractionation using membrane inlet mass spectrometry for CO<sub>2</sub>-consuming reactions

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**Abstract.** Methods for determining carbon isotope discrimination,  $\Delta$ , or kinetic isotope effects,  $\alpha$ , for CO<sub>2</sub>-consuming enzymes have traditionally been cumbersome and time-consuming, requiring careful isolation of substrates and products and conversion of these to CO<sub>2</sub> for measurement of isotope ratio by mass spectrometry (MS). An equation originally derived by Rayleigh in 1896 has been used more recently to good effect as it only requires measurement of substrate concentrations and isotope ratios. For carboxylation reactions such as those catalysed by D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39) and PEP carboxylase (PEPC, EC 4.1.1.31), this has still required sampling of reactions at various states of completion and conversion of all inorganic carbon to CO<sub>2</sub>, as well as determining the amount of substrate consumed. We introduce a new method of membrane inlet MS which can be used to continuously monitor individual CO<sub>2</sub> isotope concentrations, rather than isotope ratio. This enables the use of a simplified, new formula for calculating kinetic isotope effects, based on the assumptions underlying the original Rayleigh fractionation equation and given by:

$$1 + \Delta = \alpha = \frac{d(\ln[^{12}\text{C}])}{d(\ln[^{13}\text{C}])}.$$

The combination of inlet membrane MS and this formula yields measurements of discrimination in less than 1 h. We validate our method against previously measured values of discrimination for PEP carboxylase and RuBisCO from several species.

**Keywords:** carbon isotope, carboxylation, discrimination, mass spectrometry, phosphoenolpyruvate carboxylase, RuBisCO.

### Introduction

Measurement of the depletion of the stable carbon isotope <sup>13</sup>C with respect to the relatively abundant <sup>12</sup>C is a valuable technique for understanding plant metabolism (for a review see Brugnoli and Farquhar 2000). It can be applied at a global level to describe climatic changes (Francey and Farquhar 1982; Bird *et al.* 1994), at the tissue level to describe metabolic pathways (e.g. Whelan *et al.* 1973; Robinson *et al.* 2003; Xu *et al.* 2004), plant physiology (e.g. Farquhar *et al.*

1982; Evans *et al.* 1986; Macfarlane *et al.* 2004) and genetic difference (e.g. Farquhar and Richards 1984; Lauteri *et al.* 2004; Masle *et al.* 2005) or at the enzyme level to describe kinetic reaction mechanisms (Tcherkez and Farquhar 2005). Enzymes from plants that have been assayed include carbonic anhydrase (CA) (Paneth and O'Leary 1985), PEPC (Whelan *et al.* 1973; Reibach and Benedict 1977; O'Leary *et al.* 1981) and RuBisCO (Christeller *et al.* 1976; Roeske and O'Leary 1984; Guy *et al.* 1993).

Abbreviations used: CA, carbonic anhydrase (EC 4.2.1.1); MS, mass spectrometer or mass spectrometry; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase (EC 4.1.1.31); PGA, 3-phosphoglycerate; *R*, carbon isotope ratio; RuBisCO, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); RuBP, D-ribulose-1,5-bisphosphate.

Relative abundance of carbon isotopes is characterised by the carbon isotope ratio,  $R$ , of a substance defined as (O'Leary 1981; O'Leary *et al.* 1992):

$$R = \frac{[^{13}\text{C}]}{[^{12}\text{C}]}. \quad (1)$$

The isotope composition of a substance,  $\delta^{13}\text{C}$  or  $\delta$ , is given by (Craig 1957; Kroopnick and Craig 1976):

$$\delta^{13}\text{C} = \delta = \frac{R}{R_{\text{std}}} - 1, \quad (2)$$

where  $R_{\text{std}}$  is the isotope ratio for a given standard (marine limestone from Pee Dee Cretaceous belemnite formation, South Carolina, PDB, or an artificial version from Vienna, VPDB). The units for  $\delta$  are usually expressed in terms of parts per thousand or 'parts per mil' (‰). For a process that (irreversibly) transforms a substrate or source from one isotopic composition,  $R_{\text{S}}$ , to a product with another composition,  $R_{\text{P}}$ , the fractionation factor or kinetic isotope effect,  $\alpha$ , is (Lane and Dole 1956):

$$\alpha = \frac{R_{\text{S}}}{R_{\text{P}}}. \quad (3)$$

Isotope fractionation or discrimination,  $\Delta$ , for that process is then (O'Leary 1981):

$$\Delta = \alpha - 1 \approx \delta_{\text{S}} - \delta_{\text{P}}. \quad (4)$$

Photosynthesis results in depletion of  $^{13}\text{C}$  in plant biomass (Park and Epstein 1960; Farquhar *et al.* 1989). Isotope compositions are reasonably consistent with  $\delta \approx -28\%$  for  $\text{C}_3$  plants and  $\delta \approx -13\%$  for  $\text{C}_4$  plants (Smith and Epstein 1971; O'Leary 1981). Given that, in the absence of industrial activity, air has an isotope composition of  $-8\%$ , discrimination ( $\Delta$ ) is  $\sim 20\%$  for  $\text{C}_3$  plants and  $\sim 5\%$  for  $\text{C}_4$  plants (O'Leary and Osmond 1980; Vogel 1980; O'Leary 1981; Farquhar *et al.* 1989). This discrimination is primarily (but not entirely) due to the different kinetic isotope effects of RuBisCO and PEPC, the primary carboxylating enzymes of  $\text{C}_3$  and  $\text{C}_4$  plants, respectively (Farquhar *et al.* 1982; Brugnoli and Farquhar 2000). Models of carbon isotope discrimination due to carbon fixation have been developed for  $\text{C}_3$  plants (Farquhar *et al.* 1982; Farquhar and Richards 1984),  $\text{C}_4$  plants (Farquhar 1983),  $\text{C}_3$ - $\text{C}_4$  intermediates (von Caemmerer 1989) and, more recently, crassulacean acid metabolism (CAM) plants (Griffiths *et al.* 1990; Griffiths 1992), which are modelled as  $\text{C}_4$  plants with no bundle sheath leakage of intercellular  $\text{CO}_2$  (Farquhar 1983). They all rely on accurate determination of the carbon isotope discrimination by RuBisCO and PEPC.

The kinetic isotope effect for carboxylation has been traditionally measured as the difference between isotope compositions of source and product as described above. This involves isolation of the reaction product involved in bond formation with the substrate inorganic carbon, followed by combustion or decarboxylation to  $\text{CO}_2$  and measurement of

the isotope ratio of this and the substrate inorganic carbon. It is generally a laborious and time consuming experimental process but its careful use has provided accurate results (e.g. Christeller *et al.* 1976; O'Leary *et al.* 1981; Roeske and O'Leary 1984, 1985).

The kinetic isotope effect can also be measured without reference to the product by examining the disappearance of substrate only. The continuous conversion of a substrate into a product with differential fractionation may be expressed in terms of the ratio of two first-order rate processes as originally proposed by Rayleigh (1896)<sup>1</sup> for the enrichment by diffusion of one gas relative to another according to:

$$\frac{d[^{12}\text{C}]}{d[^{13}\text{C}]} = \alpha \frac{[^{12}\text{C}]}{[^{13}\text{C}]}, \quad (5)$$

where  $[^{12}\text{C}]$  and  $[^{13}\text{C}]$  are the concentrations of substrate  $^{12}\text{C}$  and  $^{13}\text{C}$ , respectively, and  $\alpha$  is the (constant) fractionation factor. Upon rearrangement of Eqn (5), the fractionation factor is given by:

$$\alpha = \frac{d[^{12}\text{C}]/[^{12}\text{C}]}{d[^{13}\text{C}]/[^{13}\text{C}]} = \frac{d(\ln[^{12}\text{C}])}{d(\ln[^{13}\text{C}])}. \quad (6)$$

We argue that Eqn (6) is a perfectly suitable form for obtaining the fractionation factor from the equation developed by Rayleigh (by plotting  $\ln[^{12}\text{C}]$  v.  $\ln[^{13}\text{C}]$ ) if individual isotope concentrations (and not just the isotope ratio,  $R$ ) can be monitored. In order to apply this equation form to the enrichment of argon in air, Rayleigh integrated further, obtaining an analytical solution for the 'enrichment' (in our case given by  $R$  with respect to some initial  $R$ ) as a function of gas volume remaining after diffusion through a membrane into a vacuum. He applied this integral form of the equation to published experimental data where only the final enrichments and gas volumes were known to him. Until now, in similar fashion, enzyme discrimination has been obtained by integrating Eqn (6) such that:

$$\ln[^{13}\text{C}] = \frac{1}{\alpha} \ln[^{12}\text{C}] + k, \quad (7)$$

where  $k$  is an arbitrary constant resulting from the indefinite integral. Equation (7) can be re-arranged to:

$$\ln \frac{[^{13}\text{C}]}{[^{12}\text{C}]} = \left( \frac{1}{\alpha} - 1 \right) \ln[^{12}\text{C}] + k, \quad (8)$$

or:

$$\ln R = -\Delta' \ln[^{12}\text{C}] + k, \quad (9)$$

where discrimination,  $\Delta'$ , in Eqn (9) varies from the standard definition of  $\Delta$  according to (see Appendix):

$$\Delta' = 1 - \frac{1}{\alpha} = 1 - \frac{1}{1 + \Delta} = \frac{\Delta}{1 + \Delta}. \quad (10)$$

<sup>1</sup>This reference has often been incorrectly cited as Rayleigh (1896), *Philos. Mag.* **42**, 77–107. The correct page numbering is 493–498.

While  $\Delta$  and  $\Delta'$  are similar, they are not the same and result in a difference of up to 1‰ in the case of discrimination by RuBisCO (Farquhar *et al.* 1989; see Appendix). This indefinite integral form of the Rayleigh equation is recommended by Scott *et al.* (2004a) and, indirectly, by Henry *et al.* (1999). Discrimination,  $\Delta'$ , can be determined from a line of best fit through points ( $-\ln[^{12}\text{C}], R$ ) that occur during reaction without constraining the line through the origin. This form of the Rayleigh equation is necessary when isotope ratio mass spectrometry is employed and  $[^{12}\text{C}]$  is approximated by the net concentration of  $\text{CO}_2$ . Until recently, the definite integral has been utilised, resulting in (Kroopnick and Craig 1976; Guy *et al.* 1989, 1993):

$$\ln\left(\frac{R}{R_0}\right) = -\Delta' \ln\left(\frac{[^{12}\text{C}]}{[^{12}\text{C}]_0}\right) = -\Delta' \ln f', \quad (11)$$

where  $f'$  is the fraction of substrate not consumed, based on  $^{12}\text{C}$ . This version of the Rayleigh fractionation equation depends upon an initial point ( $-\ln[^{12}\text{C}]_0, R_0$ ) with which to compare all subsequent points ( $-\ln[^{12}\text{C}], R$ ). This initial point may have undue influence on the slope if the dataset is small (Henry *et al.* 1999; Scott *et al.* 2004a). It is important to note that when the fraction of source not consumed is based on  $^{13}\text{C}$  rather than  $^{12}\text{C}$ , the form of this equation is changed to:

$$\ln\left(\frac{R}{R_0}\right) = -\Delta \ln\left(\frac{[^{13}\text{C}]}{[^{13}\text{C}]_0}\right) = -\Delta \ln f, \quad (12)$$

where  $f$  is the fraction of substrate not consumed, based on  $^{13}\text{C}$ . However, it has not always been possible to measure  $[^{13}\text{C}]$  accurately because  $^{12}\text{C}$  is the dominant isotope. Most workers have measured the fraction of substrate not consumed without regard for isotopic composition. That is, they have not distinguished between  $f$  and  $f'$  but have measured something closer to  $f'$  than  $f$ . The use of the definite integral form of the Rayleigh fractionation equation in determining enzyme discrimination (e.g. Guy *et al.* 1993; Robinson *et al.* 2003) has persisted, in our opinion, because of the need to derive analytical solutions for  $R$  for various enrichment processes (e.g. gaseous diffusion of uranium hexafluoride to enrich  $^{235}\text{UF}_6$  with respect to  $^{238}\text{UF}_6$ ) given an initial isotope ratio,  $R_0$ .

In this paper, we propose a new method for measuring the kinetic isotope effect for PEPC and RuBisCO based on the assumptions underlying the original form of the equation derived by Rayleigh and utilising continuous, real-time measurement of carbon isotope concentrations by membrane-inlet MS. This method requires no reaction quenching or sampling and no isolation of substrate inorganic carbon from the reaction solution, making it quicker and easier to perform than either of the two existing methods used previously. It is only made possible by monitoring of individual isotope concentrations rather than the isotope

ratio. We validate the method by applying it to measure the carbon isotope discrimination of enzymes where this value is well characterised.

## Materials and methods

### Purified enzymes and RuBP

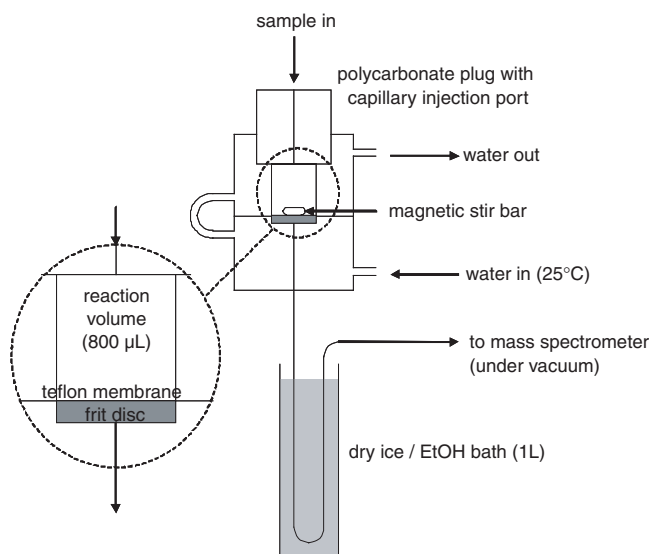
Phosphoenolpyruvate carboxylase from maize (Sigma-Aldrich, St Louis, MO), supplied as an  $(\text{NH}_4)_2\text{SO}_4$  suspension, was harvested by centrifugation at 2000 *g* and 22°C and resuspended in buffer (100 mM HEPES, 1 mM EDTA, 5 mM  $\text{MgCl}_2$ , pH 7.6) at  $\sim 10$  mg·protein mL<sup>-1</sup>. Wild type (WT) RuBisCO was purified from commercially available spinach (*Spinacea oleracea*) leaves by a procedure based on the method of Hall and Tolbert (1978) as described by Edmondson *et al.* (1990) but omitting the final gel filtration step (Morell *et al.* 1997). RuBisCO large subunit octamer from *Rhodospirillum rubrum* was obtained from IPTG-inducible, kanamycin-resistant *E. coli* cultures containing *R. rubrum* rbcM gene on plasmid pRR1 as described by Morell *et al.* (1990), where this construct originally derived from *R. rubrum* rbcM on pRR2119 (Nargang *et al.* 1984). The enzyme was purified according to Andrews and Kane (1991). RuBisCO L<sub>8</sub>S<sub>8</sub> complex from *E. coli* expressing *Synechococcus* PCC 6301 rbcLS gene was purified according to Andrews and Ballment (1983). RuBP was prepared enzymatically from D-ribose-5-phosphate according to the procedure for unlabelled RuBP of Kane *et al.* (1998) based on the method of Horecker *et al.* (1958) with the addition of a desalting step used for  $^{14}\text{C}$ -labelled RuBP by Kane *et al.* (1998).

### Mass spectrometry

Measurements of the concentrations of  $^{16}\text{O}_2$  (mass 32),  $^{12}\text{CO}_2$  (mass 44) and  $^{13}\text{CO}_2$  (mass 45) were made with a purpose-built IsoPrime stable isotope gas mass spectrometer from Micromass Instruments (Manchester, UK). This instrument has eight Faraday collector buckets arranged in a configuration where for one accelerating voltage and magnetic field setting, we can simultaneously monitor eight separate masses, including masses 32, 44 and 45. In addition, we have modified the data collection so that all collector outputs are monitored independently of the computer software supplied with the instrument. This has allowed us to create our own Visual Basic (Microsoft) program on a measurement computer for conducting the assays, calculating the data in 'real-time' and saving data to a file. Our measuring system continuously monitors the frequency outputs of the head amplifier and we divide the data into 'time-slices' so that in a typical measurement each time-point represents a 5-s average of the frequency output of each collector. A reference frequency generator from the counter card used by the measurement computer provides the time base for measurements. Measurements on the mass 32 collector were made with a 10<sup>9</sup>-ohm resistor while masses 44 and 45 were amplified with 10<sup>11</sup>-ohm resistors. All measurements were made at natural abundance for all isotopes.

### CO<sub>2</sub> uptake reactions

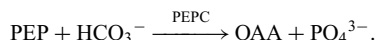
All reactions were performed in an 800-μL volume contained inside a stainless steel, water jacketed (25°C) cylindrical cell with polycarbonate plug (Fig. 1), with the reaction being stirred by a small teflon magnetic stir bar. The bottom of the cell consisted of a teflon membrane sitting on a porous polyethylene disc recessed into the base of the chamber. The frit was under vacuum ( $< 10^{-7}$  mbar) so that volatiles in the cell were drawn through the teflon membrane and frit into the vacuum line. The fraction of  $\text{CO}_2$  consumed by the vacuum is small compared with the  $\text{CO}_2$  consumed by the enzyme (1 : 40, see Appendix 1). From here, the line passed through a 1-L dry ice/ethanol bath to condense water vapour out of the gas phase. Dry volatiles were then continuously drawn into the enclosed source of the MS. The trap current was 400 μA during measurements.



**Fig. 1.** Cuvette used to measure continuous carbon isotope discrimination via membrane inlet mass spectrometry.

#### PEPC reactions

The following carboxylation reaction was monitored to measure the discrimination of PEPC:

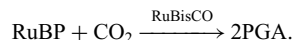


Initially, the reaction cell was filled with at least 600 µL buffer (100 mM HEPES, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, pH 7.6) that had been sparged with N<sub>2</sub> gas for at least 1 h to remove O<sub>2</sub>. A polycarbonate plug was floated on the thin film of solution that was in slight

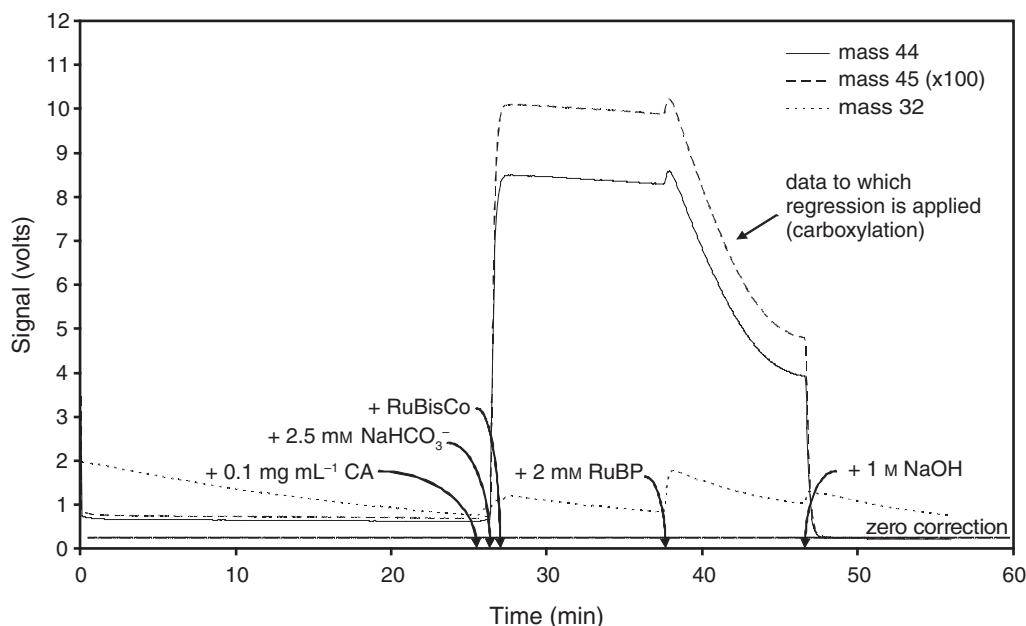
excess of the cell volume. Any residual O<sub>2</sub> was allowed to be drawn out of the cell through the vacuum line and was monitored via the MS until it was almost completely removed (~30 min). This was undertaken to reduce the competition for substrate RuBP by the RuBisCO-mediated oxygenase reaction and to avoid formation of extraneous CO<sub>2</sub> at the source as a result of combustion of carbon. The following solutions were then introduced to the cell via syringe through the capillary port in the polycarbonate plug: 10 µL × 10 mg·mL<sup>-1</sup> CA (included to facilitate equilibrium between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>) from bovine erythrocytes (Sigma-Aldrich); 20 µL × 100 mM NaHCO<sub>3</sub>; 5 µL purified PEPC. Again, any residual O<sub>2</sub> introduced with these solutions was allowed to be drawn out of the cell through the vacuum line (~15 min). The carboxylation reaction was initiated by addition of 15 µL × 100 mM PEP (Roche, Basel, Switzerland) through the capillary port. Final concentrations were always 0.1 mg·mL<sup>-1</sup> CA, 2.5 mM NaHCO<sub>3</sub><sup>-</sup> and 2 mM PEP in a total volume of 800 µL. The carboxylation reaction was monitored until almost all PEP was exhausted (~10 min) at which point 50 µL of a saturated NaOH solution was added. This resulted in >1 M NaOH in solution and the conversion of all (volatile) CO<sub>2</sub> to (non-volatile) CO<sub>3</sub><sup>2-</sup>. The MS signal at this time (averaged over 5 min) was deemed to represent a zero concentration of CO<sub>2</sub>.

#### RuBisCO reactions

The following carboxylation reaction was monitored to measure the discrimination of RuBisCO:



The procedure was essentially the same as for PEPC reactions except that the buffer contained 25 mM instead of 5 mM MgCl<sub>2</sub> and 20–100 µL purified RuBisCO extract (depending on the source) was used instead of PEPC. Again, the presence of low O<sub>2</sub> was essential in the assays and sparging of assay buffer with N<sub>2</sub> and in-cell O<sub>2</sub> draw down was employed. When O<sub>2</sub> was allowed to be drawn out of the cell through the vacuum line (~15 min), RuBisCO was also allowed time to become

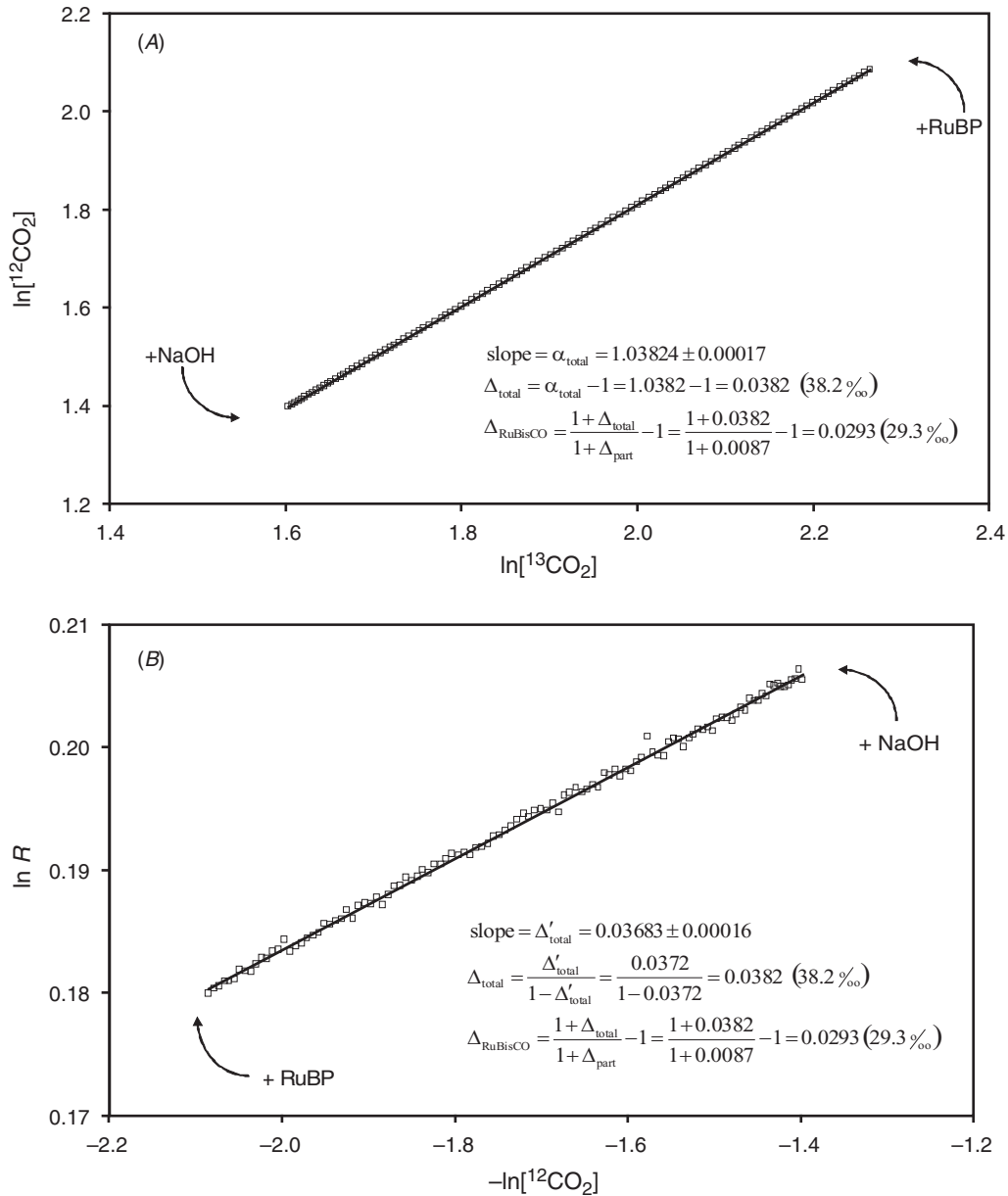


**Fig. 2.** Mass spectrometer signals for a typical carboxylation reaction catalysed by spinach RuBisCO. The addition of various reaction components are indicated together with the segment of the reaction used for regression calculations in Fig. 3.

activated by (non-substrate)  $\text{CO}_2$  and  $\text{Mg}^{2+}$  (Laing and Christeller 1976; Lorimer *et al.* 1976; Andrews 1996). The carboxylation reaction was initiated by addition of  $60 \mu\text{L} \times 25 \text{ mM}$  RuBP through the capillary port. Final concentrations were always  $0.1 \text{ mg}\cdot\text{mL}^{-1}$  CA,  $2.5 \text{ mM}$   $\text{NaHCO}_3^-$  and  $2 \text{ mM}$  RuBP in a total volume of  $800 \mu\text{L}$ . The carboxylation reaction was monitored until almost all RuBP was exhausted ( $\sim 10 \text{ min}$ ) after which  $50 \mu\text{L}$  of saturated NaOH was added. The MS signals arising from a typical experimental sequence are shown in Fig. 2.

#### Calculation of discrimination

The signals for each isotope were corrected for their zero offsets using a value for zero obtained by averaging the raw signal for each isotope for 5 min after addition of NaOH at the conclusion of the reaction. The corrected signals were then used to obtain a raw fractionation,  $\alpha_{\text{total}}$ , from Eqn (6) where  $\ln[^{12}\text{C}]$  was plotted against  $\ln[^{13}\text{C}]$  (Fig. 3). The slope was calculated by linear regression for points that occur some time after initiation of reaction (by PEP and RuBP for PEPC and RuBisCO, respectively) and some time before quenching of the reaction with



**Fig. 3.** Rayleigh fractionation plots with straight lines of best fit regressed to data (Fig. 2) generated some time after initiation of carboxylation (by addition of RuBP) and some time before quenching of the reaction (by addition of NaOH). This segment is indicated in Fig. 2. (A) The slope of the line of best fit for  $\ln[^{12}\text{C}]$  v.  $\ln[^{13}\text{C}]$  is the overall system fractionation factor,  $\alpha_{\text{total}}$ , from which the spinach enzyme discrimination,  $\Delta_{\text{RuBisCO}}$ , may be calculated. (B) The slope of the line of best fit for  $\ln R$  v.  $-\ln[^{12}\text{C}]$  is the overall system discrimination,  $\Delta'_{\text{total}}$ , from which  $\Delta_{\text{RuBisCO}}$  may be calculated.

NaOH. The slope was not constrained to pass through the origin (Henry *et al.* 1999; Scott *et al.* 2004a). The raw discrimination,  $\Delta_{\text{total}}$ , was then calculated by subtracting one from the raw fractionation, in accordance with Eqn (4). An enzyme discrimination was calculated for each reaction according to (see Appendix 1):

$$\Delta_{\text{RuBisCO}} = \frac{1 + \Delta_{\text{total}}}{1 + \Delta_{\text{part}}} - 1, \quad (13)$$

and:

$$\Delta_{\text{PEPC}} = \frac{1 + \Delta_{\text{total}}}{(1 + \Delta_{\text{part}})(1 + \Delta_{\text{eq}})} - 1, \quad (14)$$

where  $\Delta_{\text{eq}}$  is the discrimination due to the equilibrium isotope effect between  $\text{CO}_2(\text{aq})$  and  $\text{HCO}_3^-(\text{aq})$  (relative to  $\text{CO}_2$ : see Appendix 1) equal to  $-9\%$  (Mook *et al.* 1974) and  $\Delta_{\text{part}}$  is the discrimination due to partitioning of inorganic carbon between  $\text{CO}_2$  and  $\text{HCO}_3^-$  in solution given by (see Appendix 1):

$$\Delta_{\text{part}} = \frac{-\Delta_{\text{eq}}}{1 + (1 + \Delta_{\text{eq}})10^{\text{pK}_{\text{eq}} - \text{pH}} + \Delta_{\text{eq}}}, \quad (15)$$

where  $\text{pK}_{\text{eq}}$  is the equilibrium dissociation constant for  $\text{CO}_2(\text{aq})/\text{HCO}_3^-(\text{aq})$ . For the experimental conditions employed:

$$\Delta_{\text{part}} = \frac{0.009}{1 + (1 - 0.009) \times 10^{6.25 - 7.60} - 0.009} = 0.0087 \text{ (8.7\%)}$$

For both PEPC and RuBisCO, the amount of enzyme used in the assay was adjusted so as to maximise the rate of reaction without that rate being too fast for the response time of the MS. This was important because there is probably some small discrimination that occurs due to diffusion through the teflon membrane and polyethylene frit as  $\text{CO}_2$  is drawn under vacuum to the MS (see Appendix 1). In this experimental system, a reaction time of  $\sim 10$  min to exhaust substrate (either PEP or RuBP) was appropriate.

#### Previous estimates of PEPC and RuBisCO discrimination

The discriminations for PEPC and RuBisCO enzymes from the species included in this paper but determined by other workers are shown in Table 1.

## Results and discussion

The data chosen to be included in the fractionation plot were somewhat arbitrary in that immediately after addition of RuBP there was some short adjustment in solution as a new isotopic pseudo-equilibrium was achieved and, as  $\text{CO}_2$  became exhausted in solution, the signal-to-noise ratio increased. Therefore, data immediately after RuBP addition (for  $\sim 1$  min) and immediately before NaOH addition (for  $\sim 1$  min) were excluded. The fractionation plot ( $\ln[^{12}\text{C}]$  v.  $\ln[^{13}\text{C}]$ ) arising from a typical experimental sequence for RuBisCO (Fig. 3) is shown in Fig. 3A. This may be compared with the commonly used Rayleigh fractionation plot ( $\ln R$  v.  $-\ln[^{12}\text{C}]$ ) in Fig. 3B. The correlation between  $\ln[^{12}\text{C}]$  and  $\ln[^{13}\text{C}]$  appears much better than that between  $\ln R$  and  $-\ln[^{12}\text{C}]$  and while this may, at first glance, suggest more accurate determination of  $\Delta_{\text{total}}$  and  $\Delta_{\text{enzyme}}$ , it must be remembered that in Fig. 3A, data points will cluster about a line with slope that varies very little from unity while in Fig. 3B, the expanded  $\ln R$  axis amplifies the scatter of data points. Nevertheless, we show in the Appendix that the absolute error for  $\Delta_{\text{enzyme}}$  calculated from a plot of  $\ln[^{12}\text{C}]$  v.  $\ln[^{13}\text{C}]$  (Fig. 3A) is identical to that for  $\Delta_{\text{enzyme}}$  calculated from a plot of  $\ln R$  v.  $-\ln[^{12}\text{C}]$  (Fig. 3B). We therefore recommend our newer, simpler method as an alternative when individual isotope concentrations can be monitored.

Individual values for the enzyme discriminations for PEPC and RuBisCO from spinach, *R. rubrum* and *Synechococcus* PCC 6301 are included in Table 2. Estimates of discriminations by other workers under experimental conditions that most closely resemble ours are displayed in comparison to our results in Fig. 4 and show that the values

**Table 1. Discrimination for PEPC and RuBisCO enzymes from species included in this paper**

Enzyme type	Species	Common name	$\Delta$ (‰)	Conditions	Source
PEP carboxylase					
C4	<i>Sorghum bicolor</i>	sorghum	$2.7 \pm 4.4$	24°C, pH 8.5	Whelan <i>et al.</i> (1973)
C4	<i>Zea mays</i>	maize	2.03	25°C, pH 7.5	Reibach and Benedict (1977)
C4	<i>Zea mays</i>	maize	$2.9 \pm 0.5$	25°C, pH 7.5	O'Leary <i>et al.</i> (1981)
C4	<i>Zea mays</i>	maize	0.9	25°C, pH 9.0	O'Leary <i>et al.</i> (1981)
C4	<i>Zea mays</i>	maize	-2.7	25°C, pH 10.0	O'Leary <i>et al.</i> (1981)
RuBisCO					
Form 1B, C3	<i>Spinacea oleracea</i>	spinach	$29.7 \pm 0.8$	25°C, pH 7.0	Roeske and O'Leary (1984)
Form 1B, C3	<i>Spinacea oleracea</i>	spinach	$29 \pm 1$	25°C, pH 8.0	Roeske and O'Leary (1984)
Form 1B, C3	<i>Spinacea oleracea</i>	spinach	$26.4 \pm 0.6$	25°C, pH 9.0	Roeske and O'Leary (1984)
Form 1B, C3	<i>Spinacea oleracea</i>	spinach	$29.0 \pm 0.3$	pH 7.6	Guy <i>et al.</i> (1993)
Form 1B, C3	<i>Spinacea oleracea</i>	spinach	$30.3 \pm 0.8$	pH 8.5	Guy <i>et al.</i> (1993)
Form 1B, C3	<i>Spinacea oleracea</i>	spinach	26.2–29.8 (95%)	24°C, pH 8.5	Scott <i>et al.</i> (2004b)
Form IB	<i>Anacystis nidulans</i>	cyanobacterium, blue-green alga	$22.0 \pm 0.2$	pH 8.1	Guy <i>et al.</i> (1993)
Form II from proteobacteria	<i>Rhodospirillum rubrum</i>		$17.8 \pm 0.8$	25°C, pH 7.8	Roeske and O'Leary (1985)
Form II from proteobacteria	<i>Rhodospirillum rubrum</i>		19–24	pH 7.9, $[\text{Mg}^{2+}] =$ 2–25 mM	Guy <i>et al.</i> (1993)

**Table 2. Enzyme discriminations determined from Rayleigh fractionation plots with means and standard deviations (‰) for each enzyme**

PEPC ( $\Delta_{\text{PEPC}}$ )	RuBisCO ( $\Delta_{\text{RuBisCO}}$ )		
	<i>S. oleracea</i>	<i>R. rubrum</i>	<i>S. PCC 6301</i>
2.6	30.7	24.1	19.9
2.8	28.9	21.0	20.2
2.4	28.9	21.3	21.3
	31.4	20.4	21.7
	29.9	20.9	21.5
	28.0	19.7	
	26.9	21.0	
	29.3	25.2	
	26.7	22.0	
	28.3	20.9	
		24.2	
		26.1	
2.6 ± 0.2	28.9 ± 1.5	22.2 ± 2.1	20.9 ± 0.8

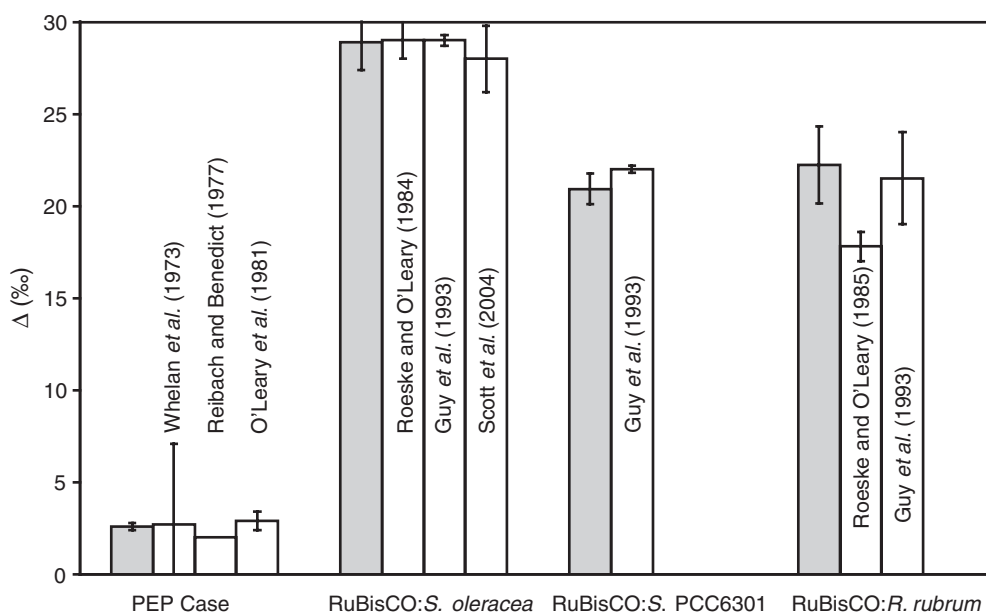
for enzyme discrimination ( $\Delta_{\text{enzyme}}$ ) obtained by our new method are indistinguishable (given error ranges) from those of other measurements for PEPC from maize and RuBisCO from spinach and *R. rubrum* (Table 1).

Our value for  $\Delta_{\text{PEPC}}$  [(2.6 ± 0.2)‰] is indistinguishable from previously measured values of (2.7 ± 4.4)‰ at 24°C and pH 8.5 (Whelan *et al.* 1973) and (2.9 ± 0.5)‰ at 25°C and pH 7.5 (O’Leary *et al.* 1981). It is also between the two measurements obtained by Reibach and Benedict (1977) of 0.51 and 3.55‰ (the average value 2.03‰ was reported). Our value for  $\Delta_{\text{RuBisCO}}$  for spinach [(28.9 ± 1.5)‰] is

also indistinguishable from previously measured values of (29 ± 1)‰ at 25°C and pH 8.0 (Roeske and O’Leary 1984), (29.0 ± 0.3)‰ at unspecified temperature and pH 7.6 (Guy *et al.* 1993) and (26.2–29.8)‰ (95% confidence interval) at 24°C and pH 8.5 (Scott *et al.* 2004b).

The two previous measurements of  $\Delta_{\text{RuBisCO}}$  for *R. rubrum* differ significantly from each other (Table 1) even though the experimental conditions under which they were measured did not [assuming that Guy *et al.* (1993) performed their measurements at ambient temperatures]. Our measured value of (22.2 ± 2.1)‰ is consistent with that obtained by Guy *et al.* (1993) but not with that of Roeske and O’Leary (1985). It should be noted that Guy *et al.* used a substrate-depletion method (in common with us) whereas Roeske and O’Leary measured isotopic composition of substrate and products. Our relatively large error range is also consistent with that of Guy *et al.* (1993), who attributed it to sensitivity of the measurement to  $\text{Mg}^{2+}$  concentration (for *R. rubrum* but not for spinach). Our error range was obtained at the one  $\text{Mg}^{2+}$  concentration (25 mM). When we repeated our measurements at 40 mM  $\text{Mg}^{2+}$ , we found no significant difference in discrimination values (data not shown).

Our upper range limit for *Synechococcus* of (20.9 + 0.8)‰ is only 0.1‰ below the lower range limit determined by Guy *et al.* (1993) of (22.0–0.2)‰. It should be noted that the *Anacystis nidulans* referred to in their paper is equated to *Synechococcus* PCC 6301. It should also be noted that the pH at which we conducted our assays (7.6) was here significantly less than their experimental pH (8.1).



**Fig. 4.** Discrimination ( $\Delta$ ) values measured by Rayleigh fractionation using membrane inlet MS in this paper (dark bars) compared with measurements from other sources (light bars) under experimental conditions that most closely resemble those in this study (from Table 1).

The discrimination values reported here for RuBisCO are all referenced to aqueous phase or dissolved CO<sub>2</sub> as substrate, in common with those reported by others in Table 1. Similarly, discrimination values for PEPC are referenced to aqueous HCO<sub>3</sub><sup>-</sup>. Plant physiological models (e.g. Farquhar *et al.* 1982; Farquhar 1983) typically use discriminations referenced to gaseous CO<sub>2</sub> because this is the form of CO<sub>2</sub> that exists outside the leaf. To convert between discrimination referenced to aqueous substrate, Δ<sub>aq</sub>, and discrimination referenced to gaseous substrate, Δ<sub>g</sub>, equilibrium isotope effects must be invoked (assuming that gaseous and aqueous substrates are in equilibrium) according to:

$$\Delta_{g,\text{RuBisCO}} = \alpha_{g,\text{RuBisCO}} - 1 = \alpha_{\text{aq,RuBisCO}} \alpha_{\text{CO}_2(\text{aq}) \rightarrow \text{CO}_2(\text{g})} - 1 \\ = (1 + \Delta_{\text{aq,RuBisCO}})(1 + \Delta_{\text{CO}_2(\text{aq}) \rightarrow \text{CO}_2(\text{g})}) - 1, \quad (16)$$

$$\Delta_{g,\text{PEPC}} = \alpha_{g,\text{PEPC}} - 1 = \alpha_{\text{aq,PEPC}} \alpha_{\text{HCO}_3^- (\text{aq}) \rightarrow \text{CO}_2(\text{g})} - 1 \\ = (1 + \Delta_{\text{aq,PEPC}})(1 + \Delta_{\text{HCO}_3^- (\text{aq}) \rightarrow \text{CO}_2(\text{g})}) - 1, \quad (17)$$

The relevant equilibrium isotope effects are given by (Mook *et al.* 1974):

$$\Delta_{\text{CO}_2(\text{aq}) \rightarrow \text{CO}_2(\text{g})} \approx +1\text{‰},$$

$$\Delta_{\text{HCO}_3^- (\text{aq}) \rightarrow \text{CO}_2(\text{g})} \approx -8\text{‰}.$$

These corrections result in discriminations referenced to gaseous CO<sub>2</sub>, Δ<sub>g</sub>, which differ from discriminations referenced to aqueous substrate, Δ<sub>aq</sub>, by about +1‰ for RuBisCO and about -8‰ for PEPC. Hence, for application in gas-phase models, discrimination by spinach RuBisCO of Δ<sub>aq</sub> ≈ 29‰ becomes Δ<sub>g</sub> ≈ 30‰ and for PEPC, Δ<sub>aq</sub> ≈ 2‰ becomes Δ<sub>g</sub> ≈ -6‰.

Figure 2 shows that each addition of reagents to the reaction cuvette results in some oxygen (mass 32) addition. This is inevitable given the low oxygen partial pressure in the cuvette after application of a vacuum and before reagent addition. Thus, introduced oxygen has the potential to react with carbon in the source and interfere with both the mass 44 and 45 signals. However, the compatibility of our results with measurements determined by other means suggests that the effect is negligible.

## Conclusions

We have shown that our new method of monitoring CO<sub>2</sub> isotope concentrations directly via membrane inlet MS provides values for discrimination by purified extracts of the carboxylating enzymes PEPC and RuBisCO, determined from the basic form of the Rayleigh equation, which are consistent with previous measurements by other workers. Our method, however, provides single estimates of discrimination inside 1 h and does not involve isolation, decarboxylation or combustion of any substrates or products. The simplicity and rapidity of the method makes it ideal for collecting a wide range of data that may give insights into kinetic

differences which may arise through the evolution of an enzyme, including adaptation to a changed environment (see Tcherkez *et al.* 2006), or through directed mutagenesis changes designed to probe mechanism.

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## References

- Andrews TJ (1996) The bait in the rubisco mousetrap. *Nature Structural Biology* **3**, 3–7. doi: 10.1038/nsb0196-3
- Andrews TJ, Ballment B (1983) The function of the small subunits of ribulosebiphosphate carboxylase–oxygenase. *Journal of Biological Chemistry* **258**, 7514–7518.
- Andrews TJ, Kane HJ (1991) Pyruvate is a by-product of catalysis by ribulosebiphosphate carboxylase–oxygenase. *Journal of Biological Chemistry* **266**, 9447–9452.
- Bird MI, Lloyd J, Farquhar GD (1994) Terrestrial carbon storage at the LGM. *Nature* **371**, 566. doi: 10.1038/371566a0
- Brugnoli E, Farquhar GD (2000) Photosynthetic fractionation of carbon isotopes. In 'Photosynthesis: physiology and metabolism'. (Eds RC Leegood, TD Sharkey, S von Caemmerer) pp. 399–434. (Kluwer Academic Publishers: Dordrecht)
- Christeller JT, Laing WA, Troughton JH (1976) Isotope discrimination by ribulose-1,5-diphosphate carboxylase: no effect of temperature or HCO<sub>3</sub><sup>-</sup> concentration. *Plant Physiology* **57**, 580–582.
- Craig H (1957) Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. *Geochimica et Cosmochimica Acta* **12**, 133–149. doi: 10.1016/0016-7037(57)90024-8
- Edmondson DL, Badger MR, Andrews TJ (1990) A kinetic characterization of slow inactivation of ribulosebiphosphate carboxylase during catalysis. *Plant Physiology* **93**, 1376–1382.
- Evans JR, Sharkey TD, Berry JA, Farquhar GD (1986) Carbon isotope discrimination measured concurrently with gas exchange to investigate CO<sub>2</sub> diffusion in leaves of higher plants. *Australian Journal of Plant Physiology* **13**, 281–292.
- Farquhar GD (1983) On the nature of carbon isotope discrimination in C<sub>4</sub> species. *Australian Journal of Plant Physiology* **10**, 205–226.
- Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 503–537. doi: 10.1146/annurev.pp.40.060189.002443
- Farquhar GD, O'Leary MH, Berry JA (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* **9**, 121–137.
- Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water use efficiency of wheat genotypes. *Australian Journal of Plant Physiology* **11**, 539–552.
- Francey RJ, Farquhar GD (1982) An explanation of <sup>13</sup>C/<sup>12</sup>C variations in tree rings. *Nature* **297**, 28–31. doi: 10.1038/297028a0
- Griffiths H (1992) Carbon isotope discrimination and the integration of carbon assimilation pathways in terrestrial CAM plants. *Plant, Cell & Environment* **15**, 1051–1062. doi: 10.1111/j.1365-3040.1992.tb01655.x

- Griffiths H, Broadmeadow MSJ, Borland AM, Hetherington CS (1990) Short-term changes in carbon-isotope discrimination identify transitions between C<sub>3</sub> and C<sub>4</sub> carboxylation during crassulacean acid metabolism. *Planta* **181**, 604–610. doi: 10.1007/BF00193017
- Guy RD, Berry JA, Fogel ML, Hoering TC (1989) Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-sensitive respiration in plants. *Planta* **177**, 483–491. doi: 10.1007/BF00392616
- Guy RD, Fogel ML, Berry JA (1993) Photosynthetic fractionation of the stable isotopes of oxygen and carbon. *Plant Physiology* **101**, 37–47.
- Hall NP, Tolbert NE (1978) A rapid procedure for isolation of ribulose biphosphate carboxylase/oxygenase from spinach leaves. *FEBS Letters* **96**, 167–169. doi: 10.1016/0014-5793(78)81085-0
- Henry BK, Atkin OK, Day DA, Millar AH, Menz RI, Farquhar GD (1999) Calculation of the oxygen isotope discrimination factor for studying plant respiration. *Australian Journal of Plant Physiology* **26**, 773–780.
- Horecker BL, Hurwitz J, Weissbach A (1958) Ribulosediphosphate. In 'Biochemical preparations'. (Ed. CS Vestling) pp. 83–90. (J. Wiley & Sons: New York)
- Kane HJ, Wilkin JM, Portis AR, Andrews TJ (1998) Potent inhibition of ribulose-bisphosphate carboxylase by an oxidized impurity in ribulose-1,5-bisphosphate. *Plant Physiology* **117**, 1059–1069. doi: 10.1104/pp.117.3.1059
- Kroopnick P, Craig H (1976) Oxygen isotope fractionation in dissolved oxygen in the deep sea. *Earth and Planetary Science Letters* **32**, 375–388. doi: 10.1016/0012-821X(76)90078-9
- Laing WA, Christeller JT (1976) Model for kinetics of activation and catalysis of ribulose-1,5-bisphosphate carboxylase. *Biochemical Journal* **159**, 563–570.
- Lane GA, Dole M (1956) Fractionation of oxygen isotopes during respiration. *Science* **123**, 574–576.
- Lauteri M, Pliura A, Monteverti MC, Brugnoli E, Villani F, Eriksson G (2004) Genetic variation in carbon isotope discrimination in six European populations of *Castanea sativa* Mill. originating from contrasting localities. *Journal of Evolutionary Biology* **17**, 1286–1296. doi: 10.1111/j.1420-9101.2004.00765.x
- Lorimer GH, Badger MR, Andrews TJ (1976) The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism and physiological implications. *Biochemistry* **15**, 529–536. doi: 10.1021/bi00648a012
- Macfarlane C, Adams MA, White DA (2004) Productivity, carbon isotope discrimination and leaf traits of trees of *Eucalyptus globulus* Labill. in relation to water availability. *Plant, Cell & Environment* **27**, 1515–1524. doi: 10.1111/j.1365-3040.2004.01260.x
- Masle J, Gilmore SR, Farquhar GD (2005) The ERECTA gene regulates plant transpiration efficiency in *Arabidopsis*. *Nature* **436**, 866–870. doi: 10.1038/nature03835
- Mook WG, Bommerson JC, Staverman WH (1974) Carbon isotope fractionation between dissolved bicarbonate and gaseous carbon-dioxide. *Earth and Planetary Science Letters* **22**, 169–176. doi: 10.1016/0012-821X(74)90078-8
- Morell MK, Kane HJ, Andrews TJ (1990) Carboxylterminal deletion mutants of ribulosebiphosphate carboxylase from *Rhodospirillum rubrum*. *FEBS Letters* **265**, 41–45. doi: 10.1016/0014-5793(90)80879-N
- Morell MK, Wilkin JM, Kane HJ, Andrews TJ (1997) Side reactions catalyzed by ribulose-bisphosphate carboxylase in the presence and absence of small subunits. *Journal of Biological Chemistry* **272**, 5445–5451. doi: 10.1074/jbc.272.9.5445
- Nargang F, McIntosh L, Somerville C (1984) Nucleotide sequence of the ribulosebiphosphate carboxylase gene from *Rhodospirillum rubrum*. *Molecular & General Genetics* **193**, 220–224. doi: 10.1007/BF00330671
- O'Leary MH (1977) Studies of enzyme reaction mechanisms by means of heavy-atom isotope effects. In 'Isotope effects on enzyme-catalyzed reactions'. (Eds WW Clelland, MH O'Leary, DB Northrop) pp. 233–251. (University Park Press: Baltimore)
- O'Leary MH (1981) Carbon isotope fractionation in plants. *Phytochemistry* **20**, 553–567. doi: 10.1016/0031-9422(81)85134-5
- O'Leary MH, Madhavan S, Paneth P (1992) Physical and chemical basis of carbon isotope fractionation in plants. *Plant, Cell & Environment* **15**, 1099–1104. doi: 10.1111/j.1365-3040.1992.tb01660.x
- O'Leary MH, Osmond CB (1980) Diffusional contribution to carbon isotope fractionation during dark CO<sub>2</sub> fixation in CAM plants. *Plant Physiology* **66**, 931–934.
- O'Leary MH, Rife JE, Slater JD (1981) Kinetic and isotope effect studies of maize phosphoenolpyruvate carboxylase. *Biochemistry* **20**, 7308–7314. doi: 10.1021/bi00528a040
- Paneth P, O'Leary MH (1985) Carbon isotope effect on dehydration of bicarbonate ion catalyzed by carbonic anhydrase. *Biochemistry* **24**, 5143–5147. doi: 10.1021/bi00340a028
- Park R, Epstein S (1960) Carbon isotope fractionation during photosynthesis. *Geochimica et Cosmochimica Acta* **21**, 110–126.
- Rayleigh JWS (1896) Theoretical considerations respecting the separation of gases by diffusion and similar processes. *Philosophical Magazine and Journal of Science* **42**, 493–498.
- Reibach PH, Benedict CR (1977) Fractionation of stable carbon isotopes by phosphoenolpyruvate carboxylase from C<sub>4</sub> plants. *Plant Physiology* **59**, 564–568.
- Robinson JJ, Scott KM, Swanson ST, O'Leary MH, Horken K, Tabita FR, Cavanaugh CM (2003) Kinetic isotope effect and characterization of form II RubisCO from the chemoautotrophic endosymbionts of the hydrothermal vent tubeworm *Riftia pachyptila*. *Limnology and Oceanography* **48**, 48–54.
- Roeske CA, O'Leary MH (1984) Carbon isotope effects on the enzyme-catalyzed carboxylation of ribulose bisphosphate. *Biochemistry* **23**, 6275–6284. doi: 10.1021/bi00320a058
- Roeske CA, O'Leary MH (1985) Carbon isotope effect on carboxylation of ribulose bisphosphate catalyzed by ribulosebisphosphate carboxylase from *Rhodospirillum rubrum*. *Biochemistry* **24**, 1603–1607. doi: 10.1021/bi00328a005
- Scott KM, Lu X, Cavanaugh CM, Liu JS (2004a) Optimal methods for estimating kinetic isotope effects from different forms of the Rayleigh distillation equation. *Geochimica et Cosmochimica Acta* **68**, 433–442. doi: 10.1016/S0016-7037(03)00459-9
- Scott KM, Schwedock J, Schrag DP, Cavanaugh CM (2004b) Influence of form IA RubisCO and environmental dissolved inorganic carbon on the δ<sup>13</sup>C of the clam-chemoautotroph symbiosis *Solemya velum*. *Environmental Microbiology* **6**, 1210–1219. doi: 10.1111/j.1462-2920.2004.00642.x
- Smith BN, Epstein S (1971) Two categories of <sup>13</sup>C/<sup>12</sup>C ratios for higher plants. *Plant Physiology* **47**, 380–384.
- Tcherkez G, Farquhar GD (2005) Carbon isotope effect predictions for enzymes involved in the primary carbon metabolism of plant leaves. *Functional Plant Biology* **32**, 277–291. doi: 10.1071/FP04211
- Tcherkez GGB, Farquhar GD, Andrews TJ (2006) Despite slow catalysis and confused substrate specificity, all ribulose biphosphate carboxylases may be nearly perfectly optimized. *Proceedings of the National Academy of Sciences USA* **103**, 7246–7251. doi: 10.1073/pnas.060605103
- Vogel JC (1980) Fractionation of the carbon isotopes during photosynthesis. In 'Sitzungsberichte der Heidelberger Akademie der Wissenschaften, Mathematisch-naturwissenschaftliche Klasse'. pp. 111–135. (Springer-Verlag: Berlin)

- von Caemmerer S (1989) A model of photosynthetic CO<sub>2</sub> assimilation and carbon-isotope discrimination in leaves of certain C<sub>3</sub>–C<sub>4</sub> intermediates. *Planta* **178**, 463–474. doi: 10.1007/BF00963816
- Whelan T, Sackett WM, Benedict CR (1973) Enzymatic fractionation of carbon isotopes by phosphoenolpyruvate carboxylase from C<sub>4</sub> plants. *Plant Physiology* **51**, 1051–1054.
- Winkler FJ, Kexel H, Kranz C, Schmidt H-L (1982) Parameters affecting <sup>13</sup>CO<sub>2</sub>/<sup>12</sup>CO<sub>2</sub> isotope discrimination of the ribulose-1,5-bisphosphate carboxylase reaction. In 'Stable isotopes'. (Eds H-L Schmidt, H Förstel, K Heinzinger) pp. 83–89. (Elsevier: Amsterdam)
- Xu C-Y, Lin G-H, Griffin KL, Sambrotto RN (2004) Leaf respiratory CO<sub>2</sub> is <sup>13</sup>C-enriched relative to leaf organic components in five species of C<sub>3</sub> plants. *New Phytologist* **163**, 499–505. doi: 10.1111/j.1469-8137.2004.01153.x
- York D (1966) Least-squares fitting of a straight line. *Canadian Journal of Physics* **44**, 1079–1086.

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### Appendix 1

#### Alternative definitions of fractionation factor and discrimination

There exist alternative definitions of fractionation factor and discrimination (Farquhar *et al.* 1989; Guy *et al.* 1989). While the fractionation factor employed by us is referenced to the product, the alternative fractionation factor is referenced to the source and results in the following definitions for fractionation factor and discrimination (e.g. Kroopnick and Craig 1976; Guy *et al.* 1989, 1993; Henry *et al.* 1999):

$$\alpha' = \frac{R_P}{R_S}, \quad (\text{A1})$$

$$\Delta' = 1 - \alpha'. \quad (\text{A2})$$

The relationships between the alternative definitions are as follows (Guy *et al.* 1989, 1993):

$$\alpha' = \frac{1}{\alpha}, \quad (\text{A3})$$

$$\Delta' = 1 - \alpha' = 1 - \frac{1}{\alpha} = 1 - \frac{1}{1 + \Delta} = \frac{\Delta}{1 + \Delta}. \quad (\text{10})$$

#### Isotope effects

A mass balance on inorganic carbon, C, in the closed system described in this paper with continuous withdrawal of CO<sub>2</sub> for measurement and consumption of CO<sub>2</sub> by enzyme can be represented (Henry *et al.* 1999):

$$\frac{d[C]}{dt} = -r - s, \quad (\text{A4})$$

where  $r$  and  $s$  (concentration per unit time) are the rates of consumption of CO<sub>2</sub> by enzyme and by the measuring device (MS), respectively. In a buffered system (pH 7.6) where [H<sup>+</sup>] is constant, where equilibrium between aqueous phase inorganic species is facilitated by CA and where there is no headspace, the total inorganic carbon present in the system is divided between aqueous CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> according to:

$$[C] = [\text{CO}_2] \left( 1 + \frac{K_{\text{eq}}}{[\text{H}^+]} \right), \quad (\text{A5})$$

where the concentration of CO<sub>3</sub><sup>2-</sup> is negligible and where  $K_{\text{eq}}$  is the equilibrium constant relating CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> concentrations given by:

$$K_{\text{eq}} = \frac{[\text{HCO}_3^-][\text{H}^+]}{[\text{CO}_2]}. \quad (\text{A6})$$

Equation (A4) becomes:

$$\frac{d[\text{CO}_2]}{dt} = \frac{-r - s}{1 + K_{\text{eq}}/[\text{H}^+]}. \quad (\text{A7})$$

The total or overall kinetic isotope effect is:

$$\alpha_{\text{total}} = \frac{d[^{12}\text{CO}_2]/[^{12}\text{CO}_2]}{d[^{13}\text{CO}_2]/[^{13}\text{CO}_2]} = \frac{r^{12}}{r^{13}} \left( \frac{1 + s^{12}/r^{12}}{1 + s^{13}/r^{13}} \right) \left( \frac{[\text{H}^+] + K_{\text{eq}}^{13}}{[\text{H}^+] + K_{\text{eq}}^{12}} \right) \frac{[^{13}\text{CO}_2]}{[^{12}\text{CO}_2]}. \quad (\text{A8})$$

For enzymes like RuBisCO, where <sup>12</sup>C and <sup>13</sup>C are competing for the same active site (O'Leary 1977; Farquhar *et al.* 1982):

$$\frac{r^{12}}{r^{13}} = \alpha_{\text{RuBisCO}} \frac{[^{12}\text{CO}_2]}{[^{13}\text{CO}_2]}. \quad (\text{A9})$$

The kinetic isotope effect for RuBisCO,  $\alpha_{\text{RuBisCO}}$ , is independent of the concentration of either isotope, a fact supported by Christeller *et al.* (1976), who found no effect on the discrimination of soybean RuBisCO by bicarbonate concentrations in

the range 2.5–50 mM. In this case, the total isotope effect in Eqn (A8) is:

$$\alpha_{\text{total}} = \alpha_{\text{RuBisCO}} \alpha_{\text{part}}, \quad (\text{A10})$$

where the fractionation due to inorganic carbon partitioning,  $\alpha_{\text{part}}$ , is given by:

$$\alpha_{\text{part}} = \left( \frac{1 + s^{12}/r^{12}}{1 + s^{13}/r^{13}} \right) \left( \frac{[\text{H}^+] + \text{K}_{\text{eq}}^{13}}{[\text{H}^+] + \text{K}_{\text{eq}}^{12}} \right) = \left( 1 + \frac{s^{12}/s^{13} - r^{12}/r^{13}}{r^{12}/s^{13} + r^{12}/r^{13}} \right) \left( \frac{[\text{H}^+] + \text{K}_{\text{eq}}^{13}}{[\text{H}^+] + \text{K}_{\text{eq}}^{12}} \right). \quad (\text{A11})$$

If the rate of withdrawal of  $\text{CO}_2$  to the MS is small ( $s \ll r$ ) or if  $s^{12}/s^{13}$  is comparable to  $r^{12}/r^{13}$ , then Eqn (A11) becomes:

$$\alpha_{\text{part}} \approx \frac{[\text{H}^+] + \text{K}_{\text{eq}}^{13}}{[\text{H}^+] + \text{K}_{\text{eq}}^{12}} = \frac{[\text{H}^+] + \text{K}_{\text{eq}}^{12}/\alpha_{\text{eq}}}{[\text{H}^+] + \text{K}_{\text{eq}}^{12}} = \frac{\alpha_{\text{eq}}[\text{H}^+]/\text{K}_{\text{eq}}^{12} + 1}{\alpha_{\text{eq}}[\text{H}^+]/\text{K}_{\text{eq}}^{12} + \alpha_{\text{eq}}}, \quad (\text{A12})$$

where  $\alpha_{\text{eq}}$  is the equilibrium isotope effect for aqueous  $\text{CO}_2/\text{HCO}_3^-$  given by (O'Leary 1981; Farquhar 1983; Farquhar *et al.* 1989; Brugnoli and Farquhar 2000):

$$\alpha_{\text{eq}} = \frac{\text{K}_{\text{eq}}^{12}}{\text{K}_{\text{eq}}^{13}}. \quad (\text{A13})$$

This will be the case if the rate of reaction,  $r$ , is much greater than the instrument consumption rate,  $s$ , and so this simplification can be applied if enzyme concentrations are great enough that reactions are fast. In Fig. 2 (a typical MS trace), the initial reaction rate (after addition of RuBP) is  $\sim 40$  times greater than the instrument consumption rate (before addition of RuBP) and so for membrane discriminations of the same order of magnitude as enzyme discriminations (or less), this simplification is valid and results in errors of less than 1%. The equilibrium discrimination for dissolved bicarbonate relative to dissolved  $\text{CO}_2$  at 25°C is given by (Mook *et al.* 1974):

$$\Delta_{\text{eq}} = \alpha_{\text{eq}} - 1 = -0.009 \text{ (} -9\% \text{)}$$

Guy *et al.* (1993) propose a similar correction factor,  $C$ , given by:

$$C = 1 - \frac{\Delta'_{\text{eq}}}{1 + 10^{\text{pK}_{\text{eq}} - \text{pH}}}. \quad (\text{A14})$$

It should be noted that they have used the alternative definition for discrimination described by Eqns (A1) and (A2), so that  $C$  can be rearranged by substitution of Eqn (10):

$$C = 1 - \frac{\frac{\Delta_{\text{eq}}}{1 + \Delta_{\text{eq}}}}{1 + 10^{\text{pK}_{\text{eq}} - \text{pH}}} = \frac{1 + (1 + \Delta_{\text{eq}})10^{\text{pK}_{\text{eq}} - \text{pH}}}{1 + (1 + \Delta_{\text{eq}})10^{\text{pK}_{\text{eq}} - \text{pH}} + \Delta_{\text{eq}}} \approx \frac{1 + \alpha_{\text{eq}}[\text{H}^+]/\text{K}_{\text{eq}}^{12}}{\alpha_{\text{eq}}[\text{H}^+]/\text{K}_{\text{eq}}^{12} + \alpha_{\text{eq}}} = \alpha_{\text{part}}. \quad (\text{A15})$$

$C$  and  $\alpha_{\text{part}}$  are essentially identical because the  $\text{pK}_{\text{eq}}$  is substantially dictated by the dominant  $^{12}\text{C}$  isotope. They are also equivalent to the correction factor employed by Winkler *et al.* (1982). In terms of discrimination due to partitioning of inorganic carbon:

$$\Delta_{\text{part}} = \alpha_{\text{part}} - 1 = \frac{-\Delta_{\text{eq}}}{1 + (1 + \Delta_{\text{eq}})10^{\text{pK}_{\text{eq}} - \text{pH}} + \Delta_{\text{eq}}} = 0.0087 \text{ (} = 8.7\% \text{ at pH 7.6)}. \quad (15)$$

Now, in terms of the total or overall discrimination observed in the system:

$$1 + \Delta_{\text{total}} = (1 + \Delta_{\text{RuBisCO}})(1 + \Delta_{\text{part}}). \quad (\text{A16})$$

Rearranging:

$$\Delta_{\text{RuBisCO}} = \frac{1 + \Delta_{\text{total}}}{1 + \Delta_{\text{part}}} - 1. \quad (13)$$

PEP carboxylase will have a different correction factor applied because it utilises bicarbonate as substrate, in which case:

$$\frac{r^{12}}{r^{13}} = \alpha_{\text{PEPC}} \frac{[\text{H}^{12}\text{CO}_3^-]}{[\text{H}^{13}\text{CO}_3^-]} = \alpha_{\text{PEPC}} \frac{\text{K}_{\text{eq}}^{12}[\text{CO}_2]}{\text{K}_{\text{eq}}^{13}[\text{CO}_2]} = \alpha_{\text{PEPC}} \alpha_{\text{eq}} \frac{[^{12}\text{CO}_2]}{[^{13}\text{CO}_2]}. \quad (\text{A17})$$

Therefore:

$$\alpha_{\text{total}} = \alpha_{\text{PEPC}}\alpha_{\text{part}}\alpha_{\text{eq}}. \quad (\text{A18})$$

At  $\text{pH} > 7$ , the correction factor is almost insignificant for PEPC because  $\alpha_{\text{part}}$  and  $\alpha_{\text{eq}}$  are almost reciprocals owing to the fact that PEPC utilises the form of inorganic carbon ( $\text{HCO}_3^-$ ) that is most prevalent. In terms of the total or overall discrimination observed in the system:

$$1 + \Delta_{\text{total}} = (1 + \Delta_{\text{PEPC}})(1 + \Delta_{\text{part}})(1 + \Delta_{\text{eq}}). \quad (\text{A19})$$

Rearranging:

$$\Delta_{\text{PEPC}} = \frac{1 + \Delta_{\text{total}}}{(1 + \Delta_{\text{part}})(1 + \Delta_{\text{eq}})} - 1 \quad (14)$$

#### Comparison of errors between different forms of the Rayleigh fractionation equation

The standard linear regression algorithm is based on the assumption that there is no error in the abscissa value. This is avoided if the ‘least squares cubic equation’ is solved (York 1966). In order to apply standard linear regression, however, we need to determine whether or not the regressed slope and associated error is dependent on the choice of abscissa and ordinate. For a set of  $n$  measurements for  $\ln[^{12}\text{C}]_i$  and  $\ln[^{13}\text{C}]_i$ , where  $i$  denotes the  $i$ th measurement, standard linear regression with  $\ln[^{13}\text{C}]$  as the abscissa results in a value for  $\alpha$  according to (Scott *et al.* 2004a):

$$\alpha = \frac{\sum_{i=1}^n (\ln[^{13}\text{C}]_i - \overline{\ln[^{13}\text{C}]}) (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})}{\sum_{i=1}^n (\ln[^{13}\text{C}]_i - \overline{\ln[^{13}\text{C}]})^2}, \quad (\text{A20})$$

where  $\overline{\ln[^{12}\text{C}]}$  and  $\overline{\ln[^{13}\text{C}]}$  are the mean values of the natural logarithms of the signals for  $^{12}\text{C}$  and  $^{13}\text{C}$ , respectively (the regressed slope is independent of the amplification ratio for either signal). The standard deviation,  $s$ , of the slope estimate for  $\alpha$  is (Scott *et al.* 2004a):

$$s_{\alpha} = \frac{\sqrt{\sum_{i=1}^n (\ln[^{12}\text{C}]_i - (\alpha \ln[^{13}\text{C}]_i + k))^2}}{\sqrt{(n-2) \sum_{i=1}^n (\ln[^{13}\text{C}]_i - \overline{\ln[^{13}\text{C}]})^2}}. \quad (\text{A21})$$

When  $\ln[^{12}\text{C}]$  is the abscissa:

$$\alpha^{-1} = \frac{\sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]}) (\ln[^{13}\text{C}]_i - \overline{\ln[^{13}\text{C}]})}{\sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})^2}, \quad (\text{A22})$$

and:

$$s_{\alpha^{-1}} = \frac{\sqrt{\sum_{i=1}^n (\ln[^{13}\text{C}]_i - (\ln[^{12}\text{C}]_i - k)/\alpha)^2}}{\sqrt{(n-2) \sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})^2}}. \quad (\text{A23})$$

The ratio of the scaled errors for  $\alpha$  and  $\alpha^{-1}$  is given by:

$$\frac{s_\alpha/\alpha}{s_{\alpha^{-1}}/\alpha^{-1}} = \alpha \sqrt{\frac{\sum_{i=1}^n (\ln[^{13}\text{C}]_i - \overline{\ln[^{13}\text{C}]})^2}{\sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})^2}}. \tag{A24}$$

On average:

$$\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]} = \alpha(\ln[^{13}\text{C}]_i - \overline{\ln[^{13}\text{C}]}), \tag{A25}$$

and so:

$$\frac{s_\alpha}{\alpha} = \frac{s_{\alpha^{-1}}}{\alpha^{-1}}. \tag{A26}$$

Hence the error is the same regardless of which of  $\ln[^{12}\text{C}]$  and  $\ln[^{13}\text{C}]$  is plotted as the abscissa and which as the ordinate. Linear regression of  $\ln R$  v.  $\ln[^{12}\text{C}]$  results in a value for  $\Delta'$  according to:

$$\Delta' = \frac{\sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})(\ln R_i - \overline{\ln R})}{\sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})^2}. \tag{A27}$$

Now  $\ln R = \ln[^{13}\text{C}] - \ln[^{12}\text{C}]$  and so:

$$\Delta' = \frac{\sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})(\ln[^{13}\text{C}]_i - \overline{\ln[^{13}\text{C}]} - \sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})^2}{\sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})^2} = 1 - \frac{1}{\alpha}. \tag{A28}$$

Hence, the slope estimates for  $\alpha$  and  $\Delta'$  are equivalent and related as described in Eqn (10). The standard deviation of the slope estimate for  $\Delta'$  is:

$$s_{\Delta'} = \sqrt{\frac{\sum_{i=1}^n (\ln R_i - (-\Delta' \ln[^{12}\text{C}]_i + k_2))^2}{(n-2) \sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})^2}} = \sqrt{\frac{\sum_{i=1}^n (\ln[^{13}\text{C}]_i - (\ln[^{12}\text{C}]_i - k_1)/\alpha)^2}{(n-2) \sum_{i=1}^n (\ln[^{12}\text{C}]_i - \overline{\ln[^{12}\text{C}]})^2}} = s_{\alpha^{-1}}. \tag{A29}$$

Therefore, the graphical method presented in this paper ( $\ln[^{12}\text{C}]$  v.  $\ln[^{13}\text{C}]$ ) yields the same absolute error as the method used previously ( $\ln R$  v.  $\ln[^{12}\text{C}]$ ).