# Analyzing the causes of method-to-method variability among Rubisco kinetic traits: from the first to the current measurements

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Running title: Method-to-method variability among Rubisco kinetics

Highlight: This study highlights the methodological causes responsible for the intraspecific variability among Rubisco kinetic traits and provides a normalized kinetic dataset for model species to be used by the scientific community.

**Abstract**

Due to the importance of Rubisco in the Biosphere, its kinetic parameters have been measured by different methodologies in a large number of studies over the last 60 years. These parameters are essential to characterize the natural diversity in the catalytic properties of the enzyme and they are also required for photosynthesis and cross scale crop modeling. The present compilation of Rubisco kinetic parameters in model species revealed a wide intraspecific laboratory-to-laboratory variability, which was partially solved by making corrections to account for differences in the assay buffer composition and in the acidity constant of dissolved $CO_2$, as well as for differences in the $CO_2$ and $O_2$ solubilities. Part of the intraspecific variability was also related to the different analytical methodologies used. For instance, significant differences were found between the two main methods for the determination of the specificity factor ($S_{c/o}$), and also between Rubisco quantification methods, Rubisco purification versus crude extracts and single-point versus $CO_2$-curve measurements for the carboxylation turnover rate ($k_{cat}^{c}$) determination. Causes for the intraspecific laboratory-to-laboratory variability for Rubisco catalytic traits are discussed. This study provides a normalized kinetic dataset for model species to be used by the scientific community. Corrections and recommendations are also provided to reduce measurement variability, allowing the comparison of kinetic data obtained in different laboratories using different assay conditions.

**Key words:** Rubisco, photosynthesis, kinetics, methodology, carboxylation, oxygenation, specificity factor, turnover rate, substrate affinity.

## Introduction

The conversion of inorganic to organic carbon in Nature is mainly driven by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which catalyzes the addition of $CO_2$ to ribulose-1,5-bisphosphate (RuBP), as the first step of the reductive pentose phosphate pathway in most autotrophic organisms. This pathway is present in chemosynthetic and anaerobic or aerobic photosynthetic bacteria, as well as algae, bryophytes and vascular plants.

Despite its importance in the Biosphere, all Rubiscos have a relatively slow carboxylation turnover rate ($k_{cat}^{c}$) and low affinity for $CO_2$ (i.e., high half-saturation constant for $CO_2$, $K_c$), as well as a poor discrimination between $CO_2$ and $O_2$ (Spreitzer and Salvucci, 2002). The reaction with oxygen leads to the photorespiratory pathway, which requires extra energy investment and causes a loss of fixed C (Bauwe *et al*., 2010).

These catalytic inefficiencies imply that Rubisco activity *in vivo* is the limiting step for the photosynthetic process in a range of environmental conditions that lead to low $CO_2$ availability and/or elevated $O_2$ concentrations at the Rubisco sites. For instance, drought, that leads to $CO_2$ diffusive limitations in $C_3$ plants, and elevated temperatures, which reduce the Rubisco specificity factor ($S_{c/o}$; carboxylation rate relative to oxygenation rate at given substrate concentrations) and the affinity for $CO_2$ (higher $K_c$), might limit the rate of photosynthesis under saturating illumination conditions (Galmés *et al*., 2016). The importance of accurate measurements of Rubisco kinetic traits is particularly evident for their implementation in the $C_3$ and $C_4$ photosynthesis models (Farquhar *et al*., 1980; von Caemmerer, 2000). These models are used to extrapolate aspects of cellular biochemistry to leaf gas exchange measurements, in order to predict the impact of Global Change on plant productivity (Sage and Kubien, 2007; Gornall *et al*., 2010).

Since the discovery of the reductive pentose phosphate pathway by Calvin and Benson (1948), substantial advances have been made in understanding the structure, activation, regulation and the reaction mechanism of Rubisco. Currently, it is well-known that a conserved trait of Rubisco is the required carbamylation (binding of non-substrate $CO_2$) of an essential residue in each catalytic site. Carbamylated Rubisco is then stabilized by the binding of $Mg^{2+}$ to produce the catalytically active form of the enzyme, capable of

RuBP binding and enolization, and its subsequent carboxylation or oxygenation (Andersson, 2008). Another conserved trait is the binding of sugar-phosphate inhibitors either to the carbamylated or decarbamylated forms of Rubisco (Parry *et al*., 2008), although the binding and release rate constants for these inhibitors have been shown to diverge among the different Rubisco forms (Pearce, 2006).

As deeper knowledge of Rubisco reaction mechanism was achieved, different methodologies were developed for the kinetic characterization of the enzyme. For the determination of the carboxylation turnover rate ($k_{cat}^{c}$), it is necessary to accurately quantify the Rubisco active site concentration in the same sample that is used for measurements of the maximum carboxylation rate. The use of the specific Rubisco inhibitor, 2′-carboxyarabinitol-1,5-bisphosphate (CABP), which tightly binds to the carbamylated active sites, enabled Rubisco active site quantification in non-pure extracts, as initially proposed by Collatz *et al*. (1979). Alternatively, Rubisco has been quantified for $k_{cat}^{c}$ determination in non-pure extracts by electrophoresis or immunoblotting with Rubisco large subunit antibody, or in Rubisco pure extracts by measuring total protein content through spectrophotometric methods (Warburg and Christian, 1941; Lowry *et al*., 1951; Bradford, 1976; Smith *et al*., 1985). The maximum carboxylation rate has been usually determined by radioactive (Paulsen and Lane, 1966) or spectrophotometric assays (Lilley and Walker, 1974), either by single-point measurements at near-saturating concentrations of $CO_2$ and RuBP (Tabita and McFadden, 1974; Anderson, 1975; Andrews and Abel, 1981; Parry *et al*., 1987; Sage, 2002; Kubien *et al*., 2008; Sharwood *et al*., 2008), or from the fitting of the curve obtained at varying $CO_2$ or RuBP concentrations (Andrews and Lorimer, 1985, Makino *et al*., 1985; Read and Tabita, 1992; Satagopan and Spreitzer, 2004; Whitney *et al*., 2011; Galmés *et al*., 2014).

The fitting of the substrate concentration vs. carboxylation rate curve obtained at varying $CO_2$ and $O_2$ concentrations also allowed the calculation of $K_c$ and $K_o$, respectively, which strongly depend on the calculations of the dissolved $CO_2$ and $O_2$ concentrations during the assay (dissociation constant for carbonic acid, $CO_2$ and $O_2$ solubility, ionic strength, pH, temperature). While $K_c$ was always obtained from direct carboxylase activity measurements under anoxygenic conditions, $K_o$ values have been frequently obtained indirectly from the inhibition of the carboxylase activity between two or more different $O_2$ concentrations (Orr *et al*., 2016; Hermida-Carrera *et al*., 2016), an approximation that

leads to a huge variability, even within the same species. This variability in $K_o$ values obtained from the mentioned approximation could be reduced by increasing the number of [$O_2$] points assayed within an appropriate range for the analyzed Rubisco, and by selecting the appropriate [$CO_2$] range for $K_c$ determination at each [$O_2$] (given that increased [$O_2$] will require an increased [$CO_2$] range for accurate fitting). The most sophisticated methods determine simultaneously Rubisco carboxylation and oxygenation rates, for example by using membrane inlet mass spectrometry (MIMS) that continuously records $CO_2$ and $O_2$ concentrations (Cousins *et al*., 2010). However, the complexity, poor reproducibility and long assay duration of the MIMS-based method has limited its implementation and broad use by the scientific community.

The Rubisco $CO_2/O_2$ specificity factor ($S_{c/o}$) represents the number of carboxylation per one oxygenation carried out by Rubisco at an equal concentration of dissolved $CO_2$ and $O_2$. It has been determined by a large number of methods, including direct or indirect measurements of substrate consumption ($O_2$, $CO_2$ and RuBP), such as the $O_2$ electrode method (Parry *et al*., 1989) or the MIMS method (Cousins *et al*., 2010); and/or quantification of product formation (3-phosphoglycerate and 2-phosphoglycolate) by methods including radiometric analysis of labeled products (Terzagui *et al*., 1986; Lee *et al*., 1993; Kostov and McFadden, 1995), liquid chromatography (Kent and Young, 1980; Jordan and Ogren, 1981; Zhu *et al*., 1992; Harpel *et al*., 1993; Kane *et al*., 1994; Uemura *et al*., 1996), nuclear magnetic resonance (NMR) spectroscopy (Wang *et al*., 1998) and mass-spectrometric analysis (Whitney and Andrews, 1998). Since $S_{c/o}$ summarizes all the previously described Rubisco kinetic traits (the carboxylation and oxygenation turnover rates, $k_{cat}^c$ and $k_{cat}^o$, and the half-saturation constants for $CO_2$ and $O_2$, $K_c$ and $K_o$), its determination also strongly depends on the accurate calculations of dissolved $CO_2$ and $O_2$ concentrations during the assay. The supply of $CO_2$ to the reaction in the form of bicarbonate or as gaseous $CO_2$ determines if the assay must be done in a closed-system (Zhu *et al*., 1992; Lee *et al*., 1993; Kostov and McFadden, 1995; Whitney and Andrews, 1998) or if it can be done under a continuous supply of a gaseous stream composed of known concentrations of $O_2$ and $CO_2$ (open-system; Kane *et al*., 1994; Uemura *et al*., 1996; Wang *et al*., 1998), which avoids changes in gas concentrations during the enzymatic reaction.

During the last decades, several studies have reported Rubisco kinetic properties in contrasting organisms by using the different methods described above, but have been

mainly focused on a few model species. The recent increase in the availability of Rubisco kinetic measurements on previously underreported phylogenetic groups have enabled a more profound analysis of Rubisco fine-tuning through evolution (Young and Hopkinson, 2017; Cummins *et al*., 2018; Tcherkez *et al*., 2018; Flamholz *et al*., 2019; Iñiguez *et al*., 2020; Bouvier *et al*., 2021). However, none of these Rubisco kinetic compilation studies have still recognized the existing laboratory-to-laboratory data variability. After a profound compilation of all available data in literature, we observed a wide variability for Rubisco catalytic traits reported for the same species among the different studies, even for the same variety/cultivar/population or bacterial strain sharing identical sequences, that could be inadvertently included in previous meta-analyses. Rubisco kinetics are mainly determined by the single copy of the large Rubisco subunit (*rbc*L) where the active site is located (Andersson and Backlund, 2008), although hybrid Rubiscos consisting of $C_3$ large subunits and a $C_4$ small subunits showing similar kinetics to $C_4$ Rubiscos have revealed the importance of the small subunit on catalysis (Ishikawa *et al*., 2011; Fukayama *et al*., 2019; Matsumura *et al*., 2020). Despite the fact that eukaryotes with the green-type chloroplast possess multiple small subunit Rubisco genes (*rbc*S) encoded by the nuclear genome, differential expression of small subunit genes naturally expressed in photosynthetic tissues seems not to alter Rubisco carboxylation kinetics (Lin *et al*., 2020). Up to date, only a phylogenetically diverse family of *rbc*S that are only expressed in non-photosynthetic specialized tissues of plants (i.e. glandular trichomes), has been shown to significantly alter Rubisco kinetics relative to native photosynthetic Rubisco (Morita *et al*., 2014; Laterre *et al*., 2017).

As there are many factors that could potentially alter the results of the Rubisco kinetic measurements, the identification of the potential causes for the variability observed in model species must be of importance for future studies. Our compilations demonstrate that the effects of different physicochemical constants and methods used in Rubisco kinetic assays could mask significant differences in Rubisco kinetics among species. Therefore, this information is an essential pre-requisite for succeeding in future studies about Rubisco evolution and environmental adaptation.

The aim of the present study is to describe and analyze the causes of method-to-method variability in the Rubisco kinetic traits which have been reported up to date for model species characteristically used to study Rubisco structure and function in plants, algae

and bacteria. This study also aims to provide a normalized kinetic dataset for model species, as well as recommendations and corrections to reduce intraspecific Rubisco kinetic variability, which could help researchers to improve photosynthesis and cross scale crop modeling predictions as well as future meta-analyses about Rubisco evolution.

## Material and methods

### In vitro *data compilation and filtration*

Peer-reviewed literature identified by Thompson-Reuters ISI Web of Science (Philadelphia, USA) and additional peer-reviewed non-English publications were thoroughly screened to obtain an exhaustive compilation of the data published so far on Rubisco kinetics. The compilation was focused on the main *in vitro* measured Rubisco kinetic traits: the $CO_2/O_2$ specificity factor ($S_{c/o}$), the maximum carboxylase turnover rate ($k_{cat}^c$), and the Michaelis-Menten affinity constant for $CO_2$ ($K_c$), and covered all domains of organisms expressing *bona fide* Rubiscos. From this compilation, only species reported by at least 6 different publications for each Rubisco kinetic trait ($S_{c/o}$, $k_{cat}^c$, $K_c$; $K_o$) were considered for the present study. A total of 243 publications were finally compiled, reporting $k_{cat}^c$, $K_c$ and/or $S_{c/o}$ values for 13 species (*Allochromatium vinosum*, *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, *Glycine max*, *Helianthus annuus*, *Nicotiana tabacum*, *Oryza sativa*, *Phaseolus vulgaris*, *Rhodospirillum rubrum*, *Spinacia oleracea*, *Synechococcus* sp., *Triticum aestivum* and *Zea mays*). When multiple values for a given kinetic trait were reported in the same publication, an average for each plant subspecies, variety or bacterial strain was calculated (see Data S1). No significant differences among varieties/strains from each model species were found when enough data were available (data not shown), although for most of the species and Rubisco kinetic traits, sample sizes were not appropriate to check for statistically significant differences among varieties/strains.

Data for $K_c$ or $k_{cat}^c$ that came from studies with non-preactivated enzyme extract with $CO_2$ and $Mg^{2+}$ were excluded from the database. $K_c$ measurements presumably not undertaken under anoxic conditions were also not considered (Wishnick and Lane, 1971; Ranty and Cavalie, 1982; Yaguchi *et al*., 1992). The data obtained with genetically modified Rubisco (including those arising from mutagenic approaches) were also excluded.

However, as the dataset is only based on *in vitro* measurements with extracted enzymes, we included data from recombinant Rubiscos expressed in *Escherichia coli* or *N. tabacum* whenever the original sequences of the large and small subunit genes originating from the donor organisms were unaltered.

The database was constructed with the following information: publication reference, species name, variety/cultivar name for plants or strain name for bacteria (when reported by the publication or provided by the authors through personal communications), Rubisco type and subunit composition, volume of the assay buffer ($v_A$), volume of the assay vial ($v_v$), assay temperature (T), pH, all assay buffer components and their concentrations (including added bicarbonate or gas partial pressure ($CO_2$ and $O_2$)), headspace gas composition, the reported ionic strength of the assay buffer, the reported acidity constant of dissolved $CO_2$ ($pK_{a1,CO2}$) and the Henry's law constants (or solubility constants) for $O_2$ and $CO_2$ used in the original publications to estimate the $O_2$ and $CO_2$ concentration in solution, when available. Finally, the use of Rubisco purified vs. crude extracts and the type of methodology followed in each assay were also annotated (see Table 1 for $S_{c/o}$ methods description, Table 2 for $K_c$ and $k_{cat}^c$ methods description and Table 3 for the description of Rubisco quantification for $k_{cat}^c$ calculation). Whenever relevant data were missing in the papers, authors were contacted, and the obtained information was included in the database (see the Acknowledgements section).

***Data correction and standardization***

All original data were corrected using the methodology described in detail by Galmés *et al*. (2016) to account for study-to-study differences in the assay buffer composition that affected the ionic strength and $pK_{a1,CO2}$, as well as the $CO_2$ and $O_2$ solubilities ($H_{pc,CO2}$ and $H_{pc,O2}$; Henry's law constants for $CO_2$ and $O_2$, respectively), in order to reduce the effects of methodological differences in Rubisco kinetic assays. Example of $pK_{a1,CO2}$, $H_{pc,CO2}$ and $H_{pc,O2}$ values for different temperatures and ionic strength/solute concentrations are provided in Tables S1 and S2.

In the case of $K_c$, the buffer composition correction explained in Galmés *et al*. (2016) for $pK_{a1,CO2}$ was applied as

$$K_{c,c} = K_{c,m} \frac{\left(1 - \frac{10^{pH\text{-}pK_{a,c}}}{1+10^{pH\text{-}pK_{a,c}}}\right)}{\left(1 - \frac{10^{pH\text{-}pK_{a,u}}}{1+10^{pH\text{-}pK_{a,u}}}\right)} \quad (1)$$

where the buffer composition-corrected value ($K_{c,c}$) and measured value ($K_{c,m}$) depend on the solution pH and buffer composition-corrected ($pK_{a,c}$, see Table S1) and used in the original study ($pK_{a,u}$) acidity constants of dissolved $CO_2$. This correction was always applied for those methods that supply $CO_2$ in the form of $HCO_3^-$, except for data obtained from mass spectrometry (MS) assays, since this method allows a direct measurement of $CO_2$ concentration in the gas phase, avoiding the need to know the $pK_{a1,CO2}$.

In some of the earlier studies, $K_c$ values were reported for bicarbonate ($K_{c,bic}$). These values were converted to $K_c$ for dissolved $CO_2$ ($K_{c,CO2}$) as:

$$K_{c,CO_2} = K_{c,bic} \left(1 - \frac{10^{pH\text{-}pK_{a1,CO2}}}{1+10^{pH\text{-}pK_{a1,CO2}}}\right), \quad (2)$$

where pH is the assay buffer pH value.

Furthermore, when not considered, $K_c$ data were also corrected to include the outgassing of $CO_2$ from the liquid reaction medium into the reaction vial headspace (see Ogren and Hunt (1978) for a discussion of the problem and Table S5 from Sharwood *et al*. (2016a) for calculating dissolved [$CO_2$] in the assay buffer considering the outgassing of $CO_2$) using the mass balance approach as briefly explained here. Given the added initial bicarbonate concentration of $C_{bic,i}$ and liquid volume of $v_a$, the total amount of inorganic C in the vial is $v_a C_{bic,i}$, and the corresponding dissolved $CO_2$ concentration calculated without considering $CO_2$ outgassing from the solution is $C_{CO2,L,i}$. Once the vial is hermetically sealed, a new equilibrium between the initially $CO_2$-free air in the vial gas-phase with volume $v_g = v_v - v_a$ and liquid phase is established, where $v_v$ is the vial volume, and the new liquid-phase bicarbonate concentration, $C_{bic,n}$, becomes:

$$C_{bic,n} = \frac{v_a C_{bic,i} - v_g C_{CO_2,L,n} H_{pc,CO_2}}{v_a}, \quad (3)$$

where $H_{pc,CO2}$ (mol mol$^{-1}$) is the dimensionless Henry's law constant for $CO_2$, and $C_{CO2,L,n}$ is the liquid-phase $CO_2$ concentration corresponding to the new equilibrium. $C_{CO2,L,n}$ corresponding to given value of $C_{bic,n}$ was calculated using both the dissociation constants for the carbonic acid and bicarbonate ($pK_{a1}$ and $pK_{a2}$, respectively). In these calculations,

300 p$K_{a1}$ and $H_{pc,CO2}$ values corresponding to the assay temperature were obtained as in
301 Galmés *et al*. (2015, 2016). p$K_{a2}$ temperature response was obtained by fitting the data of
302 Harned and Scholes (1941) by fourth order polynomial ($pK_{a2}$ = -0.000000013057$T^4$ +
303 0.000001118897$T^3$ + 0.00008433 6$T^2$ - 0.014452$T$ + 10.6256, where $T$ is in °C, $r^2$ =
304 0.99995). We note that the second dissociation step is only relevant at higher pH values
305 (at pH < 8.3, the share of total inorganic C present as $CO_3^{2-}$ is less than 1%). As $C_{bic,n}$
306 depends on $C_{CO2,L,n}$ (Eq. 1) and $C_{CO2,L,n}$ depends on $C_{bic,n}$, the calculations were carried
307 out in iterative mode. A Microsoft Excel calculator to correct for $CO_2$ outgassing effects
308 (volume correction calculator) is provided in Supplementary Information (see Data S2
309 Excel spreadsheet and Data S2 explanation tool in the supplementary data PDF file).
310
311 The outgassing correction increases with decreasing the ratio $v_A/v_v$ and with decreasing
312 pH, and was particularly significant for older studies that used large vials and relatively
313 low pH. For example, Bowes and Ogren (1972) studying the Rubisco kinetics in *G. max*
314 used a vial size of 22.5 mL with 1 mL assay buffer and the assay pH was 8.0, resulting in
315 a solution $CO_2$ concentration ratio, $C_{CO2,L,n}/C_{CO2,L,i}$, of 0.727, implying that without the
316 volume correction, $K_c$ would be overestimated by a factor of 1.38. All else being equal,
317 for a pH of 7.0, $C_{CO2,L,n}/C_{CO2,L,i}$ = 0.230 (4.35-fold overestimation of $K_c$). On the other
318 hand, in another study of Rubisco from *G. max*, Makino *et al*. (1985) used 1 mL vials
319 with 0.25 mL assay buffer (pH = 8.0), and $C_{CO2,L,n}/C_{CO2,L,i}$ = 0.956 (1.05-fold
320 overestimation of $K_c$).
321
322 In the case of $K_o$, all data were corrected, when not considered in the original study, for
323 assay solute concentration affecting the Henry's law constant for $O_2$ ($H_{pc,O2}$), as explained
324 in Galmés *et al*. (2016)

325 $$K_{o,c} = K_{o,m} \frac{H_{pc,u}}{H_{pc,s}} \quad (4)$$

326 where $K_{o,c}$ and $K_{o,m}$ are the liquid-phase $K_o$ values corrected and reported, respectively;
327 $H_{pc,u}$ is the value of the Henry's law constant for $O_2$ used at the given temperature in the
328 original study and $H_{pc,s}$ is the Henry's law constant for $O_2$ corrected for assay solute
329 concentration (see Table S2).
330
331 Data obtained by direct [$O_2$] measurements (i.e. $O_2$ electrode and MS methods) were also
332 corrected for assay solute concentration affecting $H_{pc,O2}$, since $O_2$ solubility is used for

calibrating those systems. Additionally, a correction for humidity was included that considers the circumstance that, due to water evaporation, $O_2$ partial pressure above the liquid surface inside the vial is lower than 21.28 kPa (corresponding to completely dry air) typically assumed in estimating the liquid-phase $O_2$ concentration. This correction has been called "gas pressure reduction factor" in Supplementary Data S1. Saturated water vapor pressure ($P_{ws}$) at given temperature was calculated by the Buck equation (Buck, 1981) and the dilution factor was calculated as $(P\text{-}P_{ws})/P$, where $P$ is air pressure. Typically, the effect of this correction was on the order of a few percent.

In the case of $S_{c/o}$ values from those methods that supply $CO_2$ in the form of $HCO_3^-$ (closed-system), data were corrected for the effects of buffer composition on $pK_{a1,CO2}$ and $H_{pc,O2}$, using equation 7 of Galmés *et al*. (2016)

$$S_{c/o,c} = S_{c/o,m} \frac{\left(1 - \frac{10^{pH\text{-}pK_{a,u}}}{1+10^{pH\text{-}pK_{a,u}}}\right) H_{pc,u}}{\left(1 - \frac{10^{pH\text{-}pK_{a,c}}}{1+10^{pH\text{-}pK_{a,c}}}\right) H_{pc,s}} \quad (5)$$

where $S_{c/o,c}$ and $S_{c/o,m}$ are the specificity factor values corrected and reported, respectively; $pK_{a,c}$ is the buffer composition-corrected acidity constant of dissolved $CO_2$ (see Table S1), $pK_{a,u}$ is the acidity constant of dissolved $CO_2$ used in the original study, $H_{pc,u}$ is the value of the Henry's law constant for $O_2$ used at the given temperature in the original study and $H_{pc,s}$ is the Henry's law constant for $O_2$ corrected for assay solute concentration (see Table S2).

These data were also corrected, when not considered in the original study, for the outgassing of $CO_2$ from the liquid reaction medium into the reaction vial headspace and for humidity affecting the dissolved $O_2$ concentration, as described above. $S_{c/o}$ values obtained from MS measurements were not corrected for $pK_{a1,CO2}$ despite the supply of $CO_2$ in the form of $HCO_3^-$, since this method allows a direct measurement of $CO_2$ and $O_2$ concentration in the gas phase. On the other hand, $S_{c/o}$ values from those methods supplying continuously $CO_2$ to the gas-phase (open-system, in order to achieve a certain liquid phase $CO_2$ concentration during the assay) were corrected for the effects of buffer composition on $H_{pc,CO2}$ and $H_{pc,O2}$, following equation 11 of Galmés *et al*. (2016)

$$S_{c/o,c} = S_{c/o,m} \frac{H_{pc,O2,u} H_{pc,CO2,s}}{H_{pc,CO2,u} H_{pc,O2,s}} \quad (6)$$

where $H_{pc,O2,u}$ and $H_{pc,CO2,u}$ are the values of the Henry's law constants for $O_2$ and $CO_2$ used at the given temperature in the original study, and $H_{pc,O2,s}$ and $H_{pc,CO2,s}$ are the Henry's law constants for $O_2$ and $CO_2$ corrected for assay solute concentration.

Flowcharts of how to correct and normalize the different Rubisco kinetic traits depending on the method used are reported in Figure S1. Correlations between original and corrected data for the model species can be observed in Figure 1.

Most of the compiled data come from assays performed at 25 °C and at an assay pH between 7.8 and 8.2. If the paper did not report the value of the given Rubisco kinetic trait at 25 °C, measurements taken at 20-30 °C were also included in the compilation and normalized to 25 °C using the temperature functions described in Galmés *et al*. (2015; 2016). No significant differences were found between values originally measured at 25 ºC (see Fig. S2) and those measured at a different temperature in the range of 20-30 ºC and subsequently standardized to 25 ºC (see Fig. 2) for any Rubisco kinetic trait and species analyzed (data not shown).

In the case of the maximum carboxylase turnover rate, recent papers tend to report directly $k_{cat}^c$ (amount of $CO_2$ per catalytic site and time measured in $s^{-1}$), but older papers usually provided the carboxylase specific activity ($V_{max,CO2}$, amount of $CO_2$ fixed per unit protein mass and time measured in units of μmol $CO_2$ $g^{-1}$ protein $s^{-1}$; $k_{cat}^c = V_{max,CO2}M_{Rub}(n_a)^{-1}$, where $n_a$ is the number of active sites in Rubisco molecule and $M_{Rub}$ is Rubisco molecular mass in units of g $\mu mol^{-1}$). In the latter case, the methodology of the original paper was assessed to eliminate those compiled measurements that were done in non-purified samples and Rubisco was not specifically quantified (Raghavendra and Das, 1977; Servaites and Ogren, 1977; Storro and McFadden, 1983; Castrillo, 1985; González-Moro *et al*., 1997). For the conversion of protein concentration into catalytic site concentration, the $M_{Rub}$ for each species was calculated from its *rbc*L and *rbc*S amino acid sequences that were obtained from Genbank (https://www.ncbi.nlm.nih.gov/genbank/). When multiple *rbc*S sequences were available for the given species, a weighted average molecular mass value was used. Those $k_{cat}^c$ data that were calculated assuming non-stoichiometric CABP binding (Butz and Sharkey, 1989; Sage, 2002) were recalculated for 8 CABP-binding sites per Rubisco (Blayney *et al*., 2011).

The values of all Rubisco kinetic traits are provided for liquid phase. Equations to convert the liquid phase Rubisco traits to gas-phase equivalents are provided in Galmés *et al*. (2016).

***Statistical analysis***

Significance of differences among corrected and standardized values and original reported values were tested by one- or two-way factorial analyses of variance (ANOVA), after normality (Anderson-Darling test) and homogeneity of variances (Levene's test) were confirmed. Post-hoc comparisons were performed by Fisher's LSD test. For non-normally distributed data, Kruskal–Wallis tests with Bonferroni correction for multiple comparisons were used instead of the ANOVA. Pearson correlation coefficients were calculated to characterize the significance of linear association between the different variables. The level of statistical significance of all these tests was set at $P<0.05$. All statistical analyses were performed using Statistica software v.7 (StatSoft Inc., USA).

## Results and discussion

Our compilation of the main Rubisco catalytic properties ($k_{cat}^{c}$, $K_c$, $K_o$, $S_{c/o}$) reveals that there is a wide range of values reported for the model species. The key questions for the end-users interested in simulating carbon gain of photosynthetic organisms as well as evolution of Rubisco is what are the "right" values and how can we reduce the experimental variability. The laboratory-to-laboratory kinetics variability for any given species was partially reduced by making corrections to account for differences in the assay buffer composition (that affected the ionic strength), the ratio assay volume/vial volume in closed systems, and the acidity constant of dissolved $CO_2$ ($pK_{a1,CO2}$), as well as for differences in the $CO_2$ and $O_2$ solubilities used (Fig. 2). Since $k_{cat}^{c}$ does not depend on $CO_2$ or $O_2$ concentrations for its calculation, no correction was applied for this parameter.

***Methodological differences in $S_{c/o}$ determination explained the intraspecific laboratory-to-laboratory variability in model species***

The specificity factor is the least variable Rubisco kinetic trait (Fig. 2). While $k_{cat}^{c}$ and $K_c$ can vary more than 200% in vascular plants (see Iñiguez *et al*., 2020), $S_{c/o}$ varies less than 50% (75-120; Flamholz *et al*., 2019; Iñiguez *et al*., 2020). Since it represents the ratio between $CO_2$ and $O_2$ fixation at Rubisco active site, $S_{c/o}$ measurements do not require completely activated Rubisco (Jordan and Ogren, 1983). Therefore, the assay conditions are less relevant for $S_{c/o}$ than for $k_{cat}^{c}$ and $K_c$, except for the assay temperature and the accurate determination of dissolved [$CO_2$] and [$O_2$] during the assay.

In general, the corrections to account for differences in the assay buffer composition, the acidity constant of dissolved $CO_2$, and the $CO_2$ and $O_2$ solubilities in $S_{c/o}$ were more modest than for $K_c$ and no clear reduction in data variability was obtained (Fig. 2). This can also be observed in the linear correlation between original and corrected values (Fig. 1), which reported a slope of 0.98, meaning an average correction for $S_{c/o}$ values of only 2%. However, the observed intraspecific variability might be explained by the different methodologies used for its determination. Different methods have been developed to measure $S_{c/o}$, either based on the determination of the consumption of substrates ($CO_2$, $O_2$ and RuBP) and/or the quantification of product formation (3-phosphoglycerate and 2-phosphoglycolate) for the carboxylation and oxygenation reactions.

One of the most widespread methods for $S_{c/o}$ determination is based on the measurement of $O_2$ decline during the full consumption of RuBP using a Clark-type $O_2$ electrode (Parry *et al*., 1989; Table 1). Despite its simplicity, the main problem related to this method is the requirement of an elevated concentration of Rubisco to have enough sensitivity to accurately quantify the decline of $O_2$ with the electrodes. Highly purified Rubisco is specially needed to be sure that this decline is exclusively due to Rubisco activity, and not to any other $O_2$ consuming reaction. Moreover, these electrodes cannot measure proper dissolved $O_2$ concentrations over 50% $O_2$ (gas), which might impose a limit for accurately measuring Rubiscos with elevated $S_{c/o}$ due to low Rubisco oxygenation rates (i.e. 4-5 times lower than carboxylation rates for a reaction solution equilibrated with air in vascular plants).

The other widespread method for $S_{c/o}$ determination uses radiolabeled RuBP and/or $H^{14}CO_3^-$ as substrates, and chromatographic separation of the labelled products, which can be later accurately quantified by scintillation counting (Jordan and Ogren, 1981; Zhu

*et al*., 1992; Harpel *et al*., 1993; Kane *et al*., 1994; see Table 1). A modification of this method does not involve the use of any radioactive-labelled substrate, and 3-phosphoglycerate and 2-phosphoglycolate formed in the assay medium are separated but also quantified by anion exchange chromatography (Uemura *et al*., 1996). Radioactive labeling of substrates has the advantage of providing a higher accuracy for the quantification of the reaction products, especially when their concentrations are too low to be properly quantified in a chromatogram. However, this requires either the synthesis of radiolabeled compounds which are not commercially available ([1-$^{14}$C or 1-$^{3}$H]RuBP; Kane *et al*., 1994; 1998), or to accurately determine the specific radioactivity of the substrate (when $H^{14}CO_3^-$ is used), and management of radioactive waste.

The chromatographic method also needs some Rubisco purification to reduce the possibility of further metabolism of the products of carboxylation and oxygenation by other enzymes in the extract. One of the main advantages, when done only with labelled RuBP, is that $CO_2$ and $O_2$ dissolved concentrations can be kept constant during the assay due to an open gas-purging system with an exact proportion of $CO_2$ and $O_2$ in the gas-phase, and a low amount of RuBP used, as explained by Kane *et al*. (1994). This prevents the need to calculate the dissolved $CO_2$ from added bicarbonate (which depends on pH and the $pK_{a1,CO2}$), and to calculate the dissolved $O_2$ from the barometric pressure. Therefore, open-systems might be preferable over closed-systems.

The compiled and corrected values reveal a significant difference among the methods used for the determination of $S_{c/o}$ (Fig. 3). Specifically, the values from the $O_2$ electrode method are 10-15% higher in three out of five species as compared to the different variants of the chromatographic method for product resolution and quantification (Fig. 3). Since $S_{c/o}$ values vary only 10-20% among vascular plants, this difference among the methods might be important enough to impede finding significant differences among closely related species. The chromatographic method has the advantage that both reactions are measured in the same way by quantifying their products and only accurate separation of both peak areas is required for a correct $S_{c/o}$ calculation. On the contrary, in the $O_2$ electrode method, the oxygenation reaction is measured by $O_2$ uptake while the carboxylation reaction is measured by the difference between the total RuBP consumption and $O_2$ fixation. Due to the closed-system required for the $O_2$ electrode method, the decrease in $O_2$ concentration during the reaction may limit the oxygenase

activity, whereas the $CO_2$ consumption might not alter the dissolved $CO_2$ concentration due to the addition of carbonic anhydrase. Usually, for the total RuBP consumption (Parry *et al*., 1989), the decrease in $O_2$ concentration when assaying plant Rubiscos could be of 10-15% relative to the initial $O_2$ concentration, but it can decrease up to 50% for Rubiscos with much lower $S_{c/o}$ values (i.e. bacterial Rubiscos) if [$CO_2$] in the assay buffer is not increased proportionally. This change in the $CO_2/O_2$ ratio during the reaction might be the reason for a significantly higher $S_{c/o}$ values obtained by the $O_2$ electrode method, in comparison with the chromatographic method.

Other analytical methods used for $S_{c/o}$ determination are summarized in Table 1, but no other trend was observed given the limited number of measurements for each of these methods. The few data obtained from $NaH^{14}CO_3$ incubations, determining $^{14}C$ fixation into 3-phosphoglycerate while estimating the oxygenation reaction from the total RuBP consumption (which is also a closed-system), revealed higher $S_{c/o}$ values than the other two main $S_{c/o}$ methods for *R. rubrum*, whereas for *Synechococcus* sp., $S_{c/o}$ values obtained from the $NaH^{14}CO_3$ incubation method were close to the average for the other two main methods. Despite the simplicity of the $NaH^{14}CO_3$ incubation method, the absence of a direct measurement of the oxygenation reaction, along with the need for calculate dissolved [$CO_2$] from added $NaH^{14}CO_3$, could reduce the accuracy of the method.

The nuclear magnetic resonance (NMR) spectroscopy and membrane inlet mass spectrometry (MIMS) might represent promising methods, since both carboxylation and oxygenation reactions can be measured simultaneously in real time, either by quantification of the products (NMR spectroscopy) or the gaseous substrates (MIMS) during Rubisco reaction (Table 1). However, these methods have still not been widely implemented by research groups probably due to their complexity, poor reproducibility and time-consuming nature (~ 4 hours per a single curve for MIMS; Boyd *et al*., 2015), and the need for non-routine calibrations and corrections for signal losses associated with the use of $H_2O$ as solvent, for the former, and for gaseous consumption and isotopic discrimination of the membrane, for the latter. In addition, a lower reproducibility and/or sensitivity from the MIMS method in comparison with the radioactive assay could be observed, when comparing both type of measurements from the same study at different temperatures (Boyd *et al*., 2019). A few $S_{c/o}$ data obtained by NMR spectroscopy fall within the range of other measurements available for the given species. Regarding MIMS

532 measurements, $S_{c/o}$ values reported for *T. aestivum* and *Z. mays* were the highest even
533 reported for these two species (Cousins *et al*., 2010), whereas for another non-model
534 species, *Setaria viridis,* the $S_{c/o}$ value was notably lower than the value obtained from the
535 radioactive assay (Sharwood *et al*., 2016a). Although these measurements with the MIMS
536 method were done using non-purified extracts (but included controls without RuBP
537 addition), highly purified Rubisco might be specially needed in some species with
538 elevated concentrations of antioxidants, in order to reduce non-Rubisco $O_2$ consumption.
539 More data from these promising methodologies are required to gain a conclusive insight
540 into the precision and accuracy of these methods.
541
542 ***Differences in Rubisco purification,* in vitro *carboxylation and Rubisco catalytic sites***
543 ***quantification methods determine the intraspecific laboratory-to-laboratory variability***
544 ***for $k_{cat}^c$ in model species***
545
546 Since the discovery of Rubisco in the late 50s (Mayaudon *et al*., 1957), its carboxylase
547 activity *in vitro* has been extensively determined by $^{14}CO_2$ uptake, followed by liquid
548 scintillation counting of the acid-stable products. This method has been shown to be
549 extremely sensitive and reproducible, but since it is based on a discrete measurement,
550 changes in the carboxylation rate during time cannot be observed unless parallel
551 incubations are made at shorter or longer intervals (Table 2). Because Rubisco activity
552 was shown to decline during the course of *in vitro* assays (Edmonson *et al*., 1990) due to
553 the generation of side products like xylulose-1,5-bisphosphate (XuBP) and D-glycero-
554 2,3-pentodiulose-1,5-bisphosphate (PDBP) through misfire reactions that inhibit
555 catalysis (Parry *et al*., 2008), this method was improved by reducing incubation time up
556 to one minute, and using pre-activated Rubisco and highly purified RuBP (Kane *et al*.,
557 1998). Specially, very pure RuBP free of inhibitory impurities such as PDBP has been
558 demonstrated to be critical for making accurate and reliable Rubisco catalysis
559 measurements *in vitro*, which can remain relatively linear over a 3 min assay period under
560 100% $N_2$ atmosphere (Sharwood *et al*., 2016b). However, the time-dependent self-
561 inhibition of the *in vitro* Rubisco activity has been only observed in plants, but not in
562 other phylogenetic groups so far (Pearce *et al*., 2006).
563
564 Alternatively, the spectrophotometric method allows a *real-time* recording of the
565 carboxylase activity by using an NADH-coupled enzymatic system without producing

radioactive waste (Table 2). This method is based on the conversion of 3-phosphoglycerate to glycerol 3-phosphate by means of specific coupling enzymes and an ATP-generating system. The oxidation of NADH during the assay, which is coupled with the conversion of 3-phosphoglycerate to the final product by four ATP- or NADH-requiring enzymatic reactions, is continuously monitored by changes in absorbance at 340 nm, and used for the calculation of the carboxylation activity (Lilley and Walker, 1974; Sharkey *et al*., 1991). Since the conversion of 3-phosphoglycerate to glycerol 3-phosphate through the four coupled reactions is relatively slow, elevated concentrations of the other involved enzymes relative to Rubisco are required such that the overall reaction is only limited by Rubisco carboxylation.

We did not find significant differences between $k_{cat}^{c}$ values measured by the radioactive, spectrophotometric or MIMS method (Fig. 4). However, some laboratories have found significantly lower $k_{cat}^{c}$ values (around 30% lower) for the spectrophotometric NADH-coupled assay relative to the radioactive assay, when using the same protein extracts, assay conditions and Rubisco quantification (Sharwood *et al*., 2016b; Sales *et al*., 2020). The lower values for the spectrophotometric method might be explained by substrate limitations for one or more of the enzymes in the NADH-linked assay, possibly the rate of 3-phosphoglycerate reduction (Lilley and Walker, 1974), leading to reduced estimates of $k_{cat}^{c}$ values.

Our compilation demonstrated that $k_{cat}^{c}$ has the highest laboratory-to-laboratory intraspecific variability among the studied Rubisco kinetic traits. This is related to the fact that its calculation depends on two completely different methodologies: the determination of the maximum carboxylation activity ($V_{c,max}$) and the quantification of Rubisco active sites in the same protein extract. In addition, $V_{c,max}$ can be determined by two different ways: by a single measurement at saturating substrate (dissolved $CO_2$ and RuBP) conditions or calculated from the substrate, $CO_2$, concentration vs. carboxylation rate relationship (calculating simultaneously the Michaelis-Menten constant, $K_c$, and $V_{c,max}$). In the latter case, the response curve is measured at saturating concentration of RuBP under anoxic conditions.

The single-point method led to lower values, as compared to the Michaelis-Menten curve fitting method, in some of the analyzed species (Fig. 5a), and a significant difference

among single-point vs. $CO_2$ curve estimates was found by a two-way (method and species) ANOVA ($P < 0.05$). This might be related to the fact that $V_{c,max}$ obtained from the substrate response curves represents the maximum activity at an infinite substrate concentration, which, due to the properties of the rectangular hyperbola, will be always somewhat higher than the experimental estimation at high substrate conditions typically considered saturating. Therefore, we highly recommend to calculate $k_{cat}^c$ values from the fitting of $CO_2$ curves rather than by the single-point method at saturating substrate conditions, especially if the data is to be used for modeling and carboxylation catalytic efficiency determination. Moreover, some single-point measurements have been frequently done at sub-saturating $CO_2$ concentrations, particularly in bacteria, which requires very high $CO_2$ concentrations for saturating their Rubisco carboxylation activity. This is reflected in Figure 5a, where the strongest differences between single-point and $CO_2$ curve estimates are found for the bacterial species. On the other hand, in the case of curve fitting, the range of dissolved $CO_2$ used in the assays will determine the goodness of fit, and therefore the accuracy of $k_{cat}^c$ and $K_c$ calculations.

Activation conditions before the assay, as well as whether or whether not Rubisco was purified in the proteinaceous extract could alter the results obtained for $k_{cat}^c$. Rubisco, as any other enzyme, need to be completely activated before a Michaelis-Menten curve is measured. This implies a need for previous determination of the optimum concentration of the cofactors, $CO_2$ and $Mg^{2+}$ (Christeller and Laing, 1978), which is expected to vary among different Rubisco forms and phylogenetic groups, in the same way as observed for ligand binding by Rubisco (Pearce, 2006). The time of incubation required to fully activate Rubisco without proteolytic degradation must be also tested by replicating the carboxylation assay following activation at different periods. Furthermore, the C-terminal loop of the enzyme is often a target for proteases which could result in changes in the catalytic performance, and therefore, long purification protocols might produce an irreversible decrease in $k_{cat}^c$ (Sharwood *et al*., 2008). On the other hand, rapidly prepared fresh non-purified extracts need extra caution and additional control analyses, such as non-RuBP control assays and CABP-inhibited-extract control assays, in order to corroborate that all $^{14}C$ fixed during the assay is due to Rubisco catalysis (Iñiguez *et al*., 2018). Likewise, some processing of the proteinaceous solution, such as ammonium sulfate purification steps or lyophilization, could alter the enzyme tertiary structure and lead to an irreversible partial deactivation. This was clearly shown by a significant

decrease of $k_{cat}^c$ values in purified vs. crude extracts for the analyzed species (Figure 5b; two-way ANOVA, $P < 0.05$).

Finally, the Rubisco quantification was one of the most important sources of variability in $k_{cat}^c$, since the most analyzed species showed significantly higher values when their Rubisco was quantified by an incubation with the $^{14}C$ labelled specific Rubisco inhibitor CABP, than when quantified by other methods (spectrophotometric determination of pure Rubiscos, or electrophoresis or immunodetection in non-pure extracts) (Fig. 5c). Spectrophotometric quantification of pure Rubiscos is a very inaccurate analysis that must be avoided for $K_{cat}^c$ calculation, since none of its methods for protein quantification are uniformly sensitive to all protein types. In fact, strong discrepancies among the spectrophotometric methods are widely known (summarized by Goldring, 2019) and usually bovine serum albumin is used as standard, which strongly differs in its aminoacidic composition relative to Rubisco. CABP has been shown to bind tightly to carbamylated Rubisco sites (Parry *et al*., 2008), while neither electrophoresis nor immunodetection methods are specific of carbamylated Rubisco active sites and possess less sensitivity than radiometric assays. Due to the specificity of CABP to Rubisco active sites, this quantification method has been frequently used in rapid assays with crude extracts, but care must be taken to find the proper CABP concentration that saturates Rubisco sites for a specific species (especially for organisms phylogenetically distant from plants, see Pearce *et al*., 2006 and Iñiguez *et al*., 2018) without a significant non-specific binding of CABP to other proteins (Yokota and Canvin, 1985). This would require a previous optimization of CABP binding quantification protocol for the different Rubisco forms and phylogenetic groups.

It is difficult to separate the effects of Rubisco purification from the effects of either Rubisco quantification method or $k_{cat}^c$ determination method on the compiled $k_{cat}^c$ values, since low $k_{cat}^c$ values mostly came from older publications using highly purified Rubisco, which was usually quantified by protein spectrophotometric methods and frequently measured using single-point measurements. This is shown by a positive significant correlation between the year of publication and the $k_{cat}^c$ value for many of the model species (Fig. 6), revealing a trend for older publications reporting lower $k_{cat}^c$ values. Thus, the independent effects of Rubisco purification in $k_{cat}^c$ could not be tested with the compiled data.

***Corrections for assay dissolved [$CO_2$] calculations, rather than methodology, explained the intraspecific laboratory-to-laboratory variability for $K_c$ in model species***

The corrections for $K_c$ significantly reduced data variability, especially in some species like *R. rubrum*, *N. tabacum* or *T. aestivum*, and lowered the average in most of the species (Fig. 2). The linear correlation between original and corrected values (Fig. 1) reported a slope of 0.78, indicating that, on average, $K_c$ values were corrected by 22%. This correction is highly relevant, since this percentage is equal or even higher than the differences found among $K_c$ values from different species of vascular plants (Flamholz *et al*., 2019; Iñiguez *et al*., 2020).

$K_c$ seems to be the most robust kinetic trait to be determined since similar values have been obtained for spectrophotometric, MIMS and radioactive assays for the model species (Fig. 4). Likewise, Boyd *et al*. (2019) obtained a similar temperature response and absolute $K_c$ values for the same species (*A. thaliana*) by either MIMS or radioactive assays. However, data from MIMS and spectrophotometric assays are very scarce, and much more data are needed to corroborate this assumption. The significantly lower number of studies reporting $K_c$, in comparison with $k_{cat}^c$, using the spectrophotometric method must be due to the requirement for an adaptation of the spectrophotometric cuvettes to create a complete anoxygenic atmosphere inside them and, at the same time, to avoid $CO_2$ loss from the assay solution.

No significant differences were found in $K_c$ determination between purified Rubiscos and crude extracts, either before or after corrections for $pK_{a1,CO2}$ and $CO_2$ solubility (Fig. 7 and Fig. S3). Additionally, we found reduced differences between purified and non-purified extracts and reduced coefficients of variations for the corrected data (Fig. 7) in comparison with the original values (Fig. S3) in *G. max*, *N. tabacum* and *O. sativa*, reveling the importance of doing such corrections.

The remaining variability within each species after corrections could probably be related to non-accurate determination of dissolved $CO_2$ concentrations and/or the specific radioactivity of the $^{14}CO_2$ during the assay. Moreover, leaks can lead influx of $^{12}CO_2$ and outflux of $^{14}CO_2$, altering the specific activity of $^{14}CO_2$ and the total dissolved $CO_2$, while

the influx of $O_2$ would reduce the carboxylation rate. Technical replicates are recommended to detect these issues.

Regarding $K_o$, the huge intraspecific variability observed in Figure 2, even after corrections, suggest the presence of methodological problems which preclude obtaining reliable data. This is due to the fact that most compiled $K_o$ values were obtained indirectly from the inhibition of the carboxylase activity by the radiolabel method, frequently with only two or three different $O_2$ concentrations. This methodology provokes that slight differences in $K_c$ under different oxygen concentrations can be translated into huge differences in $K_o$ among replicates, especially when it is calculated exclusively from $K_c^{0\%}$ and $K_c^{21\%}$ (half-saturation constant for $CO_2$ under 0% and 21% $O_2$, respectively). The few measurements done by the MIMS method, which directly record $O_2$ and $CO_2$ consumption during the reaction, are among the lowest values even reported for *T. aestivum* and *Z. mays* (Fig. 4). A lower $K_o$ was also obtained by the MIMS method in comparison to the radiolabel method (carboxylase inhibition) for other two species of vascular plants (Boyd *et al*., 2015; Sharwood *et al*., 2016a; Boyd *et al*., 2019). $K_o$ values obtained from oxygen evolution measurements by $O_2$ electrodes are also scarce but a trend for lower values relative to the radiolabel method (carboxylase inhibition) was also observed for some of the model species, like *R. rubrum* and *S. oleracea* (see Data S1). Therefore, more data from direct and accurate oxygen consumption measurements are specially needed to confirm this trend and to reduce the huge method-to-method variability.

## *Conclusions*

The standardization applied resulted in significant corrections in some of the Rubisco kinetic parameters, especially for $K_c$, for many of the model species, and therefore constitutes a critical step to integrate values across papers, laboratories and assay conditions. The experimental methodology followed for the determination of $S_{c/o}$ and $k_{cat}^c$ also influenced significantly the obtained data.

This study demonstrates that the proposed corrections of physicochemical characteristics used to estimate $CO_2$ and $O_2$ concentrations and the assay system (e.g. vial size/assay volume, closed vs. open-systems), as well as standardizations for method-to-method

differences, enhance the rigor of Rubisco kinetic data and might be considered in future studies when analyzing Rubisco kinetics among diverse species from compiled data.

In addition, an optimization for direct $O_2$ measurements are specially needed for reducing the huge study-to-study variability in the oxygenation kinetic traits ($K_o$ and $K_{cat}^o$), which have been frequently estimated from the inhibition of carboxylation with only two or three different $O_2$ concentration points. Furthermore, methods for the accurate quantification of Rubisco active sites and for complete Rubisco activation might be optimized for $k_{cat}^c$ determination, especially for species phylogenetically distant from vascular plants.

**Supplementary data**

Figure S1. Flowcharts of how to normalize the different Rubisco kinetic traits depending on the method used.

Figure S2. Box plot depicting compiled data for the Rubisco kinetic traits, excluding data from Fig. 2 originally measured at a different temperature than 25ºC and subsequently standardized to 25 ºC.

Figure S3. Methods and extract purification effects on Rubisco specificity factor, and the half-saturation constant for $CO_2$, respectively, from original (not corrected) values.

Data S1. Compiled and corrected Rubisco kinetic trait values from model species, including relevant information regarding the assay methods and the correction procedures.

Data S2. Excel tool explanation for vial volume correction calculator for considering $CO_2$ outgassing effects in *in vitro* Rubisco assays using bicarbonate as $CO_2$ source.

Table S1. Examples of acidity constant of dissolved $CO_2$ values for different temperatures and solution ionic strengths used for the Rubisco kinetic traits corrections.

Table S2. Examples of the Henry's law constants for $CO_2$ and $O_2$ values for different temperatures and solute concentrations used for the Rubisco kinetic traits corrections.

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## Authors contributions

JG and UN conceptualized the study. JG, UN, KM and CI compiled and standardized the data from previous studies. CI, JG, UN and KM carried out the analyses and produced the figures. CI wrote most parts of the manuscript with the contribution of all co-authors. All authors read and approved the final manuscript.

## Data Availability Statement

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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**Table 1.** Description of the different methods for the Rubisco specificity factor ($S_{c/o}$) determination, including their main advantages and disadvantages.

| **Method description** | **Carboxylation determination** | **Oxygenation determination** | **Advantages** | **Disadvantages** | **References** |
|---|---|---|---|---|---|
| $O_2$ electrode | Total RuBP consumed during incubation minus $O_2$ consumed | $O_2$ consumption by a Clark electrode | No radioactivity, simplicity | Closed system, low sensitivity, requirement for highly purified Rubisco, uncertainty about carboxylase reaction | Parry *et al*., 1989 |
| [1-$^{14}$C]RuBP or [1-$^{3}$H]RuBP incubation + Liquid Chromatography | Chromatographic resolution of (3-phospho)glycerate | Chromatographic resolution of (2-phospho)glycolate | Open gas-purging system with a certain $CO_2$ and $O_2$ concentration, high reproducibility | Use of radioactivity, complex method | Kane *et al*., 1994 |
| $H^{14}CO_3^-$ incubation | $^{14}$C fixation into 3-phosphoglycerate | Total RuBP consumed during incubation minus $^{14}$C fixed into 3-phosphoglycerate | No need for complex apparatus | Closed system, use of radioactivity, uncertainty about oxygenase reaction | Lee *et al*., 1993; Kostov and McFadden, 1995 |
| Nuclear magnetic resonance (NMR) spectroscopy | 3-Phosphoglycerate quantification by NMR spectroscopy | 2-Phosphoglycolate quantification by NMR spectroscopy | Open gas-purging system with a certain $CO_2$ and $O_2$ concentration, possibility of continuous monitoring of the simultaneous reactions | Complex apparatus, interference of phosphatases in the protein extraction, corrections needed for signal losses associated to the use of $H_2O$ as solvent | Wang *et al*., 1998 |

| | | | | | |
|---|---|---|---|---|---|
| Membrane inlet mass spectrometry (MIMS) | Continuous monitoring of $CO_2$ concentration by MIMS | Continuous monitoring of $O_2$ concentration by MIMS | Simultaneous and continuous monitoring of $CO_2$ and $O_2$ concentrations, all Rubisco kinetics can be measured at one time | Very complex method, time-consuming measurements, closed system with need for correction of membrane isotopic discrimination, low reproducibility | Cousins *et al*., 2010 |
| $NaH^{13}CO_3$ incubation + gas chromatography/ mass spectrometry (GC/MS) | Mass-spectrometric analysis of [3-$^{13}$C]glycerate | Calculated from the $^{13}$C enrichment of glycerate | No effect of further metabolism of the reaction products by other enzymes in the extract | Closed system, low sensitivity at low oxygenation rates | Whitney and Andrews, 1998 (modified from Zhu *et al*., 1992) |

**Table 2.** Description of the different methods for Rubisco carboxylase activity ($V_{c,max}$ and $K_c$ determination), including their main advantages and disadvantages.

| **Method description** | **Advantages** | **Disadvantages** | **References** |
|---|---|---|---|
| $H^{14}CO_3^-$ assay | Extremely sensitive and reproducible, direct measurement of product formation | Discrete measurement, generation of small amounts of $^{14}CO_2$ and radioactive waste | Paulsen and Lane, 1966; McFadden and Tu, 1967 |
| Spectrophotometric NADH-coupled assay | Non-radioactive, real-time measurement of carboxylation | Indirect measurement, need for coupling enzymatic reactions | Lilley and Walker, 1974; Sharwood *et al*., 2016 |
| MIMS | Real-time simultaneous measurement of $CO_2$ and $O_2$ consumptions, all Rubisco kinetics can be measured simultaneously | Very complex method, long measurements, isotopic discrimination of the membrane, low reproducibility | Cousins *et al*., 2010 |

**Table 3.** Description of the different methods for Rubisco quantification used for the carboxylation turnover rate ($k_{cat}^{c}$) determination, including their main advantages and disadvantages.

| **Method description** | **Advantages** | **Disadvantages** | **References** |
|---|---|---|---|
| CABP binding | Extremely sensitive and reproducible, quantitative measure of all Rubisco catalytic sites | Relatively time-consuming protocol, generation of radioactive waste, possibility of some unspecific binding in crude extracts | Collatz *et al*., 1979; Yokota and Canvin, 1985; Ruuska *et al.,* 1998 |
| Electrophoresis or immunodetection | More sensitive and specific than spectrophotometric determinations of purified Rubisco | Relatively time-consuming protocol, quantification includes non-active sites or other proteins with similar weight, the dynamic range for quantifying is very limited, needs conversion with Rubisco molecular weight | Campbell *et al*., 1988; Lauerer *et al*., 1993; Spreitzer *et al*., 1995; Satagopan and Spreitzer, 2008; Whitney and Sharwood, 2014 |
| Spectrophotometric determination of proteins | Very simple and fast protocols | Requires almost 100% purified Rubiscos, low specificity and sensitivity, needs conversion with Rubisco molecular weight | Warburg and Christian, 1941; Lowry *et al*., 1951; Bradford, 1976; Smith *et al*., 1985 |

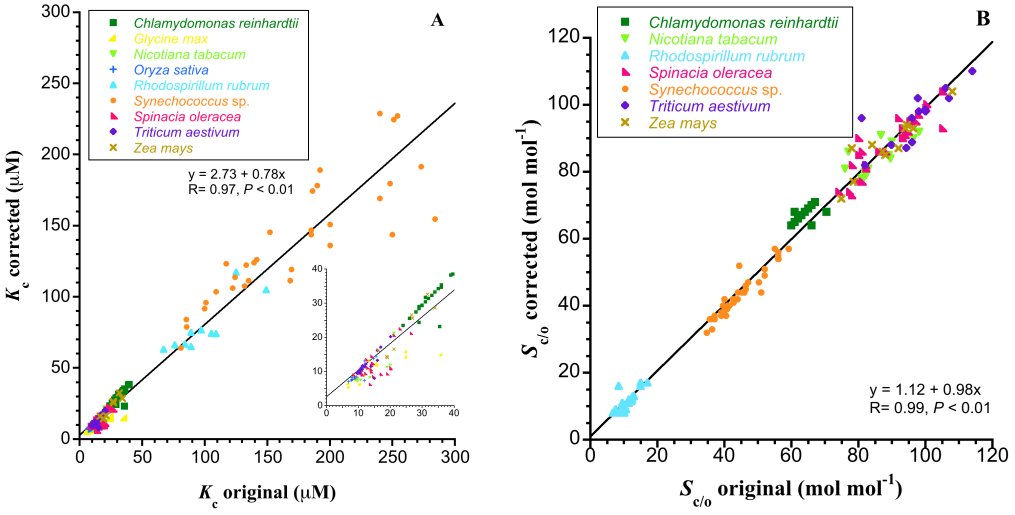


**Figure 1.** Correlations between original and corrected values (corrections for p$K_{a1,CO2}$, $CO_2$ and $O_2$ solubility, volume and humidity, as explained in the materials and methods section) for (A) the half-saturation constant for $CO_2$ ($K_c$) and (B) the $CO_2/O_2$ specificity factor ($S_{c/o}$) at 25ºC in characteristic model species with multiple assessments of Rubisco kinetic traits.

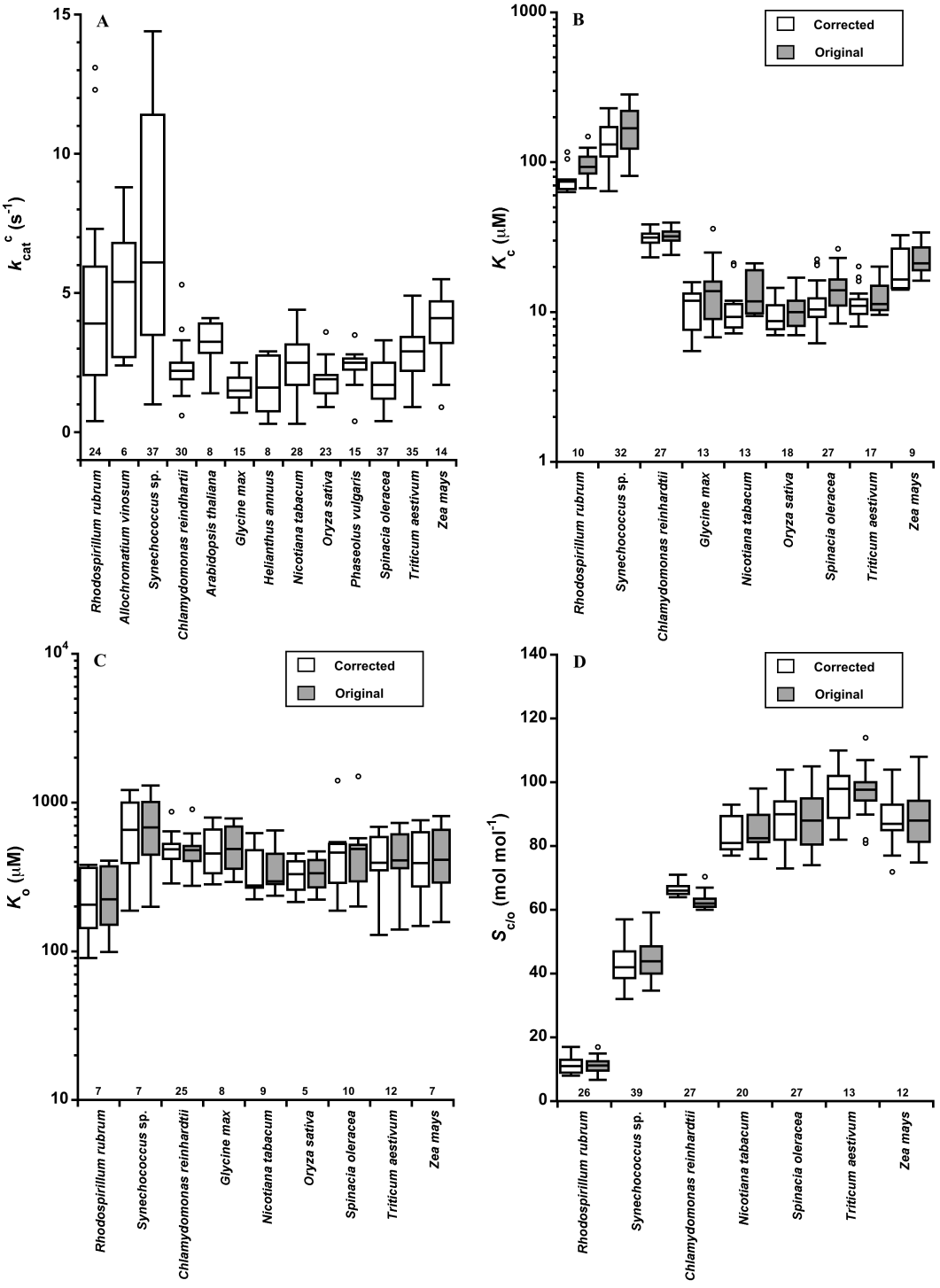


**Figure 2.** Box plot depicting compiled data for (A) the Rubisco carboxylase turnover rate ($k_{cat}^{c}$), (B) the half-saturation constant for $CO_2$ ($K_c$) and (C) for $O_2$ ($K_o$), and (D) the $CO_2/O_2$ specificity factor ($S_{c/o}$) at 25 ºC in characteristic model species with multiple assessments of Rubisco kinetic traits. For $K_c$, $K_o$ and $S_{c/o}$, original (grey) and corrected (white) values for $pK_{a1,CO2}$, $CO_2$ and $O_2$ solubility, volume and humidity, as explained in the materials and methods section, are shown for each species. For each plot, the

horizontal line represents the median, the box and whiskers represent the 25th to 75th percentile and the minimum to maximum distributions of the data, respectively, and any value outside this range is displayed as an individual point. The total number of measurements for each species (*n*) is indicated under the individual boxes.

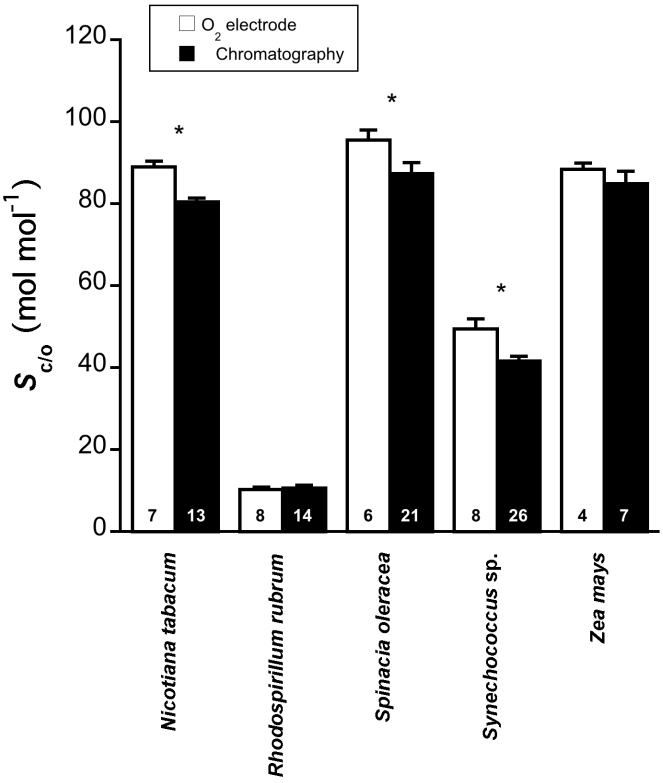


**Figure 3.** Comparison of corrected ($pK_{a1,CO2}$, $CO_2$ and $O_2$ solubility) values of Rubisco specificity factor ($S_{c/o}$) at 25ºC estimated by two different methods ($O_2$ electrode vs. chromatographic resolution of products) in model species with multiple estimates by different studies. Bars and error bars represent the mean ± standard error (*n* is indicated at the bottom of each bar). Statistically significant differences between treatments for each species are indicated by an asterisk ($P < 0.05$).

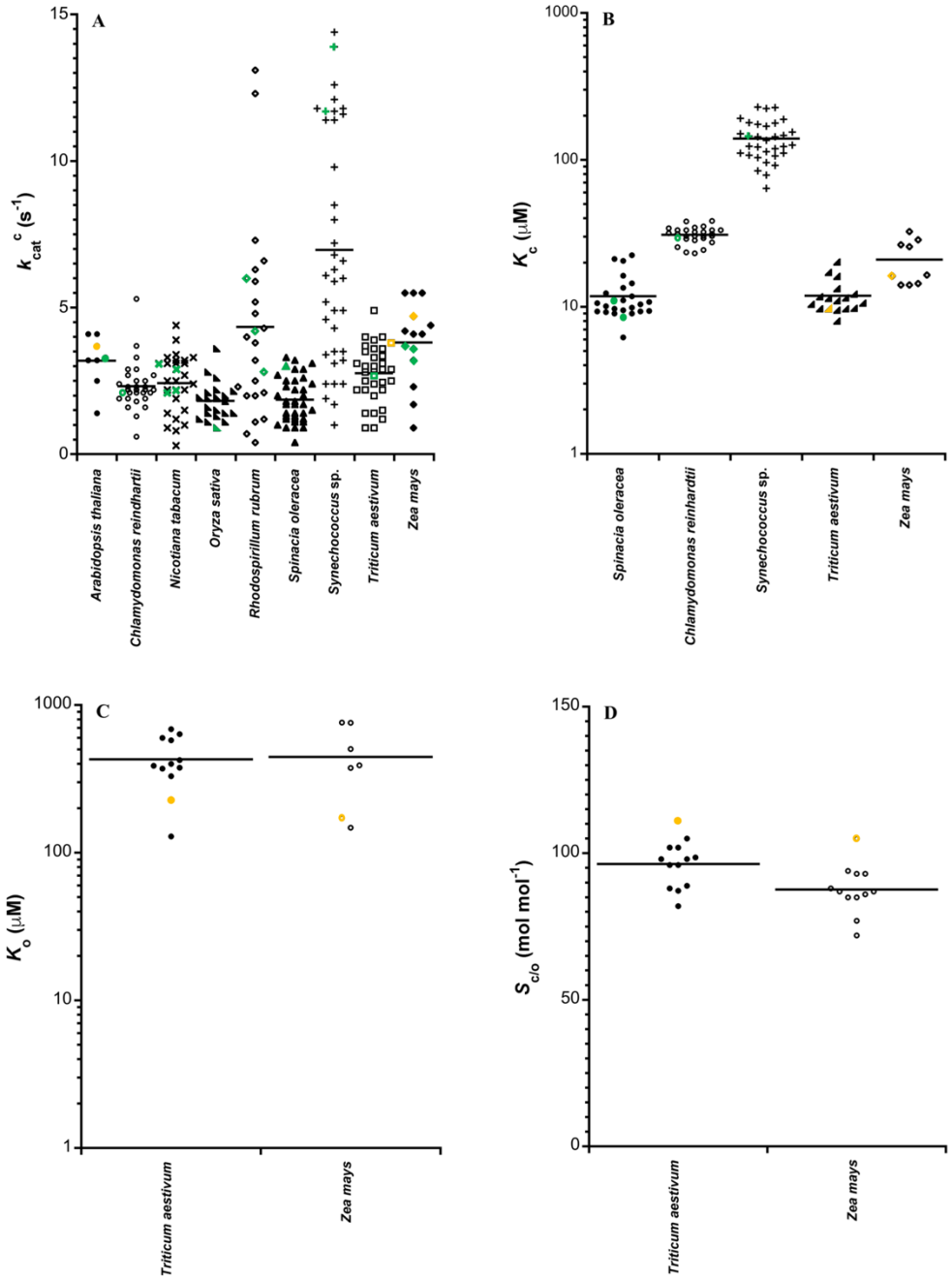


**Figure 4.** Scatterplots of (A) the Rubisco carboxylase turnover rate ($k_{cat}^{c}$), (B) the half-saturation constant for $CO_2$ ($K_c$) and (C) for $O_2$ ($K_o$), and (D) the $CO_2/O_2$ specificity factor ($S_{c/o}$) values at 25ºC. Symbols in black correspond to estimates by the radioactive assay, symbols in green correspond to estimates by the spectrophotometric assay and symbols in orange correspond to estimates by the MIMS assay. Black lines represent the mean value for each species.

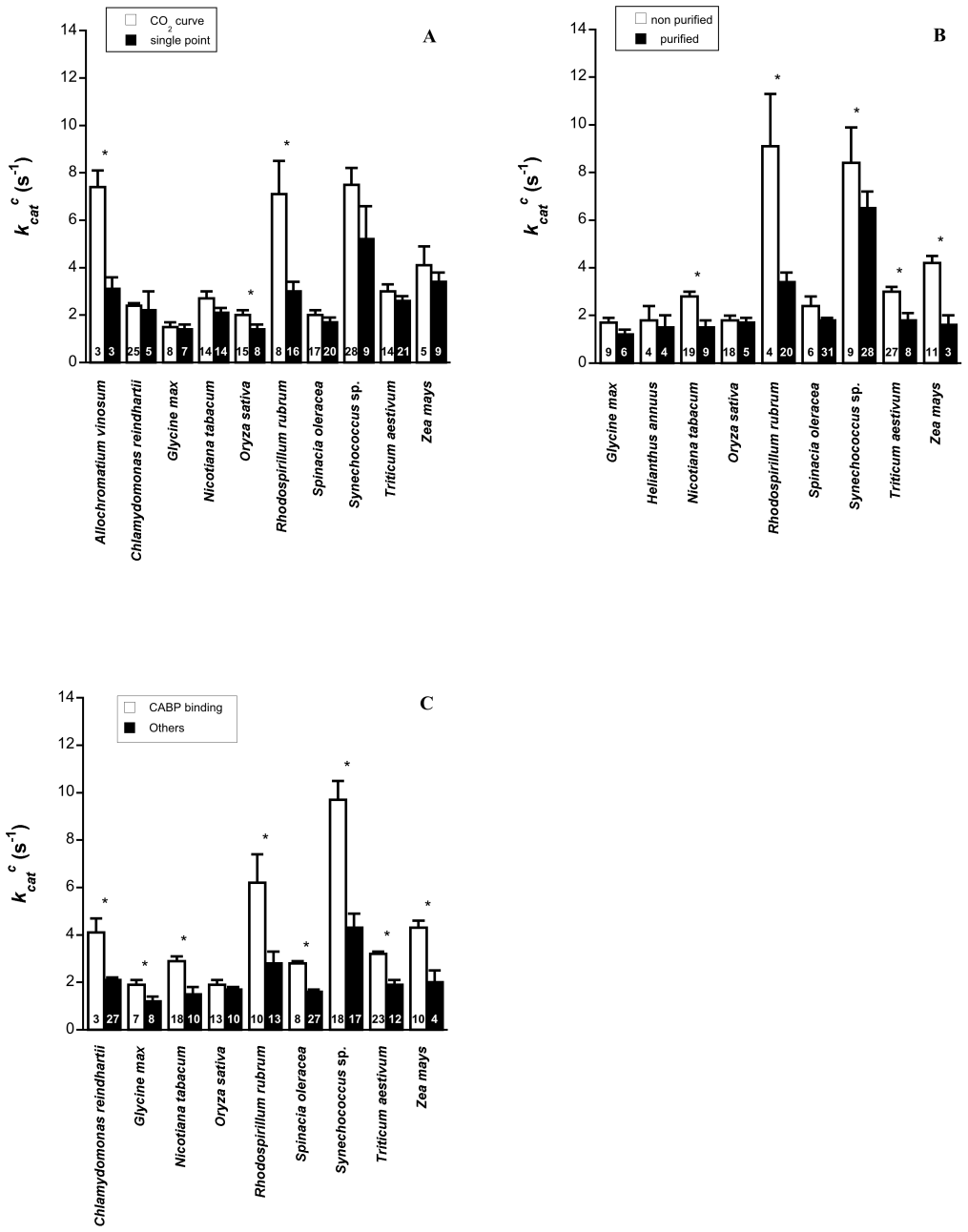


**Figure 5.** Effect of (A) single point vs. Michaelis-Menten curve, (B) enzyme purification and (C) Rubisco quantification method on the carboxylation turnover rate ($k_{cat}^{c}$) determination at 25ºC. Bars and error bars represent the mean ± standard error (*n* is indicated at the bottom of each bar). Statistically significant differences between treatments for each species are indicated by an asterisk ($P < 0.05$).

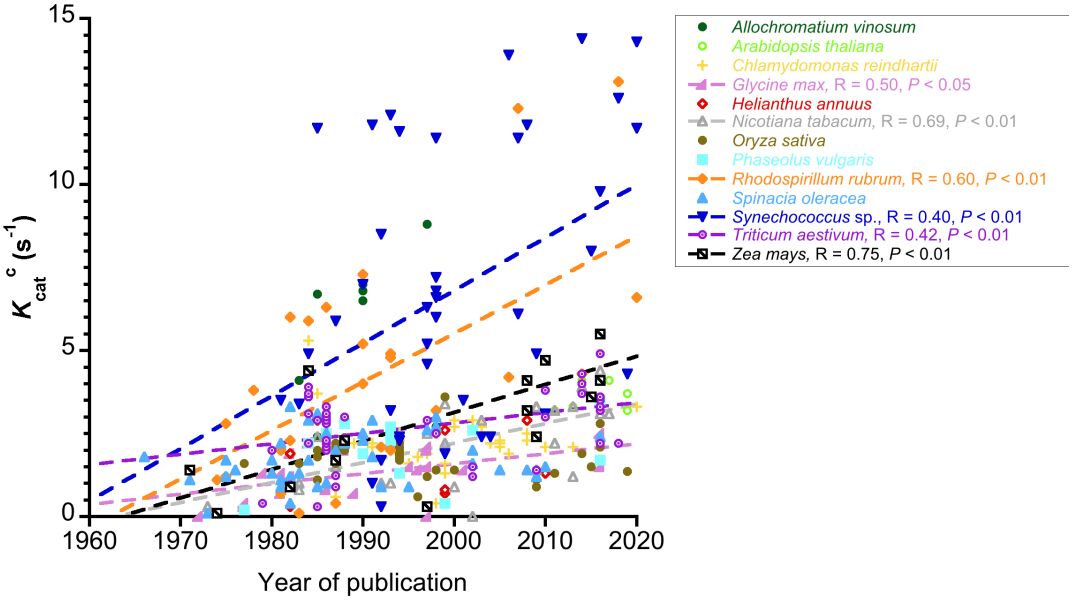


**Figure 6.** Correlations between the year of publication of each study and Rubisco carboxylation turnover rate ($k_{cat}^{c}$) at 25ºC in characteristic model species with multiple assessments of Rubisco kinetic traits. Statistically significant correlations for each species are shown ($P < 0.05$).

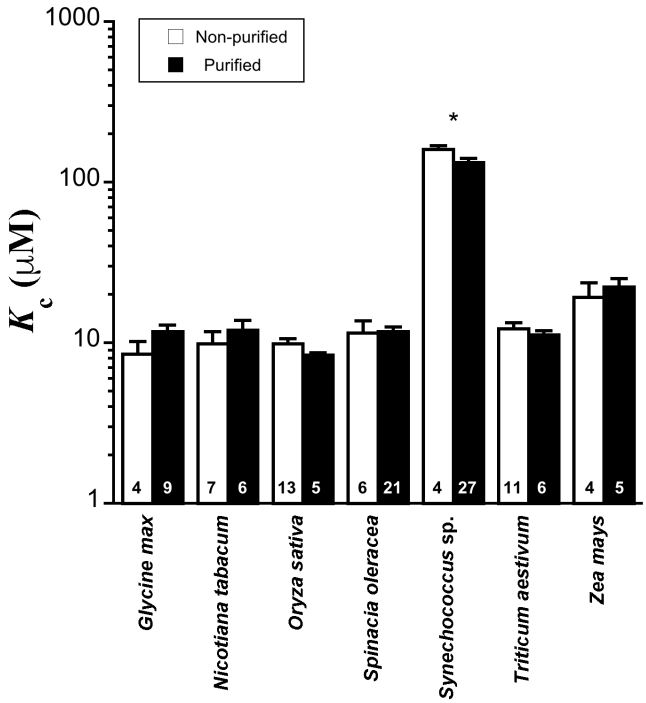


**Figure 7.** Effect of Rubisco purification on the half-saturation constant for $CO_2$ ($K_c$) determination. Bars and error bars represent the mean ± standard error (*n* is indicated at the bottom of each bar) of corrected ($pK_{a1,CO2}$ and $CO_2$ solubility) values at 25ºC. Statistically significant differences between treatments for each species are indicated by an asterisk ($P < 0.05$).

**Analyzing the causes of method-to-method variability among Rubisco kinetic traits: from the first to the current measurements**

Concepción Iñiguez[1,2*], Ülo Niinemets[3,4], Kristiina Mark[3], Jeroni Galmés[1]

## Supplementary data

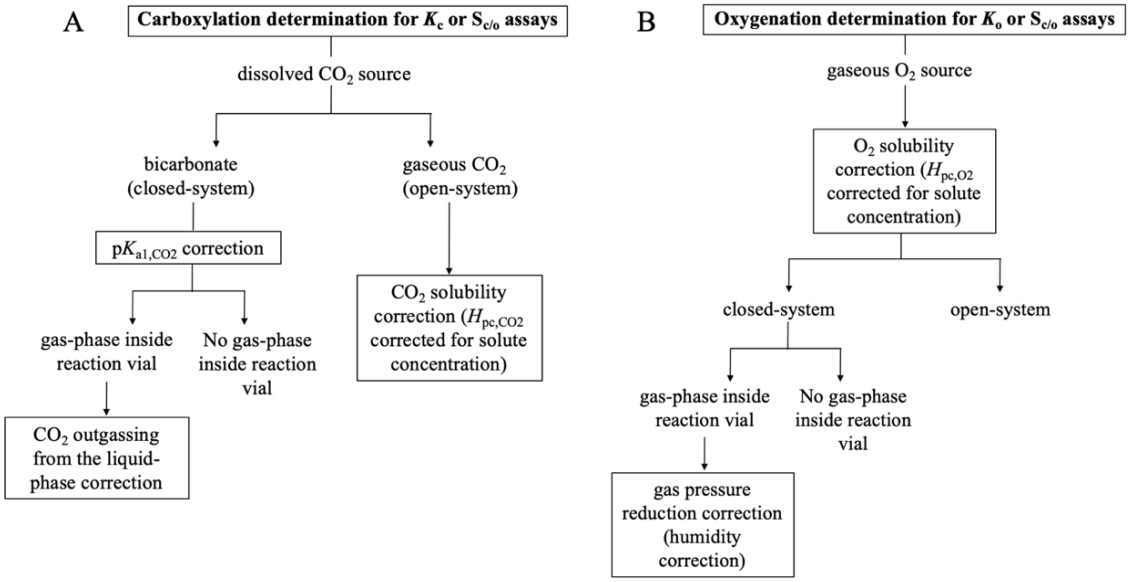


**Figure S1.** Flowcharts of how to normalize the different Rubisco kinetic traits depending on the method used. $K_c$, the half-saturation constant for $CO_2$; $K_o$, the half-saturation constant for $O_2$; $S_{c/o}$, the $CO_2/O_2$ specificity factor.

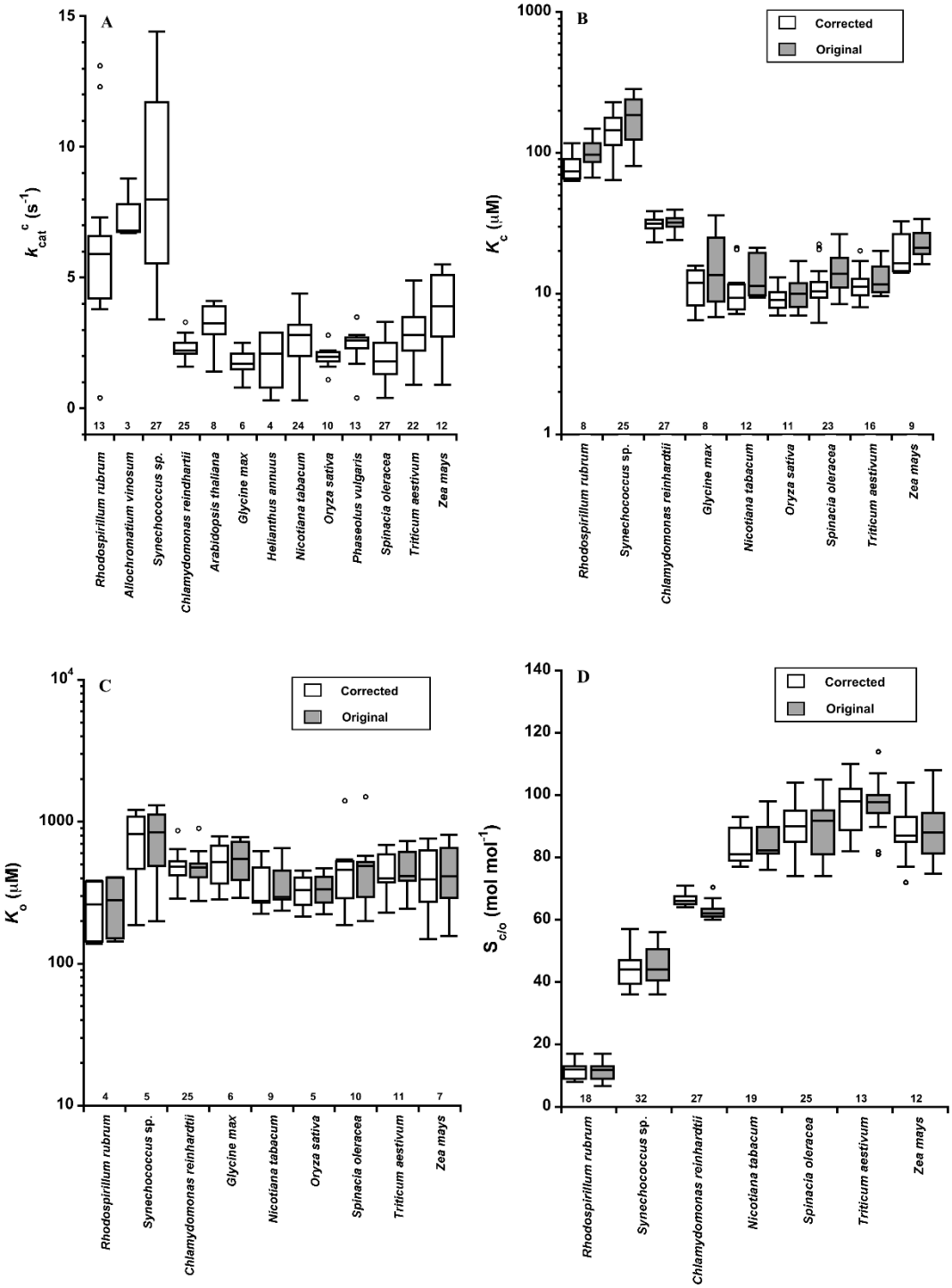


**Figure S2**. Box plot depicting compiled data for (A) the Rubisco carboxylase turnover rate ($k_{cat}^{c}$), (B) the half-saturation constant for $CO_2$ ($K_c$) and (C) for $O_2$ ($K_o$), and (D) the $CO_2/O_2$ specificity factor ($S_{c/o}$), including only those data originally measured at 25 ºC (therefore excluding data measured at a different temperature in the range of 20-30 ºC and subsequently standardized to 25 ºC, which were included in Fig. 2), in characteristic model species with multiple assessments of Rubisco kinetic traits.

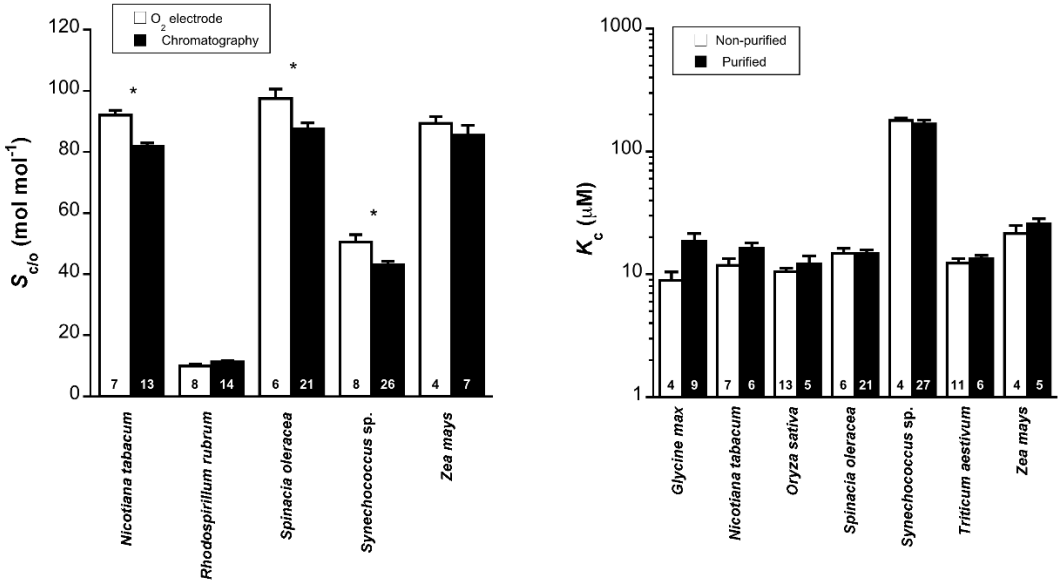


**Figure S3**. Comparison of original (not corrected) values of (A) Rubisco specificity factor ($S_{c/o}$), estimated by two different methods ($O_2$ electrode vs. chromatographic resolution of products), and (B) the half-saturation constant for $CO_2$ ($K_c$) in purified vs. non-purified extracts, at 25ºC in the model species.

**Table S1.** Examples of acidity constant of dissolved $CO_2$ ($pK_{a1,CO2}$) values for different temperatures (ºC) and solution ionic strengths (M) used for the Rubisco kinetic traits corrections. $pK_{a1,CO2}$ was calculated using equation 2 of Galmés *et al*. (2016).

| **Temperature (ºC)** | **Ionic strength (M)** | **$pK_{a1,CO2}$** |
|---|---|---|
| 20 | 0 | 6.39 |
| | 0.05 | 6.22 |
| | 0.1 | 6.16 |
| | 0.2 | 6.09 |
| | 0.3 | 6.05 |
| 25 | 0 | 6.36 |
| | 0.05 | 6.19 |
| | 0.1 | 6.13 |
| | 0.2 | 6.06 |
| | 0.3 | 6.02 |
| 30 | 0 | 6.33 |
| | 0.05 | 6.16 |
| | 0.1 | 6.10 |
| | 0.2 | 6.03 |
| | 0.3 | 5.99 |

**Table S2.** Examples of the Henry's law constants for $CO_2$ and $O_2$ ($H_{pc,CO2}$ and $H_{pc,O2}$; Pa $m^3$ $mol^{-1}$) values for different temperatures (ºC) and solute concentrations (M, NaCl equivalents) used for the Rubisco kinetic traits corrections. $H_{pc,CO2}$ and $H_{pc,O2}$ were calculated using equation 5 of Galmés *et al*. (2016).

| **Temperature (ºC)** | **Solute concentration (M, NaCl equivalents)** | **$H_{pc,CO2}$ (Pa $m^3$ $mol^{-1}$)** | **$H_{pc,O2}$ (Pa $m^3$ $mol^{-1}$)** |
|---|---|---|---|
| 20 | 0 | 2569 | 74719 |
| | 0.01 | 2573 | 74884 |
| | 0.05 | 2592 | 75542 |
| | 0.1 | 2615 | 76366 |
| 25 | 0 | 2982 | 82082 |
| | 0.01 | 2987 | 82251 |
| | 0.05 | 3009 | 82929 |
| | 0.1 | 3036 | 83780 |
| 30 | 0 | 3417 | 89639 |
| | 0.01 | 3423 | 89813 |
| | 0.05 | 3448 | 90507 |
| | 0.1 | 3479 | 91376 |

**Data S1.** Excel sheet with compiled Rubisco kinetic traits ($S_{c/o}$, $k_{cat}^{c}$, $K_c$, $K_o$) of the model species from previously published studies, including relevant information regarding the assay methods and the correction procedures, according to the 'material and methods' section, to obtain the final normalized values. n.a., not available; n.n., not needed. (Download Data S1 excel file at JXB online).

**Data S2.** Excel tool for vial volume correction calculator for considering $CO_2$ outgassing effects in *in vitro* Rubisco assays using bicarbonate as $CO_2$ source (Download Data S2 excel file at JXB online).

## Tool Explanation: Vial volume correction calculator for $CO_2$ outgassing effects in *in vitro* Rubisco assays using bicarbonate

### 1. Introduction

In *in vitro* studies on Rubisco kinetic traits using radioactive bicarbonate (typically $NaHCO_3$) as $CO_2$ source in a closed system, dissolved $CO_2$ concentration for the assay is calculated from bicarbonate aqueous equilibrium based on the first acid dissociation constant for carbonic acid (reported typically as the negative log, $pKa_1$). The Rubisco assay is typically carried out in vials including both the liquid phase (assay medium) and gas phase (headspace). Thus, a part of the dissolved $CO_2$ gasses out into the headspace until a new equilibrium is reached. The fraction of dissolved $CO_2$ gassing out into the headspace is the greater the larger is the gas phase volume relative to the total vial volume and the lower is the pH in the assay medium. Especially, in earlier studies, large vials with a small liquid-phase volume and large gas-phase volume have been used (e.g., Bowes and Ogren, 1972; Bowes *et al.*, 1975; Campbell *et al.*, 1988; Houtz *et al.*, 1985), but outgassing of $CO_2$ was frequently not considered for the calculation of dissolved $CO_2$ concentration in the assay medium. This is relevant as lack of consideration of outgassing of $CO_2$ results in major biases in estimated Rubisco kinetic traits. In particular, in a vast overestimation of the Michaelis-Menten constant ($K_c$) and underestimation of the specificity factor (Ogren and Hunt, 1978 for a discussion).

### 2. The calculator

The volume correction, $\Delta_v$, is defined here as the ratio of actual dissolved $CO_2$ concentration to the dissolved concentration without considering outgassing of $CO_2$ from the assay buffer. The calculator is provided as an MS Excel file (Supplementary Data S2-Volume correction calculator for Rubisco studies.xlsx) and it estimates $\Delta_v$ based on study-specific input data and corresponding physico-chemical characteristics of bicarbonate/carbonate/$CO_2$ equilibrium using a mass balance approach. The calculation considers both the first $pKa_1$ and second $pKa_2$ (negative logs of acid dissociation constants) of carbonic acid. At typical pH values of 7.5-8.5 used in Rubisco assays, the equilibrium is only weakly affected by the presence of carbonate, and therefore, $pKa_2$ is typically ignored. However, at higher pH values sometimes used in Rubisco assays, carbonate ions also significantly alter the total dissolved $CO_2$ concentration.

The calculator file includes example datasets with different $\Delta_v$ (values ranging from 0.896 to 0.347), and is provided for two situations depending on the magnitude of correction. For small to moderate $\Delta_v$ values (relatively low gas-phase volume, pH typically > 7.5), the $\Delta_v$ is directly calculated after assay-specific input data are entered (Method 1, lines 4-5 for sample data for Hermida-Carrera et al. (2020) and Redja et al. (1981)). In the case of large volume corrections (relatively large gas-phase volume, pH <

7.5), the simple iterative calculation mode does not always converge, and the convergence is obtained by using MS Excel Solver tool (Method 2, sample data in lines 8-10 for Bowes and Ogren (1972), Watson et al. (1999) and Alonso et al. (2009)).

**2.1. Description of the data fields in the volume correction calculator**

The data are grouped in the calculator as:

- *Sample data meta-information (Study and species; columns A and B).*

- *Assay-specific input variables (columns D-I):*
    - assay temperature (°C)
    - pH
    - total assay volume (mL)
    - liquid-phase volume (mL)
    - gas-phase volume (mL, difference between total and liquid-phase volume)
    - initial bicarbonate concentration (mM, can be an arbitrary value as the volume correction does not depend on added bicarbonate concentration).

- *Physico-chemical characteristics for bicarbonate and $CO_2$ equilibrium (columns K-N):*
    - acid dissociation constants characterizing bicarbonate/carbonate/$CO_2$ equilibrium ($pKa_1$ and $pKa_2$)
    - Henry's law constant, $H_{pc}$, expressed as the ratio of gas-phase $CO_2$ partial pressure to dissolved $CO_2$ concentration (Pa $m^3$ $mol^{-1}$)
    - Henry's law constant, $H_{cc}$, expressed as the gas-phase $CO_2$ concentration to dissolved $CO_2$ concentration (mol $mol^{-1}$)

The value of $pKa_1$ is also an input variable that is estimated for each study based on the ionic strength of the assay medium and temperature as explained in Galmés et al. (2016), whereas other equilibrium physico-chemical variables, $pKa_2$ and Henry's law constant, are estimated by the calculator. $H_{pc}$ value is calculated according to Galmés et al. (2016) using a representative value of 0.11 M for solute concentration. For $pKa_2$, the temperature dependency was derived based on Harned and Scholes (1941).

- *Calculated initial equilibrium concentrations of bicarbonate, carbonate, and dissolved $CO_2$ (columns P-T);*

- *Equilibrium amounts of bicarbonate, carbonate, dissolved $CO_2$ and gas-phase $CO_2$ based on mass balance (columns V-AA);*

- *Actual dissolved $CO_2$ concentration and corresponding $CO_2$ partial pressure (columns AC-AD);*

- The volume correction factor, $\Delta_v$ (the ratio of actual dissolved $CO_2$ concentration to the dissolved concentration without considering outgassing of $CO_2$ from the assay buffer (column AF);

- *Calculation fields for use with MS Excel Solver (Method 2, columns AH-AJ).*

**2.2. Use of the volume correction calculator**

The following data of the assay buffer are needed to use in the calculator: assay temperature (°C), pH, total assay volume (mL), liquid-phase volume (mL), bicarbonate concentration (mM) and $pKa_1$ for carbonic acid (estimated as detailed in Galmés et al. (2016)). To use the calculator, the iterative calculations mode should be enabled in MS Excel.

To estimate the volume correction factor for a new dataset, follow these steps:

1) Once the required data are assembled, fill in the input variables in corresponding cells (columns D-G, I, K) after the sample datasets for Method 1 in line 6;
2) For the remaining cells, copy-paste the data (with formulas) from the sample data line (line 5). After the copying, the volume correction for the new dataset is in cell AF6 when the iterative calculations converge (low to moderate values of volume correction).
3) If the solution does not converge (the value in cell AF6 is either <0 or >1), use Method 2.
4) For Method 2, enter an arbitrary initial estimate in cell AH6 (total C in solution) that is less than the value in cell V6 (total C).
5) Copy-paste (with formulas) the content of the cells AI8 and AJ8 into cells AI6 and AJ6.
6) Run the MS Excel Solver with the following conditions: Set objective - AJ6, To – Min, By changing variable cells - AH6. Once the fitting is finished, the value of the cell AJ6 is close to zero, and the volume correction factor corresponding to the new dataset is in cell AF6.
7) To estimate the volume correction for new data again with Method 1, delete the content of the cell AH6 and replace the input data with new values.

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