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Citation	Petersen, Sierra Victoria, and Daniel P. Schrag. 2014. "Clumped Isotope Measurements of Small Carbonate Samples Using a High-Efficiency Dual-Reservoir Technique." <i>Rapid Communications in Mass Spectrometry</i> 28 (21): 2371–2381.
Published Version	doi:10.1002/rcm.7022
Citable link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:14117133
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Clumped isotope measurements of small carbonate samples using a high-efficiency dual-reservoir technique

Sierra V. Petersen, Daniel P. Schrag

RATIONALE:

The measurement of multiply-substituted isotopologues of CO₂ derived from carbonate has allowed for the reconstruction of paleotemperatures from a single phase (CaCO₃), circumventing uncertainty inherent in other isotopic paleothermometers. Traditional analytical techniques require relatively large amounts of carbonate (3-8mg per replicate), which limits the applicability of the clumped isotope proxy to certain geological materials such as marine microfossils, commonly used for paleoclimate reconstructions.

METHODS:

Clumped isotope measurements of small samples were made on a new, high-efficiency, dual-reservoir sample preparation inlet system attached to a Thermo-Finnegan MAT 253 mass spectrometer. Sample gas produced on the inlet is introduced from a 10mL reservoir directly into the source via a capillary. Reference gas fills an identical 10mL reservoir installed between the reference bellows and capillary. Gas pressures in both reservoirs are initially balanced, and are allowed to decrease together over the run.

RESULTS:

Carbonate samples from 1mg to 2.6mg produced Δ_{47} values equivalent to the traditional two-bellows method with identical single-sample precision (1 SE = 0.005-0.015‰) and external standard error (SE = 0.006-0.015‰, n=4-6). The size of sample needed to achieve good precision is controlled by the sensitivity of the mass spectrometer and the size of the fixed reservoirs and adjacent U-trap installed on our inlet.

CONCLUSIONS:

Our demonstration of high-precision clumped isotope measurements of small aliquots of carbonate allows for the application of this proxy to a wider range of geological sample materials, such as marine microfossils, that until now have been near-impossible given sample size limitation.

The measurement of multiply-substituted isotopologues of CO₂ derived from carbonate materials has allowed for the reconstruction of paleotemperatures in a variety of geologic settings. By deriving a temperature from a single phase (CaCO₃), the clumped isotope paleothermometer circumvents the uncertainty inherent to traditional paleothermometers that require information about the isotopic composition of additional phases (H₂O). The carbonate clumped isotope paleothermometer is based on the temperature-dependent ordering of the heavy isotopes ¹³C and ¹⁸O within the carbonate lattice.^[1] At colder temperatures, these heavy isotopes “clump” to produce mass-63 CaCO₃ (Ca¹³C¹⁸O¹⁶O₂) at a level above that expected by a random (stochastic) distribution of these isotopes. When the carbonate is converted to CO₂ via acid digestion, this mass-63 anomaly manifests itself as excess mass-47 CO₂, denoted by the quantity Δ_{47} (see Eq.

1).^[1] A Δ_{47} value of zero indicates a fully random distribution of isotopes. Over normal Earth surface temperatures, Δ_{47} varies between about 0.55 and 0.8 in the absolute reference frame.^[2] Eqn. (1) defines Δ_{47} where the $R^x = {}^x\text{CO}_2/{}^{44}\text{CO}_2$ and R^{x*} is the corresponding stochastic distribution ratio.

$$\Delta_{47} = [(R^{47}/R^{47*} - 1) - (R^{46}/R^{46*} - 1) - (R^{45}/R^{45*} - 1)] \times 1000 \quad (1)$$

The temperature dependence of Δ_{47} has been demonstrated for a variety of carbonate materials including synthetic, biogenic, and inorganic carbonates^[1, 3, 4], as well as by theoretical calculations.^[5, 6] Although this is still a new proxy, researchers have already used it to determine paleo-altitude of growing mountains^[7], reconstruct hydrological and ecological conditions in Africa during the time of early humans^[8], and measure the body temperature of dinosaurs^[9], along with many other applications.

Traditional mass spectrometry practices used to measure stable isotopes ($\delta^{18}\text{O}$ and $\delta^{13}\text{C}$) require micrograms of carbonate material for a single measurement. This facilitates the creation of high-resolution records made up of many measurements of small (<1mg) carbonate samples such as foraminifera. In comparison, the clumped isotope technique requires 5-15mg of carbonate per replicate^[3], limiting the possible applications of this proxy. In a few cases, this sample size limit has been reduced. *Zaarur et al.*^[10] adjusted the capillary cross section and flow rate and installed a new bellows potentiometer, and were able to measure samples of 3-4mg per replicate. Using a Kiel device to measure tiny aliquots of carbonate (0.2mg) for a few minutes each and averaging the data from 6-10 aliquots (equivalent to 1.2-2mg), *Schmid and Bernasconi*^[11] were able to calculate a Δ_{47} value with a precision of 0.015-0.040‰ (1 SE), while at the same time producing a high-resolution stable isotope record. By averaging together 5-13 of these runs (equivalent to 6-26mg), they were able to achieve an external precision of 0.005-0.010‰ (1 SE). The total mass of carbonate required for this is similar to ~4 replicates at 3-4mg (equivalent to 12-16mg), and both methods are an improvement over traditional sample requirements (>24mg for 3 replicates).^[3] *Meckler et al.*^[12] showed that with additional corrections an external error of 0.007-0.009‰ (1 SE) could be achieved with 4.5-6mg of total sample.

The clumped isotope proxy has the potential to be very useful in the field of paleoceanography due to its ability to separate the influences of temperature and the isotopic composition of seawater on $\delta^{18}\text{O}$ of marine carbonates.^[1] Foraminifera, a commonly used sample material for paleoceanographic studies, have been shown to follow the same temperature- Δ_{47} relationship as other biogenic carbonates.^[13, 14] However, current analytical techniques make it difficult to acquire enough sample material for replicate analysis of foraminifera due to their small size. A few studies have successfully measured the clumped isotope composition of foraminifera using the traditional large-sample methods^[13, 15] and the Kiel device method described above.^[14] New methods to reduce sample size requirements will make this proxy more widely accessible as a tool for paleoceanography.

Here we present a new method of measuring individual aliquots as small as 1 mg. A high-efficiency dual-reservoir inlet system allows analysis of smaller samples by reducing the “wasted” gas left remaining in the bellows and the sample vial. Gas pressures decrease slowly from a fixed-volume sample reservoir and from an identical reference reservoir installed between the reference bellows and the change-over block. In

this study we test the sample size limit of this inlet configuration by measuring carbonate standards from 1.0mg to 2.6mg and demonstrate internal precision of 0.005-0.015‰ (1 SE) and external standard error of 0.006-0.015‰ (1 SE) for 4-6 replicates, in line with the traditional dual-bellows configuration (1 SE = 0.002-0.018‰).^[4, 10, 16,17]

EXPERIMENTAL:

Samples and standards

To calibrate the newly constructed sample-preparation inlet, carbonate standards of known composition were measured repeatedly at a range of sizes. Two high-temperature carbonates (CM2, NBS19) with similar Δ_{47} values, but different $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$, were measured. One low-temperature coral (RTG) with a higher Δ_{47} value was measured for comparison. All isotopic compositions are reported relative to V-PDB. All errors on Δ_{47} reported in this section are external standard errors (1 SE) calculated on many measurements, with the number of measurements in parentheses.

- CM2 – An in-house Carrara Marble standard with isotopic composition $\delta^{13}\text{C} = 2.29\text{‰}$ and $\delta^{18}\text{O} = -1.77\text{‰}$.^[18] Previous analysis of this standard at Harvard using the traditional two-bellows method and large (8 mg) sample sizes yielded a Δ_{47} value of $0.385 \pm 0.005\text{‰}$ (n=40)^[2] in the absolute reference frame.^[2]
- RTG – A coral specimen from Raratonga, used as a cooler-temperature in-house standard, with isotopic composition $\delta^{13}\text{C} = -2.20\text{‰}$ and $\delta^{18}\text{O} = -4.11\text{‰}$.^[18] Limited previous analyses of this standard in the two-bellows configuration produced a Δ_{47} value of $0.720 \pm 0.007\text{‰}$ (n=11).^[18]
- NBS-19 – An IAEA Carrara Marble standard with isotopic composition $\delta^{13}\text{C} = 1.95\text{‰}$ and $\delta^{18}\text{O} = -2.20\text{‰}$. Previous analyses at Harvard produced a Δ_{47} value of $0.373 \pm 0.007\text{‰}$ (n=7)^[2] in the absolute reference frame.^[2] Other labs found similar values ($\Delta_{47} = 0.399 \pm 0.005\text{‰}$ (n=12) at Johns Hopkins,^[2] $0.404 \pm 0.006\text{‰}$ (n=40) at Yale,^[2] and $0.373 \pm 0.004\text{‰}$ (n=20) and $0.359 \pm 0.004\text{‰}$ (n=19) at 25°C and 90°C at Goethe-University^[16]).

To correct the raw Δ_{47} data to the absolute reference frame^[2], heated and equilibrated gases were measured through the same sample-preparation inlet in between carbonate sample runs. A large number of heated and equilibrated gases (10-15) were run at the beginning of each measurement period to establish the calibration lines. During the measurement period, a gas standard was run every 1-2 days (every 3-8 samples). To prepare heated gases, aliquots of four gases (2 tank gases and reacted CM2 and RTG) of distinct composition were transferred into quartz tubes, which were heated to 1000°C for 2 hours. This procedure randomizes the isotope distribution to produce a near-stochastic arrangement, which we measure to correct for a number of mass spectrometer source effects.^[19] To prepare equilibrated gases, aliquots of the same four gases were transferred into Pyrex tubes containing ~1mL of deionized water. The tubes were placed in water baths held at 10°C and 35°C and allowed to equilibrate over a minimum of 2 days. Each

tube is removed immediately before analysis and the gas is extracted within minutes, before the CO₂ can equilibrate to room temperature (see description below).

Sample Preparation

Samples were prepared through a newly constructed high-efficiency dual-reservoir sample preparation inlet (Fig. 1). This inlet follows the procedure outlined by *Dennis & Schrag*^[4] for creating and cleaning CO₂. Gas is introduced into the inlet in two different ways. For carbonate samples and standards, CO₂ is created by reaction with anhydrous phosphoric acid held at 90°C in a common acid bath and is continuously frozen into a large U-trap submerged in liquid nitrogen (LN₂). Reaction time is 6 minutes, and extends well beyond the point when visible bubble formation ceases. On its way to the U-trap, the gas passes through a trap held at -80°C to remove any trace amounts of water. For gas standards (heated gases and equilibrated gases), CO₂ is introduced into the inlet via a cracker. As with reacted carbonate samples, the CO₂ passes through a -80°C trap to remove water and is frozen into the large U-trap on the far side. For equilibrated gases, within 2-3 minutes of being removed from the water bath (10°C or 35°C), the base of the Pyrex tube is submerged in LN₂, freezing both the water and CO₂ and inhibiting the equilibration of the CO₂ with water at room temperature. LN₂ is then replaced by a -80°C trap before the cracking step to release the CO₂ while the water stays frozen, and is kept at that temperature while the CO₂ is transferred to the U-trap. This whole process takes less than 10-15 minutes and the exposure of CO₂ to water at room temperature is short enough to avoid significant re-equilibration observed in other experiments.^[20] For all sample types, once the freezing step is complete, the CO₂ is allowed to warm up to room temperature within the large U-trap and the volume of gas created or transferred is roughly determined by an analog pressure gauge.

To remove trace contaminants, the gas is then passed through a Pyrex U-trap (outer diameter ½”) packed with Porapak Q (PPQ) material held at -10 to -12°C by immersion in cooled ethanol. Gas is frozen on the far side into a small U-trap (outer diameter = ¼”, internal volume ~6mL) immersed in LN₂. During this step, the pressure gauge on the large U-trap gradually decreases, demonstrating that the gas is leaving the large U-trap. When the pressure gauge nears baseline and stops decreasing (~4-7 minutes depending on sample size), this step is deemed complete. The small U-trap is closed off and the clean CO₂ is allowed to warm up to room temperature. Finally, the gas is expanded from the small U-trap into the 10mL sample reservoir and allowed to equilibrate for 3 minutes. This completes the sample preparation procedure, which in total takes ~30-40 minutes per sample. While one sample is being analyzed on the mass spectrometer, and before the next sample is processed, the PPQ trap is baked for 20-35 minutes at ~150°C to remove any collected contaminants.

Mass spectrometry

While the CO₂ is equilibrating between the small U-trap and the 10mL sample reservoir, the gas is introduced directly from the reservoir into the source. A more precise determination of yield can be estimated at this point (compared to the rough estimation from the inlet pressure gauge) using the initial beam intensity and the pressure reading off the MAT 253 vacuum gauge (Fig. 3). Reference gas from the bellows fills an identical 10mL reference reservoir installed between the bellows and the change-over block (Fig.

1, Fig. S1). The reference bellows are manually adjusted until the intensities of the two m/z 47 beams are balanced (on average to within 55 mV, and within 12 mV for samples with initial m/z 47 < 1000mV). The reference and sample reservoirs are then closed off from the bellows and the small U-trap, respectively, so the volume from which gas enters the source is identical on both the sample and reference sides. These reservoirs remain closed for the entire run, unlike in the dual-bellows method, where the reservoirs are replenished at the beginning of each acquisition during the pressure adjustment phase.

In order to perfectly balance these volumes, 87 clean glass beads (3mm diameter, ~1.2mL total volume, Fig. S1) were placed permanently in the sample-side reservoir. These beads are necessary to balance the difference between the internal volumes of the adjacent MAT 253 and inlet valves. The inlet valve has a larger internal volume, so the volume of the sample reservoir needs to be decreased accordingly. The specific number of beads was determined by gradually adding beads to the sample side until the beam intensities decreased at the same rate. Any minor offsets between the initial gas pressures set by manual bellows adjustment are eliminated during the course of the run as the reservoir with the higher gas pressure decreases more quickly and eventually matches the other reservoir closely.

The MAT 253 at Harvard is equipped with 5 Faraday cups with resistors of $3 \times 10^7 \Omega$, $3 \times 10^9 \Omega$, and $1 \times 10^{10} \Omega$ for masses 44 through 46 and $1 \times 10^{12} \Omega$ for masses 47 and 48. The capillaries on the mass spectrometer have been changed from the factory-fitted stainless steel variety to a deactivated fused-silica capillary (~1m in length, 110 μ m inner diameter) to prevent the exchange of CO₂ and H₂O within the capillaries (Fig. S1). One sample run lasts about 2 hours and 20 minutes and is comprised of 7 acquisitions of 14 cycles each, with an integration time of 26 seconds per cycle and an idle time of 12 seconds, equivalent to 2548 seconds of total integration time on each sample. Raw voltage data is processed as outlined by *Huntington et al.*^[19] to get raw Δ_{47} values. Carbonate unknowns are then corrected to the absolute reference frame using the heated and equilibrated gas data^[2], followed by an additional Δ_{48} correction described below.

In the traditional two-bellows measurement configuration, beam intensities on both the sample and reference side are set to a target value at the beginning of each acquisition (e.g. m/z 47 = 2V or 8V for the Harvard instrument^[4,18]). Over the course of one acquisition, the sample and reference beam intensities decrease somewhat (the amount depends on how many cycles per acquisition and the starting beam intensity), but are returned to the target value at the start of the next acquisition by compressing both the bellows (Fig. 2). In this set up, all of the cycles are performed at, or closely below, the target voltage (Fig. 2). By staying near the target voltage, issues of nonlinearities in the source are avoided, and there is no risk of gas fractionating as it decreases to a very low pressure. A 10mL reservoir was installed between the bellows and the capillary on both the reference and sample side of the mass spectrometer to increase the volume from which the gas enters the source (Fig. S1), therefore reducing the rate at which the gas pressures (and beam intensities) decrease.^[4] At m/z 47 = 2V, $\Delta_{47\text{-raw}}$ can be measured to a precision of 0.005-0.010‰ (1 SE) for a single sample run^[4], reflecting beam stability and shot noise limits.^[21]

In our dual-reservoir configuration, each cycle is measured at progressively lower beam intensity (Fig. 2). Over the course of a 2-hour 20-minute run, the m/z 47 beam intensity decreases by 40-60%, at a rate proportional to the gas pressure in the reservoirs

(Fig. 2). While this gas pressure decreases, the beam intensities on the sample and reference sides remain balanced. The ratios between the beams also remain constant, so the calculated isotope ratios do not show a trend with beam intensity. Despite the beam intensity changing significantly over the run, this configuration can achieve similar precision to the dual-bellows configuration for a single measurement, demonstrating isotope-ratio stability over a large voltage range. For a single sample run in this measurement configuration, $\Delta_{47\text{-raw}}$ can be measured to a precision of 0.005‰ to 0.016‰ (1 SE), depending on the sample size, consistent with the shot noise limit^[21] (see supplementary material for further discussion).

RESULTS

Demonstration of yield at small sample sizes

Sample yield was measured by calculating the increase in pressure recorded by the source vacuum gauge after introducing the sample gas into the source. This yield estimate represents the volume of gas reaching the source per milligram of carbonate reacted, and includes both yield from the acid digestion step and any loss of gas that may have occurred through the cleaning process. The data shows a clear linear relationship between this pressure increase and the mass of carbonate reacted (Fig. 3a), as is expected from the ideal gas law. In June 2013, installation of a smaller U-trap in front of the sample reservoir decreased the volume of “wasted” gas left in the U-trap and increased the pressure in the source for the same mass of carbonate reacted (Fig. 3a). Even at the smallest sample sizes, the linear relationship is maintained, indicating good yield is being achieved. This corroborates the inlet pressure gauge, which indicates that nearly all the gas is transferred away from the large U-trap during the PPQ cleaning step.

Sample size can also be compared with the initial m/z 47 beam intensity (Fig. 3b). For comparison to other instruments, m/z 44 beam intensity is about 5/8 of m/z 47. A source tuning after a power outage in early October 2013 strongly increased the sensitivity (mV beam intensity/mol of gas) of the mass spectrometer, which allowed us to decrease our sample size further, while maintaining the same level of precision (Fig. 3b, Smaller U-trap, tuning 2). This tuning was done using the Isodat autofocus routine. The increase in sensitivity came mainly from a decrease in the Extraction parameter, and corresponded to a significant decrease in the slope of the gas calibration lines (0.009 to 0.006) but minimal changes in the intercepts. After this shift in sensitivity, sample sizes ranging from 1.0mg to 2.5mg produced initial m/z 47 beam intensities between 650mV and 5000mV.

Diagnosing an unknown fractionation

We observe in all our data a strong correlation between Δ_{48} and Δ_{47} , both raw ($\Delta_{47\text{-raw}}$) and fully corrected to the absolute reference frame^[21] ($\Delta_{47\text{-RFAC}}$), with Δ_{47} increasing by $\sim 0.05\%$ for every 1% increase in Δ_{48} (Fig. 4). This relationship is present in carbonate standards and heated gases, and the slope of this relationship is similar among all sample types (Table S1). The slope is also nearly unchanged before and after the correction to the absolute reference frame (Fig. S2, Table S2), and across significant changes in source tuning (Fig. S3, Table S3). We observe no significant correlation

between Δ_{48} and $\delta^{18}\text{O}$ or $\delta^{13}\text{C}$ (Fig. S4), or between sample size and $\delta^{18}\text{O}$ or $\delta^{13}\text{C}$ (Fig. S5). Sample size does seem to influence the magnitude of the fractionation, with the smallest carbonate samples often (but not always) having higher Δ_{48} and Δ_{47} values (Fig. S6). The slope of the fractionation is mildly dependent on the average Δ_{47} value of the sample type, with the slope getting slightly steeper (0.04 to 0.08) as the degree of clumping decreases (Table S1).

Previously, high Δ_{48} values were interpreted as contamination by hydrocarbons, chlorocarbons, or sulfur compounds, which produce a mass interference on mass-47 and mass-48.^[22] Conventionally, samples should plot within the envelope of calibration gas points in δ^{48} vs. Δ_{48} space. Points outside of this range are thought to be contaminated and would be thrown out in a typical study.^[19] In our data, we observe Δ_{48} values both within and outside of the envelope of calibration gas data (ex. Fig 5), for samples we consider to be clean, such as reference gas run through the inlet (Fig. 4). These high Δ_{48} values have corresponding high Δ_{47} values offset from the known value (zero in the case of reference gas run against itself) and in line with the relationship described above. The result is that we observe a larger range in Δ_{48} for “clean” samples than previously deemed acceptable.

We do not know the cause of this fractionation, but we can rule out some potential explanations. We see the relationship between Δ_{47} and Δ_{48} in heated gases originating from multiple tanks and in the reference gas run as a sample, all of which are pure CO_2 . This suggests our fractionation is not due to sample contamination. The fractionation is also not produced during the acid digestion step because it is observed in standard gases that do not get reacted (and are measured both with and without an acid bath attached to the inlet). Variations in yield (Fig. 3) also do not cause the fractionation because residuals on this yield do not correlate with Δ_{48} (Fig. S7).

One possible explanation is that this fractionation is produced in the PPQ cleaning step. Previous studies measuring extremely small samples (<15 μmol CO_2 equivalent to <1.5mg CaCO_3) observed a fractionation in Δ_{47} associated with the GC cleaning step producing Δ_{47} values up to 0.2‰ enriched compared to larger samples.^[23] This increase in Δ_{47} is on the same order of magnitude as the fractionation observed in our data (Fig. 4). However, the authors of that study observed no correlation between Δ_{47} and either mass-48 or mass-49 excesses.^[23]

We observe different relationships between Δ_{48} and Δ_{47} when gas does or does not pass through the PPQ trap. Reference gas introduced directly into the small U-trap (therefore bypassing the PPQ step) did not show a strong relationship in Δ_{48} vs. Δ_{47} (Fig. S8). However, reference gas frozen directly into the small U-trap (instead of expanded) shows a fairly strong correlation in Δ_{48} vs. Δ_{47} , but with a slope almost twice as steep as when the same gas passed through the whole inlet (Fig. S8). Both the amount of gas frozen into the U-trap and the voltage at which the gas was run did not correlate with the magnitude of the fractionation, although none were as small as our smallest samples (Fig. S9). Replacing the PPQ material did not change the slope (Fig. S3). The duration of baking the PPQ trap before passing a new sample through was observed to have some influence (longer baking = lower Δ_{48}), but it was inconsistent.

The sample size dependence of this fractionation points to a possible influence of the pressure-dependent negative baselines (PBL) observed in many other studies^[24,25,26], which would have the strongest influence on samples run at the lowest signal intensity. We did not measure PBLs in this study, but the correction to the absolute reference frame

implicitly takes the PBL effect into account if reference gases and samples are run at the same voltage. In this study, calibration gases span a range of sizes that generally overlaps with the range of sample sizes measured (Fig. S10). Some of our smallest samples are outside this range. However, looking at a subset of samples and calibration gases run over a narrow voltage range (initial m/z 47 = 3300 – 3800mV), we still observe the same relationship, both before and after correction to the absolute reference frame (Fig. S2, Table S2). This suggests that the size (and running voltage) of the reference and sample gases is not causing the observed fractionation.

Heated gases show a smaller range of fractionation than the carbonate samples (Fig. 4). The volume of CO₂ in each aliquot of heated or equilibrated gas was often larger than the volume of CO₂ produced by a typical carbonate sample (especially compared to our smallest samples) due to the method of preparation of aliquots from the reference tanks. After passing through our sample preparation inlet (described below), heated and equilibrated gases were chopped down to a volume comparable to the carbonate samples so they could be run at similar beam intensities (Fig. S10). This was done by expanding the gas into the T-junction adjacent to the small U-trap (Fig. 1), or by closing off the reservoir, evacuating the small U-trap, and expanding the gas back into the U-trap. The smaller magnitude of fractionation in the heated gases suggests that the volume of gas passing through the PPQ trap may play a role in setting the magnitude of fractionation.

Another possibility is that this fractionation is caused by our dual-reservoir configuration and the way the beam intensities decrease throughout the run. However, Δ_{48} and Δ_{47} show little trend over the 2-hour 20-minute run, and the Δ_{48} , whether low or high, is identifiable in the first few cycles, which are no different than the first few cycles of a traditional two-bellows analysis. Regardless of the cause of the fractionation, it is consistent, well defined, and can be corrected.

Correcting for a fractionation in Δ_{47} and Δ_{48}

The observed relationship between increased Δ_{48} and Δ_{47} causes an undesirable, large scatter in the Δ_{47} of measured carbonate samples and standards. If a “true” Δ_{48} value is known for each sample type (e.g. CM2, RTG), the Δ_{47} data can be simply corrected back to this value along the observed slope. In the past, the “true” (uncontaminated) Δ_{48} value has been determined in relation to the behavior of heated and equilibrated gases in δ^{48} vs. Δ_{48} space.^[19] Heated and equilibrated gases show a positive linear relationship between δ^{48} and Δ_{48} , and replicates of a single carbonate sample form an intersecting line of steeper slope (see Fig. 5a). The deviation between the Δ_{48} of an individual replicate and the line determined by the heated/equilibrated gases is denoted $\Delta(\Delta_{48})$ and is calculated based on Eqn. (2).

$$\Delta(\Delta_{48}) = \Delta_{48} - (\delta^{48} * \text{Slope}_{\text{HG/EG}} + \text{Int}_{\text{HG/EG}}) \quad (2)$$

$\text{Slope}_{\text{HG/EG}}$ and $\text{Int}_{\text{HG/EG}}$ are the slope and intercept of the line fitted to all the heated and equilibrated gas points in δ^{48} vs. Δ_{48} space (Fig. 5a, black points/line). The “true” Δ_{48} value for each carbonate sample type (CM2, RTG, unknown) is then determined to be the value at which $\Delta(\Delta_{48}) = 0$ (i.e. the point of intersection between the sample line and the heated/equilibrated gas line). This can be calculated for each sample type using Eqn. (3).

$$\text{“true” } \Delta_{48} = (\text{Int}_{\text{CARB48}} * \text{Slope}_{\text{HG/EG}} - \text{Int}_{\text{HG/EG}} * \text{Slope}_{\text{CARB48}}) / (\text{Slope}_{\text{HG/EG}} - \text{Slope}_{\text{CARB48}}) \quad (3)$$

$\text{Slope}_{\text{CARB48}}$ and $\text{Int}_{\text{CARB48}}$ are the slope and intercept of the line fitted to all the data of a single carbonate sample type in δ^{48} vs. Δ_{48} space (Fig. 5a, colored points/lines).

$\text{Slope}_{\text{CARB48}}$ and $\text{Int}_{\text{CARB48}}$ can be determined individually for each sample type by fitting a line to all replicates of that sample. The individual δ^{48} vs. Δ_{48} slopes for carbonates agree with each other within error when sample types have enough replicates (Table S5) suggesting all carbonates are following the same slope and the lines are parallel.

Therefore, data sets of all carbonate sample types can be fitted simultaneously for a common slope and individual intercepts. This is the same as the mathematical procedure done to solve for the slope and intercepts of the heated and equilibrated gases in δ^{47} vs. Δ_{47} space when correcting data to the absolute reference frame.^[2] By solving all data sets together, this decreases the influence of any individual outlying point, and improves the fit to a sample for which you have few replicates (e.g. NBS19). Slopes and intercepts resulting from the group fit are the same as those from individual fits for CM2 and RTG within error, but not for NBS19, which has only 2-5 replicates per measurement period (See Table S5). The close agreement between slopes of different sample types, and the benefits when measuring unknowns with few replicates lead us to prefer the group fit for this step. See the supplementary info for an in-depth discussion of group fit vs. individual fit. δ^{48} vs. Δ_{48} lines in this study have been calculated using a group fit of CM2, RTG, and NBS19 together. The resulting single $\text{Slope}_{\text{CARB48}}$ and multiple $\text{Int}_{\text{CARB48}}$ values are inserted into Eqn. (3) to calculate “true Δ_{48} ”.

Once the “true” Δ_{48} value is determined for each sample type, the scattered Δ_{47} data can be corrected to this value using Eqn. (4), following the observed relationship between Δ_{47} and Δ_{48} (see Fig. 5b).

$$\Delta_{48}\text{-Corrected } \Delta_{47} = \Delta_{47\text{-corr}} = \Delta_{47\text{-RF/AC}} - (\Delta_{48} - \text{“true” } \Delta_{48}) * \text{Slope}_{\text{CARB47}} \quad (4)$$

$\Delta_{47\text{-RF/AC}}$ is the Δ_{47} value corrected to the absolute reference frame and adjusted for the acid digestion fractionation.^[2] We choose to apply this correction to the data already corrected into the absolute reference frame since any nonlinearities caused by source effects should be removed in the $\Delta_{47\text{-RF/AC}}$ data. However, the slope of the carbonate lines in Δ_{48} vs. Δ_{47} space ($\text{Slope}_{\text{CARB47}}$) are very similar for the reference frame-corrected and raw data (Fig. S2, Table S2, Table S3), so the choice of order of operations does not have a large effect (mean difference of 0.001‰).

$\text{Slope}_{\text{CARB47}}$ can be found by fitting replicates of a single sample type in Δ_{48} vs. Δ_{47} space, or by fitting data sets of all carbonate sample types simultaneously for a common slope. When correcting unknown data with a small number of replicates (e.g. NBS19), it is preferable to do the group fit again (see discussion in supplement). When correcting samples or standards with a large (>10-15) number of replicates, the individual fit should accurately capture $\text{Slope}_{\text{CARB47}}$ for that sample type. For carbonate samples, $\text{Slope}_{\text{CARB47}}$ is roughly equal to 0.05 and does not change much through time (Fig. S3). We do observe a slight but consistent difference between the slopes of CM2 and RTG (Table S3), which may be Δ_{47} -dependent. To preserve the difference in slope between CM2 and RTG while still getting the benefits of the group fit for NBS19, we chose to fit RTG individually, and CM2 and NBS19 together for this step. The small number of NBS19 points have a

minimal effect on the group fit, making the resulting slope identical within error to the CM2 individual fit. A comparison of data corrected using different fit procedures is shown in the supplement. In future studies of unknowns, it is preferable to have multiple replicates of each sample that can be corrected together (i.e. run in the same measurement period) to constrain $\text{Slope}_{\text{CARB47}}$ for each sample individually. Where this is not possible, samples of similar Δ_{47} should be fit together. This can either use a data set of all unknowns, where at least a few samples have multiple replicates, or it could include a standard of similar Δ_{47} composition, run many times. Wherever possible, the behavior of a new unknown sample type should be independently constrained to help determine which standard is appropriate for a group fit. Group fits with a standard material following a different slope can result in corrected data that is skewed too high or too low.

Corrected carbonate data

Applying this correction dramatically reduces the scatter in our Δ_{47} data and shifts the mean value to within error of the published values (see Table 1). A full comparison of corrected and uncorrected data can be found in the supplement (Fig. S11, Table S4). For CM2, the correction shifts the mean Δ_{47} from $0.429 \pm 0.010\text{‰}$ to $0.378 \pm 0.003\text{‰}$ ($n=108$). For RTG, the correction shifts the mean Δ_{47} from $0.773 \pm 0.010\text{‰}$ to $0.723 \pm 0.004\text{‰}$ ($n=76$). These values compare nicely with the published values of $0.385 \pm 0.005\text{‰}$ ($n=40$) for CM2 and $0.720 \pm 0.007\text{‰}$ ($n=11$) for RTG.^[2] The offset between the uncorrected and corrected mean values reflects the number of points in a certain data set above or below the “true Δ_{47} ” value. This is not a fixed value and would not be expected to be the same for two similar standards (CM2 and NBS19) or even two subsets of the same standard (such as the two subsets of NBS19 in Table 1). $\delta^{13}\text{C}$ values agree well with published data, whereas $\delta^{18}\text{O}$ values are consistently too light by 0.15-0.2‰ from known values (Table 1). When measuring unknowns in the future, stable isotope data of unknown samples will be corrected for this systematic offset using carbonate standards, as has been done in other studies.^[27]

Figure 6 shows all the corrected data for CM2 and RTG. The data is separated into 0.1mg bins and samples in each bin are treated as replicates of one sample. In each bin, the average and standard error are computed (Fig. 6, filled symbols). For both CM2 and RTG, there is no systematic increase in scatter of individual points or in the external standard error of the binned groups as the sample size is reduced. The RTG data points show more scatter than the CM2 data points ($\Delta_{47\text{-corr}}$ 1 s.d. = 0.036 vs. 0.031, all replicates). This might be explained by increased heterogeneity of sample material between replicates. The RTG coral material was more coarsely ground than the CM2 marble, and shallow-water corals are known to be heterogeneous in Δ_{47} .^[3,28] In addition, fewer replicates of RTG were run in many of the mass bins compared to CM2 (Table S4), leading to larger standard errors for the same mass bins.

A smaller number of replicates of NBS19 were run, producing a mean Δ_{47} value of $0.366 \pm 0.007\text{‰}$ ($n=9$). This compares well with the value of $0.373 \pm 0.005\text{‰}$ calculated from previous measurements of this standard at Harvard.^[2] Replicates of NBS19 fall into two mass groups – a larger group (2.3-2.5mg) and a smaller group (1.2-1.4mg). The means of these two groups ($0.365 \pm 0.011\text{‰}$ ($n=5$) and $0.367 \pm 0.010\text{‰}$ ($n=4$), respectively) were within error of each other and of the published value (see Table 2).

Errors (1 SE) on raw Δ_{47} of individual samples increase as the sample size decreases following the shot noise limit^[21] (see supplemental material). At 1mg, error bars on $\Delta_{47\text{-raw}}$ for individual replicates are 0.014-0.016‰ for both CM2 and RTG. At 1.5mg, the same error bars are 0.009-0.011‰. When these raw 1 SE errors are propagated through the reference frame and Δ_{48} corrections, they get significantly larger and the influence of sample size disappears (see supplement for details of error propagation calculations). The average error increases from 0.009‰ to 0.034‰. The majority of this increase comes from the reference frame correction, which is responsible for 0.019‰ of the 0.025‰ increase, whereas the Δ_{48} correction contributes only 0.007‰ on average. The Δ_{48} correction had the largest impact during measurement period #1, where it contributed a 0.013‰ increase to average error, due to the limited number of carbonate samples run during this period (n=15), causing greater uncertainty in the carbonate slopes and intercepts needed for the correction. The reference frame correction is smallest in measurement period #2 (only 0.006‰), where the most standard gases were run. Other measurement periods (#1, #3) were cut short due to mechanical issues (e.g. power outages, mechanical issues) before the desirable number of gas standards were run, resulting in larger uncertainties in the heated and equilibrated gas lines and the empirical transfer function during these measurement periods.

DISCUSSION

Precision and sample size

The benefit of this technique is the ability to measure Δ_{47} on small aliquots of carbonate. All applications of clumped isotopes require at least 3 replicates per unknown to reduce the uncertainty on the mean. For geologic applications, errors on the order of 0.010‰ are needed to get meaningful results. To improve the overall sample requirements (combined mass of all replicates), it's necessary to balance the sample size per replicate and the number of replicates needed to achieve the necessary precision.

In applying this method, we recommend 5-6 replicates of mass 1.2-1.4mg per unknown (equivalent to 6 to 8.4 mg of CaCO_3) to minimize the total amount of sample material needed while achieving acceptable precision. Our measurements generally show 0.007-0.010‰ errors in this range. For CM2, mass bins in this range give standard errors of 0.007-0.008‰ for 5 or 6 replicates. The smaller NBS19 mass bin (1.2-1.4mg) has a standard error of 0.010‰ for 4 replicates. For RTG, the 1.2-1.3mg mass bin has a standard error of 0.009‰ for 4 replicates. The one exception is the larger RTG mass bin (1.3-1.4mg), which has a higher error (0.023‰), but only 4 replicates. The addition of 1-2 more replicates would improve the error in these mass bins. Even with a larger number of replicates, this is about half the mass required for the best existing traditional techniques (~4 replicates at 3-4mg^[10]) and is similar to the lower limit of measurements made with the Kiel device technique^[11,12] (6-26mg).

Detecting contaminated samples

One issue that arises is how to detect contaminated samples if a perfectly clean sample can have high Δ_{48} due to this unexplained fractionation. We suggest that contamination would be unlikely to affect Δ_{47} and Δ_{48} in the exact same ratio as our

fractionation. Contaminated samples would therefore deviate from the observed relationship, likely in the direction of higher Δ_{48} . In this study, we observe 7 samples that significantly deviate from the fractionation line in the direction of high Δ_{48} (Fig. S12), and believe them to be contaminated. These few points have been excluded from all plots and values shown here.

In practice, a threshold or envelope around the fractionation line should be set and points outside this range should be tossed out, similar to how the heated gas line in δ^{48} vs. Δ_{48} space has been used previously. When measuring unknowns, residuals of the unknown data around the unknown's group fit line can be compared to residuals of carbonate standards. Scatter in excess of the typical carbonate standard (especially in the high Δ_{48} direction) should be deemed contamination. This method of determining contamination has the risk of failing to eliminate some "mildly contaminated" samples. Inclusion of these samples would result in lower Δ_{47} and temperatures that were too hot.

A lower limit on sample size

We have shown that for sample sizes as low as 1mg, the average of multiple replicates can faithfully reproduce the mean value and give standard errors within an acceptable range (0.005-0.010‰), similar to that achieved by traditional methods.^[4, 10, 16, 17]

The inlet sample preparation procedure does not seem to limit the sample size that can be run because for all sample sizes we obtain good yields consistent with the ideal gas law (Fig. 3). Instead, we are limited by the beam intensities at which the sample is run, which are intimately linked to the shot noise limit and the maximum achievable precision on each replicate. As voltages drop below ~500mV on m/z 47, we observe less stability in the isotope ratios through the run, despite obtaining correct mean values when many replicates of this size are measured. This may indicate the lower limit at which the mass spectrometer can operate. The gas pressure (and therefore beam intensity) produced by a given mass is affected by a few parameters: 1) the size of the small U-trap; 2) the sensitivity of the MAT 253; and 3) the size of the sample and reference reservoirs.

Installing a smaller U-trap in front of the sample reservoir decreased the samples size necessary to achieve an internal precision of 0.010‰ and 0.007‰ from 3.1mg to 1.6mg and from 3.7mg to 2.4mg, respectively. Currently, the internal volume of the small U-trap is about 6mL. If this were decreased further to 3mL, it would further decrease the above sample sizes to 1.4mg and 2.1mg, respectively. In an ideal case, the volume of the U-trap would be as small as possible while still allowing the sample to freeze and expand without fractionating.

After tuning the source using the Isodat autotune function, we observed an increase in average sensitivity from 1.94×10^{10} to 2.66×10^{10} mV on m/z 47 per mbar source vacuum pressure. This is equivalent to an increase from m/z 47 = ~2900mV to ~4100mV at ~35mbar pressure in the bellows. This caused the sample size necessary to achieve an internal precision of 0.010‰ and 0.007‰ to decrease further from 1.6mg to 1.4mg and from 2.4mg to 2.0mg, respectively. An instrument with higher sensitivity could measure even smaller samples and achieve the same level of precision.

Changing the size of the sample (and reference) reservoir(s) could also increase the signal from a given volume of sample gas, by the same mechanism as decreasing the size of the small U-trap. However, decreasing the reservoir size also increases the rate at

which gas depletes. The 10mL reservoir used in this study is the smallest available from Swagelok (Fig. S1), but this technique should work equally well for a smaller reservoir. For very small reservoir sizes, the gas may deplete away before the 2-hour run completes. In other methods using microvolumes, either the gas is not run for a very long time^[11,12] or additional corrections for fractionation in stable isotopes were necessary.^[29] We selected the 10mL reservoir size to balance the sample yield and the depletion rate.

Our high-efficiency dual-reservoir configuration, in its current state, can handle samples as small as 1 mg. At this low limit, we begin to see more instabilities in the isotope ratios. A similar inlet with a smaller U-trap, a slightly smaller reservoir, or higher sensitivity of the mass spectrometer could push the sample size even lower using this measurement technique.

CONCLUSIONS

This study demonstrates that sample sizes as small as 1mg can be measured using a newly constructed, high-efficiency, dual-reservoir inlet and 5-6 replicates of 1.2-1.4mg can reliably produce external standard errors in the acceptable range (0.007-0.010‰). By eliminating the sample bellows and installing matching fixed reservoirs from which gas gradually enters the source, the volume of “wasted” gas typically left in a sample vial or bellows is reduced and yield is increased. With some adjustments, this measurement configuration could be successful for even smaller sample sizes. This achievement will facilitate the application of the clumped isotope proxy to sample materials for which it is difficult to acquire a large amount of material, such as foraminifera, and will expand the usage of this proxy in fields such as paleoceanography.

SUPPLEMENTARY INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

The authors thank Henry and Wendy Breck for support and G. Eischeid, S. Manley, and F. Chen for laboratory assistance.

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Sample	$\delta^{13}\text{C}$ (‰)	$\delta^{18}\text{O}$ (‰)	$\Delta_{47}\text{-RFAC}$ (‰)	$\Delta_{47}\text{-corr}$ (‰)
CM2 (n = 108)	2.22 ± 0.004	-1.95 ± 0.008	0.429 ± 0.010	0.378 ± 0.003
<i>CM2 published</i> ^[2]	2.29 ± 0.006	-1.77 ± 0.010		0.385 ± 0.005 (n=40)
RTG (n = 76)	-2.19 ± 0.006	-4.34 ± 0.007	0.773 ± 0.010	0.723 ± 0.004
<i>RTG published</i> ^[18]	-2.2 ± 0.062	-4.11 ± 0.019		0.720 ± 0.007 (n=11)
NBS19 (n = 9)	1.95 ± 0.019	-2.34 ± 0.037	0.372 ± 0.021	0.366 ± 0.007
<i>NBS19 published</i> ^[2]	1.95	-2.2		0.373 ± 0.007 (n=7)

Table 1: Summary of all carbonate samples of sizes 1.0-2.6mg measured from September 2013 to March 2014 compared to published values. $\delta^{13}\text{C}$ and corrected Δ_{47} values compare well to published values. $\delta^{18}\text{O}$ values are consistently 0.15-0.2‰ too light. Future analysis of unknown carbonates will be corrected for this fixed offset. All listed errors are 1 standard error (1 SE) estimates on the mean of all points of that sample type, with the number of replicates listed in parentheses.

Date	Sample	Mass (mg)	δ^{47} (‰)	$\Delta_{47\text{-raw}}$ (‰)	$\Delta_{47\text{-RF/AC}}$ (‰)	δ^{48} (‰)	Δ_{48} (‰)	$\Delta_{47\text{-corr}}$ (‰)
12/10/13	NBS19	2.32	17.317	-0.587±0.008	0.307±0.017	12.926	0.235	0.379±0.022
12/13/13	NBS19	2.41	17.494	-0.547±0.007	0.346±0.016	14.026	1.147	0.366±0.021
01/13/14	NBS19	2.37	17.463	-0.564±0.006	0.329±0.040	14.220	1.407	0.327±0.045
02/05/14	NBS19	2.47	17.491	-0.546±0.006	0.348±0.039	13.564	0.658	0.390±0.044
03/25/14	NBS19	1.23	17.497	-0.449±0.012	0.414±0.036	15.537	2.729	0.356±0.039
03/25/14	NBS19	1.36	17.401	-0.484±0.010	0.379±0.037	14.217	1.502	0.387±0.039
03/27/14	NBS19	2.32	17.499	-0.553±0.006	0.327±0.037	13.889	1.022	0.363±0.039
03/27/14	NBS19	1.27	17.480	-0.477±0.011	0.385±0.037	15.229	2.431	0.343±0.040
03/27/14	NBS19	1.33	17.102	-0.345±0.010	0.517±0.034	17.634	4.151	0.380±0.037
ALL				-0.504±0.025	0.372±0.021			0.366±0.007
Group 1 (1.2-1.4mg, n=4)				-0.439±0.032	0.331±0.007			0.367±0.010
Group 2 (2.3-2.5mg, n=5)				-0.555±0.009	0.424±0.032			0.365±0.011
<i>Published Value</i>								0.373±/± 0.007 (n=7)

Table 2: Raw data used in the correction of NBS19 data points measured from September 2013 to March 2014. Errors on $\Delta_{47\text{-raw}}$ are 1 SE on all cycles measured for an individual run. Errors on $\Delta_{47\text{-RF/AC}}$ and $\Delta_{47\text{-corr}}$ have taken the raw error and propagated it through the reference frame and Δ_{48} correction, respectively. Average errors (1 SE) on δ^{47} , δ^{48} , and Δ_{48} are 0.011‰, 0.053‰, and 0.052‰, respectively. Errors reported on mean values represent external 1 SE of that sample group.