Fast and reproducible δ^{13}C-CO_2 analysis from 1 mL of ambient atmospheric air using continuous-flow IRMS: from sampling to storage to analysis.

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Abstract. The stable carbon isotopic ratio (δ^{13}C) of atmospheric carbon dioxide (CO_2) is a key tracer for understanding terrestrial carbon dynamics, yet its extended application remains limited due to analytical and sampling restrictions. Here, we present an accessible methodology for high-precision δ^{13}C-CO_2 analysis of ambient atmospheric CO_2 from 1 mL air samples, tailored to the challenges of growth chamber experiments using microcosm model systems and other volume-limited systems. Our approach combines simple vial conditioning, dual-sealing using malleable self-adhesive butyl-rubber compound to prevent gas leakage, low-temperature storage (-80°C), and cryogenic pre-concentration coupled to continuous-flow isotope-ratio mass spectrometry (IRMS). The workflow is rapid, low-cost, relies on standard material, and avoids laborious sample preparation steps (i.e. purification), enabling other laboratories to reproduce the method easily. Using this approach δ^{13}C-CO_2 measurement achieves a precision of ± 0.1 ‰, with negligible isotopic drift for storage durations up to 1-week when vials are kept under low-temperature condition inside zip-lock bags filled with dry CO_2-free air. Longer storage times or storage at ambient temperature reduces both precision and accuracy, emphasising the importance of short-term storage at negative temperature. This methodology allows high sampling frequency, low-impact δ^{13}C–CO_2 measurements on 1 mL samples, while maintaining analytical performance, operational simplicity, accessibility and accessibility. It provides a practical solution for studies constrained by sample volume, time or cost.

1 Introduction

The stable carbon isotopic ratio (δ^{13}C) of atmospheric CO_2 is a powerful tool for tracing carbon sources and sinks, quantifying biogeochemical processes, and constraining global carbon cycle models (Bowling et al., 2008; Ciais et al., 2014; Farquhar et al., 1989). Because key processes such as photosynthesis and respiration fractionate carbon isotopes differently, isotopic measurements provide insights into the balance and dynamics of terrestrial carbon fluxes. These isotopic differences are essential for constraining global carbon cycle models and improving predictions of carbon–climate feedbacks (Tans et al., 1993).

Advances in isotope ratio mass spectrometry (IRMS) and automated gas handling systems have considerably improved the efficiency, cost-effectiveness and accessibility of δ^{13}C-CO_2 analysis. Continuous-flow techniques and the development of more stable and sensitive detectors now allow rapid processing of large numbers of samples with high analytical precision (Brand, 1996; Fisher et al., 2006; West et al., 2006). These improvements have made stable isotope analysis more widely applicable in ecosystem and atmospheric sciences. However, most existing methods still require sample volumes of several millilitres (Tu et al., 2001), which limits their applicability in experiments where only small amounts of air can be collected or where repeated sampling from the same volume is required, and therefor represents a major bottleneck.

Laser-based instruments have emerged as alternatives for in situ δ^{13}C-CO_2 analysis (Berryman et al., 2011; Steur et al., 2021; 2023, van Geldern et al., 2014). However, these systems also typically require 10 to 20 mL of sample to achieve sufficient sensitivity and high precision. Simple dilution of smaller samples (e.g., 1 mL into 10-20 mL) is not always a viable solution because although such instruments can in principle measure CO_2 at tens of ppm, the loss of precision becomes substantial and changes in the gas matrix introduced by dilution can generate significant isotopic offsets. Thus, despite operational advantages, laser-based approaches remain unsuitable for high-precision δ^{13}C-CO_2 analysis of sub-1 mL atmospheric air sample.

These constraints are particularly evident in growth chamber experiments, where small model systems (e.g. microcosms with plants and soils) are incubated under tightly controlled atmospheric conditions. One common method to disentangle CO_2 sources is the Keeling plot approach, which estimates the isotopic signature of ecosystem respiration by examining the linear relationship between δ^{13}C-CO_2 and the inverse of CO_2 concentration during mixture between atmospheric and ecosystem reservoirs (Keeling, 1958; Pataki et al., 2003). The strength of such analyses relies on precision and temporal resolution of δ^{13}C-CO_2 measurements, which depend on capturing subtle isotopic variations in constrained headspace volumes, often under limits of sampling frequency and minimal system disturbance (Midwood and Millard, 2011; Pataki et al., 2003; Sperlich et al., 2022; Werner et al., 2006). In microcosm setups with many small pots, withdrawing large air volumes can disrupt experimental conditions or prevent repeated sampling (Gillespie et al., 2020; Guillot et al., 2019; Siegwart et al., 2023). Thus, there is a growing need for analytical methods capable of δ^{13}C-CO_2 analysis in very small air volumes (~1 mL) at ambient CO_2 concentration, while maintaining high analytical precision. Such methods would allow for high-resolution, minimally invasive sampling across space and time, facilitating isotopic monitoring in highly replicated experimental designs.

Although precise δ^{13}C–CO_2 methods exist for small CO_2 volumes, they typically rely on repeated aliquot injections from large reservoir (1–5 L) (Brand et al., 2016; Rothe et al., 2005; Werner et al., 2001), or specialised setups involving extensive sample preparation (e.g., purification, tube-cracker devices) (Schmidt et al., 2011; Walker et al., 2021). These approaches are not compatible with volume-limited experimental systems and are often too time-consuming and complex for routine use. To address these limitations, we developed an analytical workflow for measuring δ^{13}C–CO_2 of ambient atmospheric CO_2 in 1 mL samples, using continuous-flow IRMS. The method uses commercially available equipment, simple and reproducible steps, low cost and standard consumables, and short processing time, making it accessible for a wide range of laboratories. The workflow is based on cryogenic pre-concentration adapted from small carbonate samples analysis (Fiebig et al., 2005), and optimised for ambient CO_2 concentrations. This setup reduces required sample volume by an order of magnitude while maintaining high analytical performance, achieving precision of ±0.1 ‰ on $\delta^{13}C–CO_2$.

In addition to challenges associated with analysing small atmospheric sample volumes, the storage of gas samples prior to analysis represents a major limitation. Isotopic composition can drift due to preferential diffusion of lighter isotopes, leakage, or physicochemical interactions with storage materials, especially over extended period. Such storage-induced artefact have been documented for several isotopes, including ^{13}C, ^{18}O, ^{15}N, and ^{2}H (Hardie et al., 2010; Kuehfuss et al., 2014; Laughlin and Stevens, 2003; Mortazavi and Chanton, 2002; Nauer et al., 2021; Nelson, 2000; Paul and Skrzypek, 2006). Consequently, many existing protocols require analysis within hours of sampling, which restricts sample handling flexibility, limits laboratory collaboration and complicates field-based studies.

While our protocol differs in implementation, it is built on the same underlying principles as those described by Steur et al., 2023 for moisture removal and leak prevention (i.e. rapid pre-conditioning with dry CO_2-free air and reinforced sealing). Our choices focus on a workflow that is simple, rapid, and low-cost with standard consumable, allowing routine implementation in laboratories processing large samples numbers or working under logistical constraints. As part of our methodological development, we specifically evaluated the influence of: 1) vial pre-conditioning with dry CO_2-free air, 2) simple vs. double septum configurations, 3) a dual sealing layer at the bottom and top of the cap, 4) storage temperature and 5) storage duration. These tests enable the design of preservation strategy that extends the storage time for small air samples without compromising isotopic integrity, thereby improving both flexibility and robustness in $\delta^{13}C$-CO_2 measurements.

In this paper, we present an integrated methodology—from sampling to storage to IRMS analysis—and demonstrate its application in controlled-chamber experiments as well as its suitability for field deployments. We evaluate its precision, defined as the closeness of agreement among repeated measurements performed under stable conditions calculated as the standard deviation of the replicates (Belouafa et al., 2017; Squara et al., 2020), and its accuracy, defined as the closeness of the analytical results to the values of the secondary standard. We also assess its relevance for high-frequency isotopic monitoring, and discuss its broader potential for advancing carbon-cycle research. The method achieves a precision of ± 0.1 ‰ on $\delta^{13}C$-CO_2 using only 1 mL of ambient atmospheric air, providing a robust tool for studying carbon dynamics in highly constrained experimental settings.

Targeting a precision of ± 0.1 ‰ for $\delta^{13}C–CO_2$ is a pragmatic choice grounded in both scientific relevance and analytical feasibility. This level of precision is widely recognised in atmospheric and ecologicak/ecosystem studies as sufficient to resolve biologically meaningful variations, typically 0.3 to 1 ‰, associated with processes such as respiration or mixing in chamber and field conditions. It is coherent with the precision commonly achieved by continuous-flow IRMS systems using 10–12 mL sample volumes (Breecker et al., 2014; Giammanco et al., 2017; Joos et al., 2008; Leitner et al., 2023; Pataki et al., 2003; Tu et al., 2001). Achieving this same analytical threshold with only 1 mL of air, one order of magnitude less than standard protocols, offers a balance between measurement precision, sample size and operational simplicity, making the method particularly well suited to high-throughput or volume-limited experimental applications.

2 Material and methods

2.1 Vial conditioning: the basics

We used 5.9 mL flat bottom soda exetainers (Labco Limited, UK) with single chlorobutyl septum as sampling vials for CO_2 analysis. After closing, the vials were conditioned on a custom-built manifold designed for trace-level CO_2 sampling (Fig. 1). This manifold, constructed using Swagelok® fittings and valves (Swagelok Company, USA), consists of twelve interconnected ¼-inch tees (SS-400-3), linked to two manual valves: a two-way valve (SS-43GS4) for vacuum control and a three-way valve (SS-43GXS4) for pressurised nitrogen supply and pressure equilibration. Luer needles (27G ¾", Terumo Agani, China) were inserted into Ultra-Torr® fittings (SS-4-UT-6-400), which were connected to each tee. Batches of 12 vials were evacuated simultaneously to a pressure of 0.02 mbar bar over 8 minutes (two-way valve open, three-way valve closed; Fig. 1), then filled with nitrogen gas (Alphagaz 1, Air Liquid, France) at 0.5 bars (above ambient pressure) for 20 seconds to establish a slight overpressure (two-way valve closed, three-way valve open to the bottom; Fig. 1). The use of nitrogen reduces the O_2 mole fraction of the exetainer bellow ambient when the air sample is added, thus, minimising nitrous oxide production in the IRMS source, and therefore potential interference with CO_2 measurements. Helium and argon were both tested but proved unsatisfactory: helium was avoided due to its high diffusivity through the septum during storage, while argon was considered impractical for routine use because of handling constraints and cost. The overpressurised vials were then vented for 5 seconds to restore atmospheric pressure (two-way valve closed, three-way valve open to the top; Fig. 1). This evacuation–refill cycle was repeated four times, ensuring near-complete removal of any residual CO_2 or contaminants through geometric dilution, resulting in a reproducible CO_2-free starting atmosphere. After the fourth cycle, the vials were equilibrated to ambient pressure and ready for gas sampling. A control set of vials (blanks) initially showed no detectable CO_2 signal under standard analytical settings. To more rigorously evaluate blank integrity, we reprocessed the blank chromatograms using higher sensitive detection parameters—a minimum peak height of 5 mV and start/end thresholds of 1 mV s^{-1}, well below the standard settings used in this study (50 mV peak height, 20 mV s^{-1} start, 40 mV s^{-1} end). Under these stringent conditions, small CO_2 peaks (10–15 mV) were detected in approximately one-third of the blanks (i.e. no peak detected in the other ones under those settings). While these detection thresholds are more conservative than those commonly applied in comparable atmospheric CO_2 studies (e.g., Leitner et al., 2023), the low-level background identified remains far below typical analytical detection limits.

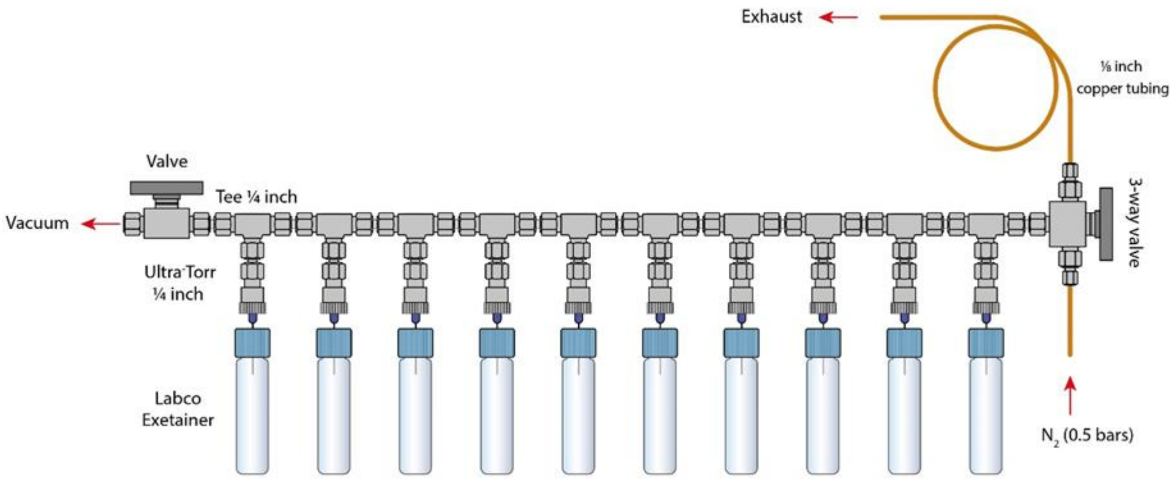

Figure 1: Schematic of the vial conditioning manifold. For clarity only 10 vials positions are shown. The conditioning procedure includes: (1) vacuum of the vials to 0.02 mbar (~8 minutes; two-way valve open, three-way valve closed), (2) filling with N_2 gas at 0.5 bars with overpressure (~20 seconds; two-way valve closed, three-way valve open to the bottom), and (3) restoration to atmospheric pressure (~5 seconds; two-way valve closed, three-way valve open to the top). A 1/8-inch copper tube is connected to the exhaust outlet to prevent ambient CO_2 from diffusing into the vials during pressure equilibration. Steps 1, 2 and 3 were repeated four times.

2.2 Gas sampling

Gas sampling was done using a 1 mL syringe (Soft-Ject, Germany) equipped with a Luer needle 27G ¾ (Terumo Agani, China). The syringe was first flushed three times with ambient air to remove any residual gases. It was then flushed 3 times with the targeted gas, by injecting and withdrawing it inside the container (chamber, flask or jar for example) without sampling or releasing, to avoid any contamination from prior samples. The syringe was then filled with a volume of air slightly exceeding the targeted volume (1 mL) before being withdrawn from the container. The volume in the syringe was carefully adjusted to 1 mL by expelling the excess air. The air sample was subsequently injected into a simple septum vial through the septum. After injection, a malleable self-adhesive butyl-rubber compound (Teroson® RB 81, Henkel, Germany) with excellent resistance to gas and moisture transfer was manually reapplied to cover the entire surface of the septum and vial cap, to prevent any gas leakage.

2.3 Isotopes analysis

The overall principle of the analytical setup is shown in Figure 2A. Isotopic analyses of CO_2 were performed using a continuous-flow isotope ratio mass spectrometer (IRMS; Delta V Plus, Thermo Fisher Scientific, USA) coupled to a Gas Bench II preparation system and a ConFlo IV. The analytical setup, included an automated cryogenic trap (a U shaped 1/16 stainless steel tube fixed to a pneumatically operated lifting unit and placed above a 2 litre dewar of liquid nitrogen), initially designed for carbonate analysis (Fiebig et al., 2005), but adapted here for $\delta^{13}C$-CO_2 measurements of atmospheric CO_2 concentration in small air samples. First, the method (i.e. protocol refinements in sample handling and peak integration) was adjusted to analyse CO_2 at atmospheric concentrations (~420 ppm), providing ~0.2 µgC per run, i.e. an order of magnitude less carbon than in Fiebig et al. 2005 (1.2–3.6 µgC from carbonates). Second, the analysis was performed directly on the atmospheric gas matrix (CO_2, N_2, O_2, Ar, CH_4, N_2O) rather than after flushing the sample vial with helium and analysing CO_2 in a nearly pure helium stream. The chromatographic conditions were accordingly modified, with the PoraPlot Q column operated at 35 °C instead of 70 °C to optimise separation peak shape while ensuring stable retention of atmospheric air components.

The PAL autosampler initiated each run by moving the needle to the appropriate exetainer, which was then continuously flushed with helium, using a single flushing needle that is fixed to the PAL, to displace atmospheric gases. The automated protocol then began: the air sample was introduced into the Gas Bench II via an automated injection system, where water vapor was removed using a Nafion® membrane. Ten seconds after flushing, the cryogenic trap was lowered into liquid nitrogen, and 20 s later, the Valco valve switched to “load” mode for 360 s to allow CO_2 to condense while non-condensable gases were flushed by the helium stream (Brand et al., 2010).

Simultaneously, five rectangular reference CO_2 peaks were injected through the open split by the ConFlo IV, with $\delta^{13}C\text{-}CO_2$ values reported relative to the Vienna Pee Dee Belemnite (VPDB) scale (Fig. 2B). The fifth peak served as the reference for sample calibration. At 390 s, the Valco valve returned to “inject” mode, and after a 20 s delay, the stainless tube of the cryogenic trap was lift out from liquid nitrogen, releasing the trapped CO_2 into the helium carrier stream via sublimation. The gas then passed through a second Nafion® trap to remove residual water, followed by a gas chromatography column (PoraPlot Q, 25 m, 0.32 mm, 10 µm film, Agilent) held at 35°C, which enabled separation of CO_2 from gases such as O_2, N_2 and N_2O, thereby ensuring stable ionisation conditions in the mass spectrometer. CO_2 was finally introduced into the Delta V Plus via an open split in the ConFlo IV. The analysis also included a CO_2 blanking procedure to correct for any background signal originating from the analytical system itself (Paul et al., 2007).

In contrast to traditional injection systems (Spötl, 2004; Spötl and Vennemann, 2003), this configuration produces a single, well-defined CO_2 peak, as the entire analyte is injected in the ion source in once (Fig. 2B).

Memory effects were evaluated, where highly contrasting samples were analysed in a random order. Alternating injections of very high CO_2 concentrations (10,000–20,000 ppm) with highly enriched isotopic signatures (up to 3.5 AT%), followed by samples at atmospheric concentration (~ 420 ppm) and depleted signatures (~ –10 ‰), did not produce any detectable carry-over (data not shown). These results indicate that memory effects are negligible under our analytical conditions and do not require additional correction.

The $\delta^{13}C\text{-}CO_2$ values were reported relative to the Vienna Pee Dee Belemnite (VPDB) standard and calibrated using working standard CO_2. Three ambient air working standards (lately named WS1, 2 or 3; synthetic air mixtures supplied by Air Liquide (“mélange Crystal”), France) were routinely used for $\delta^{13}C\text{–}CO_2$ calibration. These in-house standards contain CO_2 (WS1 380 ± 0.9 ppm and –38.72 ± 0.10 ‰; WS2: 450 ± 0.85 ppm and –35.20 ± 0.10 ‰; WS3: 450 ± 0.85 ppm and –36.26 ± 0.10 ‰), 20 % O_2 and balance N_2. They were calibrated in-house against two secondary reference gases (R1: 451 ± 0.11 ppm, –41.72

± 0.02 ‰; R2: 381.17 ± 0.06 ppm, –41.67 ± 0.01 ‰) that were themselves traceable to NOAA primary standards maintained on the RAMCES platform (LSCE, Gif-sur-Yvette, France). All gases were supplied in high-pressure cylinders equipped with Air Liquide HBS 200-3-2.5 stainless steel two-stage pressure regulators to ensure stable outlet pressure and prevent fractionation.

Regular intercomparison of the working standards confirmed their stability over time, with no detectable drift beyond analytical precision. Three vials containing 1 mL of the same CO_2 working standard (WS1 or WS2 or WS3) were analysed at the beginning and end of each daily run to monitor and correct for instrumental drift and to ensure analytical precision. Drift remained below 0.05 ‰ over the longest sequences (4–5 h) with no detectable time-dependent trend. Analysis time per sample was 17 minutes, and analytical precision of $\delta^{13}C\text{-}CO_2$ was consistently lower or equal to ± 0.1 ‰.

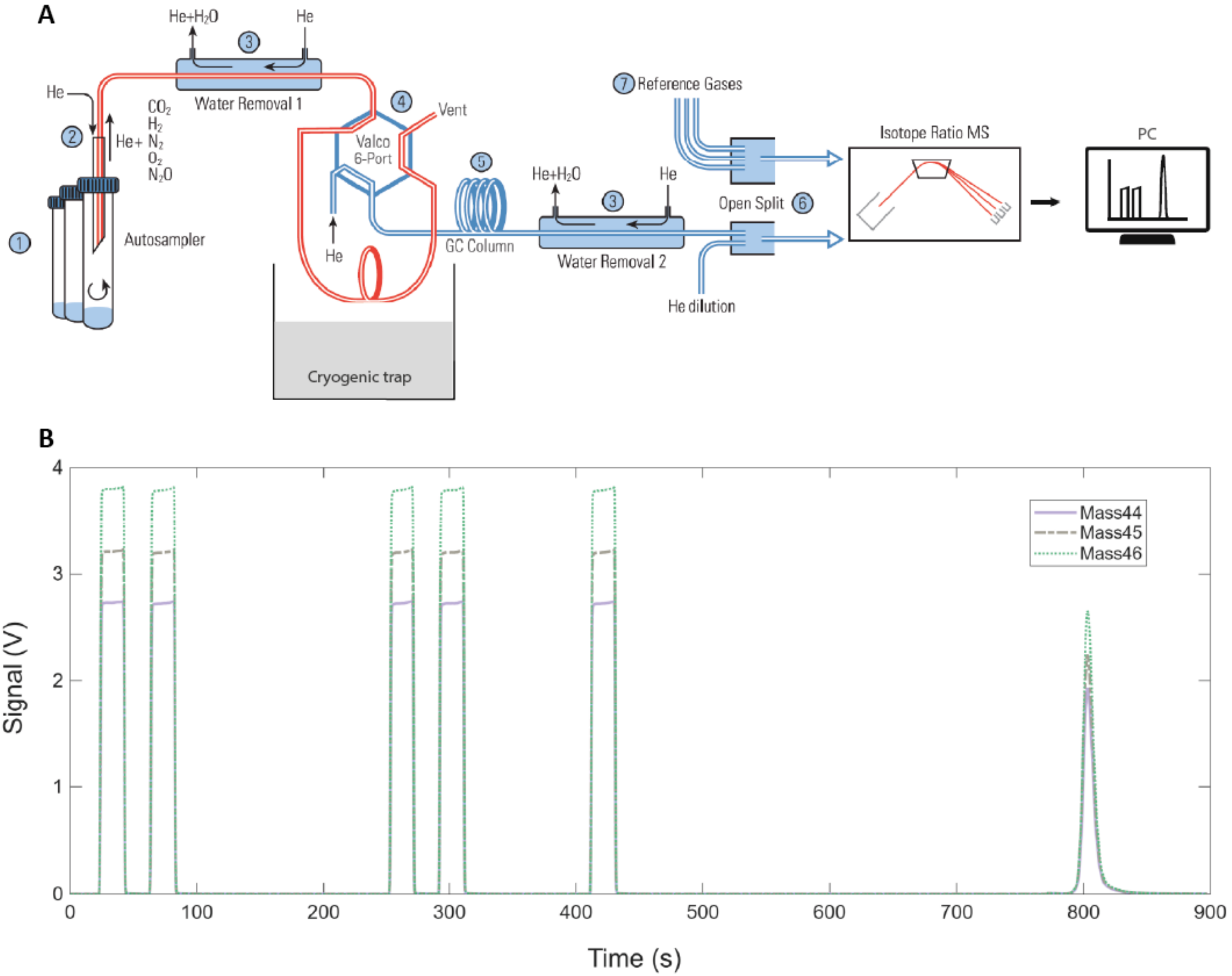

Figure 2: (A) Schematic diagram of the analytical setup for δ^{13}C–CO$_2$ measurements adapted from Gas Bench II operating manual (Thermo Fisher Scientific., 2018) (1) Sample vials loaded in the autosampler of the GasBench II; (2) injection of sample air into the helium carrier stream; (3) water-removal traps inside the GasBench; (4) 6-port Valco valve connected to the cryogenic trap; (5) separation on the GC column; (6) transfer to the ConFlo IV interface via an open-split with helium dilution; (7) introduction of reference gases through the ConFlo IV before isotope analysis by IRMS Delta V Plus. (B) Representative chromatogram obtained from the analysis of 1 mL of atmospheric air. The ion intensities for m/z 44 (purple solid line), 45 (brown dot dash line), and 46 (green dotted line), corresponding to the different isotopologues of carbon dioxide. The peaks observed between 0 and 450 seconds correspond to successive injections of a CO$_2$ reference gas, while the single peak around 800 seconds corresponds to the atmospheric air sample.

2.4 Statistical analyses

All statistical analyses and visualisations were performed using R software (R-4.4.2, R Core Team, 2015). To assess significant differences in δ^{13}C-CO$_2$ values between two sample groups, Welch's t-tests were applied when variances were unequal, while

Student’s t-tests were used when the assumption of equal variances held. For datasets involving more than two groups with unequal variances, a Welch’s ANOVA was performed, followed by a Games–Howell post-hoc test to evaluate pairwise differences. Standard deviations were calculated for each group to evaluate the precision of the measurements. Impacts on accuracy were assessed by testing whether the mean δ^{13}C–CO_2 values differed significantly from the known values of the working standards, using a one-sample t-test.

3 Results

3.1 Pre-conditioning flush with dry CO_2-free air on vial

To minimise background contamination and prevent isotopic fractionation due to degassing, each vial was systematically flushed for 8 seconds with dry CO_2-free air at a flow rate of 13.5 L min^{-1}, corresponding to approximately 40 vial-volume renewals per second. This pre-conditioning step was performed manually by briefly removing and reinserting the septum cap while applying the continuous dry CO_2-free air flow into the vial, in order to remove residual CO_2 and stabilise internal pressure before subsequent conditioning. Flushing alone is not sufficient, however; the following four N_2 evacuation–refill cycles are essential to completely remove remaining CO_2 after vial closing.

To assess the actual impact of this flushing step, we conducted a comparative experiment using vials prepared either with or without this dry CO_2-free air flush. In both cases, 1 mL of WS1 was introduced after conditioning (sections 2.1 & 2.2), and samples were immediately analysed by IRMS (section 2.3). In the absence of the flushing step precision is equal to 0.54 ‰ (largely higher than the targeted 0.1 ‰). In contrast, flushing prior to conditioning markedly improving both precision and accuracy, with precision down to ± 0.09 ‰ and δ^{13}C-CO_2 values aligning even more closely with the nominal standard (Fig. 3).

A Welch’s t-test comparing the means of the flushed versus non-flushed samples confirmed that the flushing step significantly improved precision (p_value = 0,00056). One-sample t-tests against the nominal WS1 δ^{13}C-CO_2 value further showed that only the flushed samples were not statistically different with the standard, demonstrating that the flushing step is essential for achieving both high precision and accurate δ^{13}C-CO_2 measurements.

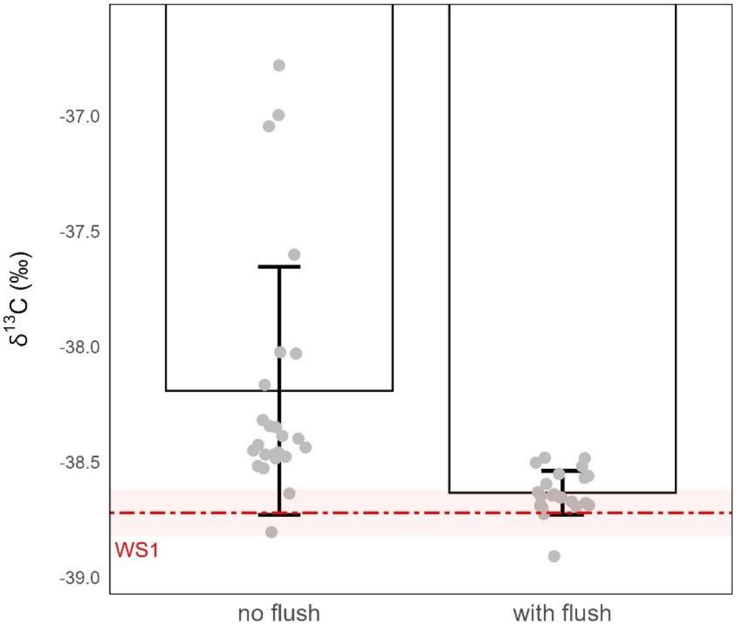

Figure 3: Effect of the initial dry CO_2-free air flush on $\delta^{13}C$-CO_2 analysis in 1 mL gas samples. $\delta^{13}C$-CO_2 values (‰) measured from vials conditioned either without a flushing step (left) or with an 8-second dry CO_2-free air flush (right) prior to gas introduction. The red dashed line indicates the expected isotopic value of the WS1 and the shaded red band its precision (–38.72 ± 0.10 ‰). Grey circles represent individual replicate measurements. Bars show the mean ± standard deviation for each condition. Without flushing, the measured precision was ± 0.54 ‰ (n = 24), whereas with flushing, precision was ± 0.09 ‰ (n = 23).

3.2 Analytical performance of 1 mL ambient atmospheric air samples

A typical chromatogram produced by the method is described in Figure 2B. This chromatogram exhibits a single well-defined CO_2 peak with sharp Gaussian symmetry and excellent signal-to-noise ratio. The retention time remains stable across replicate injections, indicating consistent flow dynamics and thermal stability of the GC column. Baseline separation is maintained, with no detectable co-elution or interfering species in the target m/z acquisition window. Peak integration was performed within fixed boundaries, and total CO_2 signal area was sufficient to ensure accurate $\delta^{13}C$-CO_2 calculation despite the low analyte mass (amplitude from 2 to 2.5 V). Across a series of replicate measurements (n = 24), performed on independently prepared vials (no storage, dry CO_2-free air flush), the analytical precision was consistently equal to ± 0.10 ‰.

In situations where CO_2 concentrations largely exceed atmospheric levels—such as in soil incubations where soil respiration leads to accumulation of CO_2 in the air space, experiments involving ^{13}C-labelled glucose additions or microbial incubations—the same analytical protocol can be applied to sample volumes as low as 50 µL without compromising precision (Siegwart et al., 2023). The enhanced signal associated with elevated CO_2 concentrations ensures that even these small volumes generate chromatograms with sufficient peak intensity and resolution for accurate isotopic analysis.

3.3 Effect of septum configuration on short-term storage stability

To assess the impact of septum configuration on the preservation of isotopic integrity during short-term storage, we compared vials sealed with a single septum to those fitted with a double septum (Labco Limited, UK; section 2.1). In both cases, 1 mL of WS1 was injected into the vials (section 2.2), which were then stored for 24 hours at room temperature. Isotopic analyses (section 2.3) revealed no statistically significant difference between the two configurations (t-test, $p > 0.05$, assuming equal variances; Fig. 4). In both cases, the precision was larger than 0.1 ‰ (± 0.20 ‰ for simple septum vs ± 0.16 ‰ for double septum), and the measured δ^{13}C-CO$_2$ values were systematically offset by approximately +1 ‰ relative to the nominal WS1 standard, indicating both isotopic drift and poor accuracy during storage at room temperature.

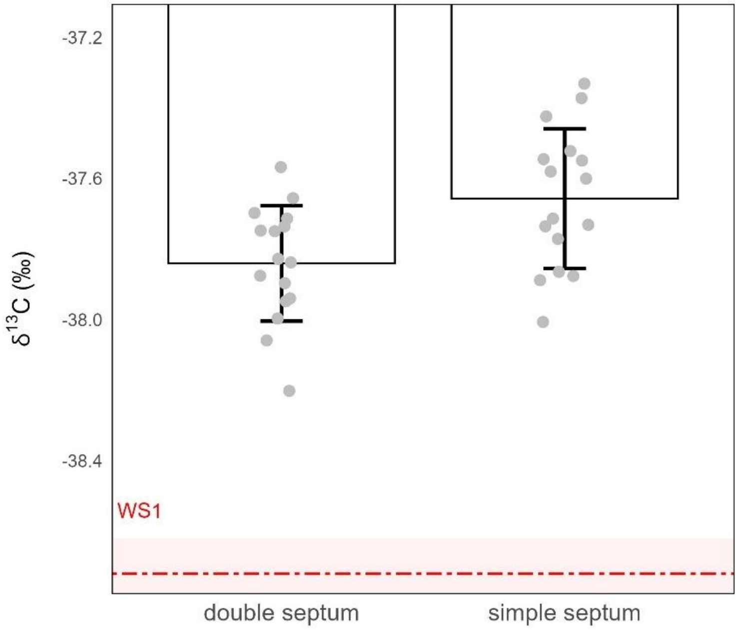

Figure 4: Effect of septum configuration on δ^{13}C-CO$_2$ stability during 24-hour storage at ambient temperature. δ^{13}C-CO$_2$ values (‰) measured in 1 mL WS1 in vials sealed with either a single septum (right) or a double septum (left). The red dashed line represents the expected isotopic value of WS1 and the shaded red band its precision (–38.72 ± 0.10 ‰). Grey circles represent individual replicate measurements. Bars show the mean ± standard deviation for each condition. Precision was ± 0.16 ‰ for the double septum (n=16) and ± 0.20 ‰ for the simple septum (n=16).

Although the double septum offered slightly better precision, it did not correct the bias and comes at a higher price. For these reasons, and in the absence of any significant benefit for both accuracy and precision, the single septum configuration was retained for subsequent analyses. These results suggest that while septum configuration alone does not mitigate storage-related isotopic shifts, alternative sealing or preservation strategies must be explored to improve the long-term stability of small-volume air samples.

3.4 Effect of dual-sealing on short-term storage performance

To further improve sample integrity during short-term storage, we evaluated the impact of applying butyl-rubber compound not only on the top of the caps but also around the lower part of the cap, directly beneath the sealing ridge and near the thread area. This dual-sealing technique aims to significantly reduce the risk of gas leakage, diffusion and associated isotopic drift, particularly over longer storage periods. As in previous tests, 1 mL of WS3 was injected into each vial (section 2.1 & 2.2), which was then stored for 24 hours at room temperature. Although dual sealing noticeably improved precision—reducing the standard deviation from ≥ 0.22 ‰ under simple sealing to ≤ 0.11 ‰ with dual sealing, both configurations exhibited a clear loss of accuracy, under storage at room temperature. Mean δ^{13}C-CO$_2$ values were significantly shift related to the nominal WS3 value (Fig. 5).

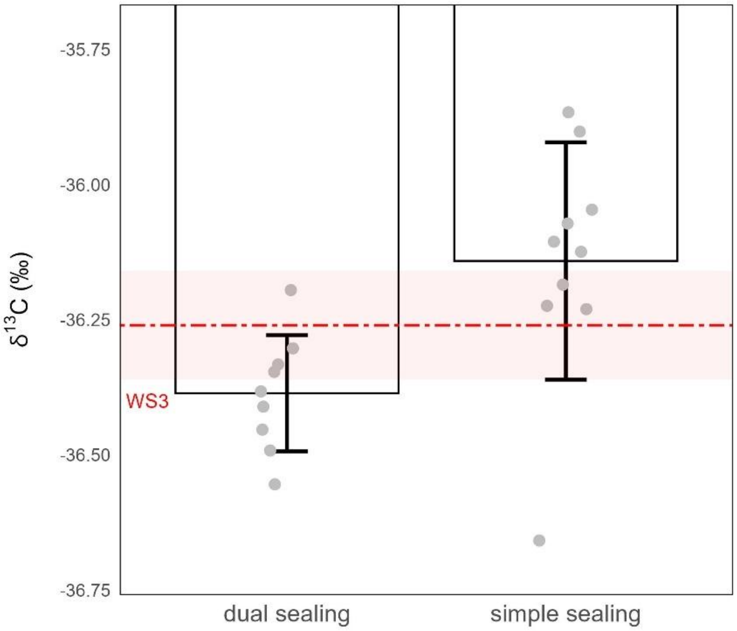

Figure 5: Effect of sealing improvement on δ^{13}C-CO$_2$ stability during 24-hour storage at ambient temperature. δ^{13}C-CO$_2$ values (‰) measured in 1 mL WS3 in vials with simple- (right) or dual-sealing (left). The red dashed line represents the expected isotopic value of WS3 and the shaded red band its precision (–36.26 ± 0.10 ‰). Grey circles represent individual replicate measurements. Bars show the mean ± standard deviation for each condition. Precision was ± 0.22 ‰ with simple sealing (n=10) and ± 0.11 ‰ with dual-sealing (n=9).

Welch's t-tests (unequal variances) confirmed that the difference in mean δ^{13}C-CO$_2$ values between the two conditions was statistically significant (p_value = 0.00776) indicating improved precision under dual sealing. However the difference between WS3 nominal value and δ^{13}C-CO$_2$ suggests some depreciation of accuracy under storage at room. Nevertheless, although this dual-sealing method improves performance, the analytical precision of δ^{13}C-CO$_2$ remained above the target threshold of ± 0.1

‰, indicating that additional measures—such as temperature control or more robust sealing systems—may be necessary to achieve optimal storage stability.

3.5 Influence of storage temperature on isotopic signal stability of small-volume air

To assess whether the target precision of ± 0.1 ‰ for δ^{13}C-CO$_2$ measurements could be achieved on 1 mL air stored samples, we investigated the effect of storage temperature on isotopic stability (Kornfeld et al., 2012). Vials were flushed with CO$_2$-free air, dual-sealed with butyl-rubber compound, filled with 1 mL of WS2, and stored for 24 hours under three different conditions: room temperature (~20°C), -20°C, and -80°C. To minimise the risk of contamination, the vials were placed in sealed zip-lock bags filled with dry CO$_2$-free air ensuring that any potential ingress would not introduce isotopically enriched CO$_2$.

The results reveal a strong temperature dependence in both precision and accuracy (Fig. 6). At room temperature, δ^{13}C–CO$_2$ values showed substantial isotopic drift, being enriched by nearly +1 ‰ relative to WS2, with a dispersion far exceeding the 0.1 ‰ target (precision ± 0.50 ‰). Storage at –20 °C and –80 °C both removed this systematic bias, restoring accuracy relative to nominal WS2 value. However, at –20 °C the precision remained above the target (± 0.24 ‰), while, in sharp contrast, storage at –80 °C fully preserved isotopic integrity, reducing precision to ± 0.10 ‰ and thus meeting the required analytical precision.

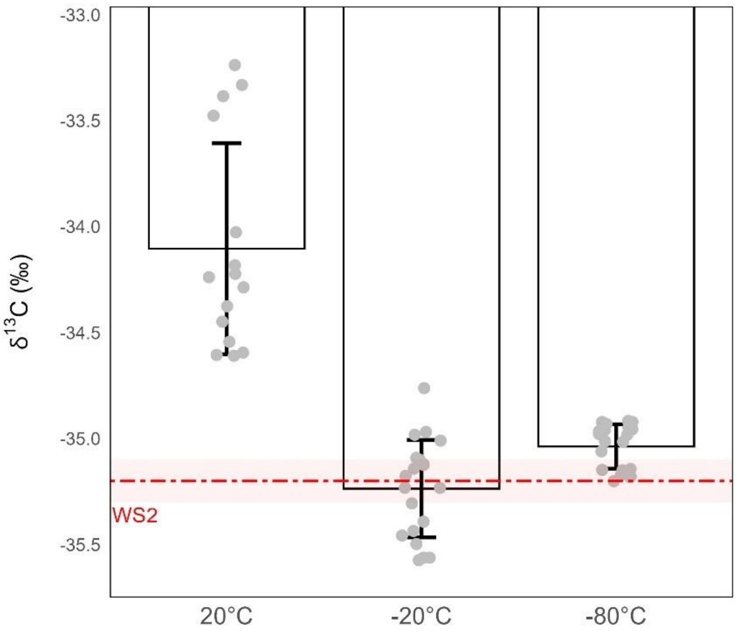

Figure 6: Effect of storage temperature on δ^{13}C-CO$_2$ stability during 24-hour storage at ambient temperature (left), -20°C (middle), -80°C (right). δ^{13}C-CO$_2$ values (‰) were measured from 1 mL WS2. The red dashed line represents the expected isotopic value of WS2 and the shaded red band its precision (–35.20 ± 0.10 ‰). Grey circles represent individual replicate measurements. Bars show

the mean ± standard deviation for each condition. Precision was ± 0.50 ‰ for sample stored at room temperature (n=15), ± 0.24 ‰ for sample stored at-20°C (n=20) and ± 0.10 ‰ for sample stored at -80°C (n=20).

To verify that contamination or leakage did not bias the results at low CO_2 levels, a control set of empty vials (flushed, dual-sealed and conditioned but not filled with WS) was stored at -80 °C and analysed using the more sensitive peak-detection settings described in section 2.1. Similarly, under these stringent conditions, small peaks of 10–15 mV were detected in approximately one-third of the blanks, exhibiting trace-level background far below the CO_2 signal of real samples. These results confirm both the integrity of the sealing protocol and the absence of significant contamination during storage at –80 °C. Possible mixing effects associated with this background signal are addressed and discussed later (section 4.1).

Based on this finding, a key remaining question concerns the maximum duration over which samples can be stored at -80°C without significant isotopic drift.

3.6 Effect of storage duration at -80°C on isotopic stability

To determine the maximum duration over which small-volume air samples can be stored at -80°C, without compromising isotopic signal, we analysed vials containing 1 mL of WS2 and WS3 stored for 1, 2 and 4 weeks. All samples were flushed with dry CO_2-free air and dual-sealing was applied prior to be stored at -80°C in zip-lock bags full of dry CO_2-free air.

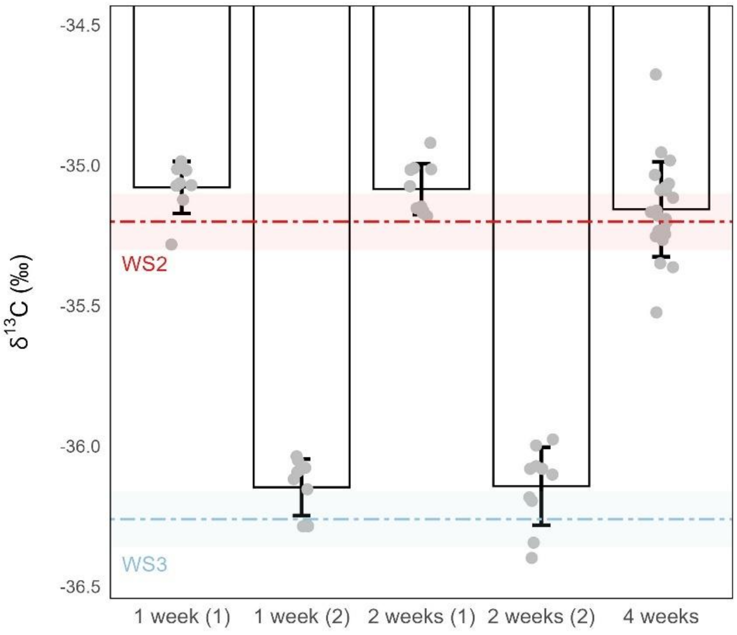

Figure 7: Effect of storage duration on $\delta^{13}C-CO_2$ stability. Sample were stored at -80°C for 1- (left), 2- (middle) or 4-weeks (right). $\delta^{13}C-CO_2$ values (‰) was measured on 1 mL WS2 or WS3. The red and light blue dashed lines represent the two expected isotopic value of the working standard's and the shaded red and blue bands their precision (respectively –35.20 ± 0.10 ‰ and –36.26 ± 0.10 ‰). Grey circles represent individual replicate measurements. Bars show the mean ± standard deviation for each condition.

Precision was ± 0.10 ‰ (n=8) and ± 0.09 ‰ (n=10) for sample stored 1-week (respectively sub samples (1) and (2)), ± 0.13 ‰ (n=10) and ± 0.09 ‰ (n=10) for sample stored 2-weeks (respectively sub samples (1) and (2)) and ± 0.17 ‰ (n=22) for sample stored 4-weeks.

The results showed that after 1 week of storage, both precision and accuracy remained within acceptable limits, with precision at or below ± 0.1 ‰ (± 0.10 ‰ and ± 0.09 ‰; Fig. 7) and mean $\delta^{13}C$ values indistinguishable from their respective working-standard targets. After 2 weeks, precision became more variable—one sample set remained within the ± 0.1 ‰ threshold (± 0.09 ‰), while the other exceeded it (± 0.13 ‰)—and small deviations from the nominal values began to emerge. By 4 weeks, precision further deteriorated to ± 0.17 ‰, accompanied by clear shifts away from the reference $\delta^{13}C$-CO_2 values, indicating that both precision and accuracy were no longer maintained (Fig. 7).

4 Discussion

We developed a simple, rapid, and standard material based analytical workflow for measuring the carbon isotopic ratio ($\delta^{13}C$-CO_2) of atmospheric CO_2 from small air samples (1 mL) with high precision (± 0.1 ‰), a threshold commonly required to resolve biologically meaningful isotopic variations in ecosystem and atmospheric studies. The method integrates straightforward vial preparation, optimised storage conditions, and continuous-flow IRMS measure using a Gas Bench II, a ConFlo IV and a cryogenic trap. This approach minimises sample disturbance while requiring only a fraction of the air volume typically used in $\delta^{13}C$–CO_2 analyses (i.e. 12 mL; Breecker et al., 2014; Giammanco et al., 2017; Joos et al., 2008; Leitner et al., 2023; Pataki et al., 2003; Tu et al., 2001). It was specifically designed for experimental systems with constrained headspace—such as growth chambers, respiration closed systems chambers, soil headspace—where repeated, minimally invasive gas sampling is essential (Siegwart et al., 2023). Because millilitre-scale samples may contain low amounts of CO_2 they are intrinsically more sensitive to potential mixing with residual CO_2 in the vials; this effect is minor in our dataset but is discussed in more detail in the next section.

Our results demonstrate that a precision of ± 0.1 ‰ on $\delta^{13}C$-CO_2 analysis can be achieved from as little as 1 mL of atmospheric air using standard equipment and minimal preparation. This capability opens new possibilities for high sampling frequency, minimally invasive monitoring in experimental setups where sample volume is the primary limiting factor.

4.1 Methodological innovations

A key advancement of this workflow is the combination of simple but critical steps that, individually, may not suffice to ensure analytical precision but, when combined, result in good performance. First, flushing vials with dry CO_2-free air prior to conditioning significantly improved precision and accuracy of $\delta^{13}C$-CO_2 analysis. This step appears to remove residual CO_2 that may otherwise fractionate or mix with the sample gas, introducing variability. While the importance of vial preconditioning has been acknowledged (Hardie et al., 2010), our data explicitly quantify its effect on sub-millilitre air samples, showing a reduction in standard deviation from ± 0.54 ‰ to ± 0.09 ‰.

Very low CO_2 background signals (~ 10–15 mV) were occasionally detected in blanks under highly sensitive detection settings despite the instrumental blanking (Paul et al., 2007; Siegwart et al., 2023). Although these signals remain below standard analytical thresholds (~50 mV), they indicate the possible presence of trace atmospheric CO_2 that could, mix with the sample if micro-leakage occurs explaining some of the variability observed in enriched δ^{13}C–CO_2. In practice, the combination of vial flushing, effective sealing, and short-term low-temperature storage appear sufficient to prevent any measurable impact on δ^{13}C–CO_2 and to maintain acceptable analytical precision. These observations underscore the need to control both residual CO_2 and potential atmospheric mixing when working with sub-millilitre air samples (Hardie et al., 2010; Brand et al., 2010; Spötl and Vennemann, 2003).

Second, we evaluated the role of sealing strategy and material integrity. Despite initial assumptions, the use of double septa did not improve performance, applying butyl-rubber compound on top and at the bottom of the cap significantly reduced variability during short-term storage. This highlights the importance of micro-leakage and diffusion processes at small sample volumes, consistent with earlier studies (Kuehfuss et al., 2014), and provides quantitative guidelines for achieving high-precision measurements in this volume range.

4.2 The critical role of temperature in preserving isotopic integrity

Low temperature storage proved essential for maintaining δ^{13}C-CO_2 stability (Fig. 6). While dual-sealing improved short-term stability at room temperature (Fig. 5), only storage at -80°C preserved both precision within the desired ± 0.1 ‰ target and accuracy relative to the working standard (Fig. 6). At this temperature, diffusion, permeation and adsorption–desorption kinetics in elastomeric materials are strongly reduced, effectively preventing isotopic drift and CO_2 loss—consistent with observations from cryogenic CO_2–polymer interaction studies (Ghiara et al., 2025) and low-temperature membrane–cryogenic capture systems (Sreenath and Sam, 2023).

This marked temperature sensitivity reflects the susceptibility of CO_2 to interact with vial surfaces and sealing materials, when stored at ambient conditions, where polymer mobility and gas diffusivity remain high. In contrast, ultra-low-temperature storage effectively immobilises these processes, ensuring that the isotopic composition remains unchanged over time. This has direct implications for field and laboratory workflows, as it enables flexible sampling schedules and batch processing without compromising data quality.

This method of storage is particularly advantageous for long-term analyses, where maintaining the stability of the isotopic signal is essential. Under these conditions, the vials can be stored for extended periods (Fig. 7), ensuring the integrity of the samples.

4.3 Limits of storage time and implications for experimental design

Although low temperatures substantially reduce isotopic drift and preserve sample integrity, (Fig. 6), our data indicate that this protection is not indefinite. After one week at -80°C, δ^{13}C-CO_2 precision remains acceptable, but beyond two weeks, a gradual deterioration in precision is observed and after four weeks, isotopic signals were significantly altered, limiting the window for reliable analysis (Fig. 7). This temporal limit suggests that, while the method supports short-term storage of small atmospheric volume samples, it cannot fully substitute for real-time or near-term analysis. High-frequency sampling campaigns in remote or difficult-to-access locations must therefore plan to analyse samples within 7–10 days post-collection, or risk losing analytical resolution.

Interestingly, the observed shifts in δ^{13}C-CO_2 were sometimes bidirectional, with both increases and decreases relative to the target values. This pattern suggests that deviations are unlikely to result solely from systematic drift and may instead reflect residual diffusion through vial materials, minor pressure differences in the headspace, or limited isotopic fractionation associated with CO_2 adsorption and desorption on vial surfaces or elastomeric seals. Studies on CO_2 interactions with polymeric and elastomeric materials demonstrate that adsorption/desorption and permeation processes can occur even at low temperatures, affecting both gas concentration and isotopic composition. Specifically, CO_2 can reversibly adsorb onto polymer and elastomer surfaces, leading to small but measurable changes in headspace composition (Aoki et al., 2022; Schukraft et al., 2022), while diffusion and permeation through elastomers is strongly temperature-dependent but not entirely suppressed under cryogenic conditions (Ghiara et al., 2025; Sreenath and Sam, 2023). The magnitude of these effects can be amplified in small-volume containers due to the limited absolute quantity of CO_2, potentially causing minor isotopic fractionation (Aoki et al., 2022; Schukraft et al., 2022; Ghiara et al., 2025; Sreenath and Sam, 2023).

While bidirectional shifts point away from a single dominant mechanism, many cases exhibit a trend toward ^{13}C enrichment. This pattern may indicate external contamination and/or preferential leakage of lighter ^{12}C-CO_2 from the vials, further supporting the importance of seal integrity and isotope-selective gas exchange in driving these anomalies. Such effects, negligible at larger volumes, become more pronounced at millilitre scale due to the small absolute quantity of CO_2 reinforcing the need to limiting storage duration to no more than 1–2 weeks for high-precision δ^{13}C-CO_2 analysis.

4.4 Broader applications and future directions

The method developed here significantly broadens the scope of stable isotope applications in ecological and environmental research, particularly in settings where sample volume or accessibility is limited. In systems such as rhizosphere or microscale soil flux chambers—where CO_2 concentrations often exceed ambient levels—the method can be further miniaturised, allowing for precise isotopic analysis from sample volumes as small as 50 µL (Siegwart et al., 2023). This capability offers promising opportunities for short-term in situ investigations of carbon dynamics in highly confined or sensitive environments.

While conceptually adaptable to other systems (e.g., δ^{18}O-CO_2), this extension requires careful calibration and validation. δ^{18}O–CO_2 is particularly sensitive to leakage, diffusive fractionation, and CO_2–H_2O exchange, and was not analysed in the present study because no certified standards were available (Steur et al., 2023). δ^{18}O–CO_2 can, however, serve as a useful indicator of analytical issues, and assessing its precision (e.g., standard deviation across experiments) could help identify potential causes of inaccuracy. Its application for routine measurements would therefore require further methodological development and controlled testing.

Initial tests were conducted for off-site sampling at locations remote from the IRMS facility (data not shown). Vials were prepared according to the protocol described in Section 2.1 and transported back and forth at low temperatures using dry CO_2-free air and isothermal boxes containing dry ice or cold packs. Short-term storage (up to 48 h) at –80 °C indicated that $\delta^{13}C$–CO_2 integrity is maintained, suggesting that the method can accommodate flexible sampling schedules without compromising data quality. Longer storage periods, or analyses of other isotopes (e.g., $\delta^{18}O$–CO_2), may behave differently and require further evaluation.

Future work may focus on improving long-term sample stability, for instance by testing alternative sealing materials, as well as automating sample preparation. Potential future applications, such as continuous in situ monitoring or $\delta^{18}O$–CO_2 analysis, provide interesting research directions but require additional validation. By focusing on applications that have been directly tested—short-term $\delta^{13}C$–CO_2 measurements of small-volume samples—the section now presents a realistic and evidence-based perspective. However, we still caution against long-term storage for applications in which very high $\delta^{13}C$–CO_2 accuracy is essential, particularly those involving strict mass-balance approaches, isotope-based mixing models, or paleoclimatic proxies Paul et al., 2023).

Conclusion

This study presents an integrated and accessible workflow for stable carbon isotope ($\delta^{13}C$-CO_2) of atmospheric CO_2 from gas samples as small as 1 mL, achieving an analytical precision of ± 0.1 ‰. By combining simple but rigorous vial preparation, a low-cost dual-sealing strategy using a malleable butyl-rubber compound, low-temperature storage, and cryogenic pre-concentration coupled to continuous-flow IRMS, the method addresses key limitations related to small sample volumes, isotopic drift, and storage stability. Importantly, this method relies on standard based/commercially available materials and equipment, keeping the procedure rapid, reproducible, and financially accessible to a wide range of laboratories.

This approach, requiring only millilitre-scale samples enables high-frequency monitoring of atmospheric $\delta^{13}C$–CO_2 supporting investigation of dynamic carbon processes such as autotrophic/heterotrophic respiration, photosynthesis, and carbon fluxes driven environmental factors like temperature, humidity, or vegetation composition (Bowling et al., 2008; Keeling, 1958; Pataki et al., 2003). In addition to controlled environments and microcosm systems, the method is also well-suited for laboratory experiments requiring specific setups—such as those involving soil fauna, or long-term incubations—where minimal disturbance and small sampling volumes are essential. This expands its applicability to studies where experimental constraints make conventional gas-sampling approaches impractical.

Beyond controlled environments, and microcosm systems the method also opens opportunities for field-based applications where sample volume, logistics or sampling frequency are limiting. Potential uses include remote or high-altitude sites, tropical forests or frozen soil.

The ability to store samples at -80°C for up to a week without measurable isotopic drift further enhances logistical flexibility, particularly for multi-site studies, though longer storage periods reduce precision and highlight the need for short-term preservation.

In summary, this workflow provides a robust, precise, and practical solution for $\delta^{13}C\text{-}CO_2$ analysis from small atmospheric air samples, improving the spatial and temporal resolution of carbon cycle research across diverse experimental and natural contexts.

Data availability

Authors contributions

JS and CP designed the project. JS, CP, and MLT carried out experiments. JS and CP analysed the data from IRMS and AM performed statistical analyses. JS, CP, MLT and AM wrote the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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