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Abstract

A common method to estimate the carbon isotopic composition of soil-respired air is to use Keeling plots (d13C versus 1/CO2 concentration). This approach requires the precise determination of both CO2 concentration ([CO2]), usually measured with an infrared gas analyser (IRGA) in the field, and the analysis of d13C by isotope ratio mass spectrometry (IRMS) in the laboratory. We measured [CO2] with an IRGA in the field (n=637) and simultaneously collected air samples in 12 mL vials for analysis of the 13C values and the [CO2] using a continuous-flow isotope ratio mass spectrometer. In this study we tested if measurements by the IRGA and IRMS yielded the same results for [CO2], and also investigated the effects of different sample vial preparation methods on the [CO2] measurement and the thereby obtained Keeling plot results. Our results show that IRMS measurements of the [CO2] (during the isotope analysis) were lower than when the [CO2] was measured in the field with the IRGA. This is especially evident when the sample vials were not treated in the same way as the standard vials. From the three different vial preparation methods, the one using N2-filled and overpressurised vials resulted in the best agreement between the IRGA and IRMS [CO2] values. There was no effect on the 13C-values from the different methods. The Keeling plot results confirmed that the overpressurised vials performed best. We conclude that in the cases where the ranges of [CO2] are large (>300 ppm; in our case it ranged between 70 and 1500 ppm) reliable estimation of the [CO2] with small samples using IRMS is possible for Keeling plot application. We also suggest some guidelines for sample handling in order to achieve proper results.
Can we use the CO₂ concentrations determined by continuous-flow isotope ratio mass spectrometry from small samples for the Keeling plot approach?

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A common method to estimate the carbon isotopic composition of soil-respired air is to use Keeling plots (δ¹³C versus 1/CO₂ concentration). This approach requires the precise determination of both CO₂ concentration ([CO₂]), usually measured with an infrared gas analyser (IRGA) in the field, and the analysis of δ¹³C by isotope ratio mass spectrometry (IRMS) in the laboratory. We measured [CO₂] with an IRGA in the field (n = 637) and simultaneously collected air samples in 12 mL vials for analysis of the ¹³C values and the [CO₂] using a continuous-flow isotope ratio mass spectrometer. In this study we tested if measurements by the IRGA and IRMS yielded the same results for [CO₂], and also investigated the effects of different sample vial preparation methods on the [CO₂] measurement and the thereby obtained Keeling plot results. Our results show that IRMS measurements of the [CO₂] (during the isotope analysis) were lower than when the [CO₂] was measured in the field with the IRGA. This is especially evident when the sample vials were not treated in the same way as the standard vials. From the three different vial preparation methods, the one using N₂-filled and overpressurised vials resulted in the best agreement between the IRGA and IRMS [CO₂] values. There was no effect on the ¹³C-values from the different methods. The Keeling plot results confirmed that the overpressurised vials performed best. We conclude that in the cases where the ranges of [CO₂] are large (>300 ppm; in our case it ranged between 70 and 1500 ppm) reliable estimation of the [CO₂] with small samples using IRMS is possible for Keeling plot application. We also suggest some guidelines for sample handling in order to achieve proper results.

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Soil respiration is considered to be the main contributor to ecosystem CO₂ efflux in temperate ecosystems.¹² Little is known, however, about the respiratory components of soil CO₂ effuxes, due to problems associated with partitioning soil respiration into autotrophic (roots and rhizosphere) and heterotrophic respiration (microorganisms decomposing litter and soil organic matter).¹³ Modelling and analysis of the soil CO₂ efflux and its response to climatic conditions require separate estimations of these flux components. Litter and soil organic matter (SOM) decomposition are key processes in the carbon cycle and it has been shown that leaf litter is decomposed faster than SOM.¹⁰ However, the contribution of litter decomposition to soil respiration has still not been well investigated. The carbon isotopic signatures of soil-respired CO₂ have been shown to be useful tools for the partitioning of soil respiration.⁷,⁸ As the soil matrix constitutes a porous system, respired CO₂ is mixed with atmospheric CO₂. Hence the isotopic signature of gas samples represents a mixture of both CO₂ sources. In order to differentiate the sources, the so-called Keeling plot approach is frequently applied to estimate the δ¹³C values of the respired CO₂.⁹,¹² In this approach the reciprocal values of the CO₂ concentrations and the δ¹³C values of gas samples are regressed linearly. Usually, the [CO₂] is measured simultaneously with the sampling of soil air. The most commonly used method is to measure with an infrared gas analyser (IRGA) in the field (e.g.⁶,¹³). Alternative ways to measure the [CO₂] in the lab were introduced by Bowling et al.,¹⁴ who used an IRGA, and by Breecker and Sharp,¹⁵ who used a dual-inlet mass spectrometer coupled to a gas chromatograph. Yet, isopotic ratio mass spectrometry (IRMS) determines the [CO₂] in addition to the isotopic ratio and it has been shown in a lab study¹⁶ that there is no difference between IRMS and the use of an IRGA when estimating the [CO₂]. Therefore, replacement of IRGA field measurement with IRMS analysis in the lab should be possible. This would make the field work less costly and
easier, providing that the measurements are reliable and that the results lead to precise Keeling plots. Furthermore, it is not always possible to make IRGA measurements, e.g. soil tubes with a small volume would draw too much air from the sampling volume and alter the soil [CO₂]. Consequently, we compared the [CO₂] values from IRMS measurements and the IRGA values of several hundred soil air samples (n = 637) from a field study during the 2007 growing season. With this study we pursued three objectives: (1) to test whether IRMS measurements yield the same results as the IRGA, (2) to investigate the possible effects of different vial preparation methods on the [CO₂] measurement, and (3) to investigate the effects of the different preparation methods on the obtained Keeling plot results (= ¹³C values of the source).

EXPERIMENTAL

Field sampling and IRGA measurements

The experimental sites were located in grassland 400 m above sea level. One year before the start of the experiment we installed PVC rings (diameter 20 cm × 5 cm) permanently on 12 plots to measure soil CO₂ fluxes as well as the ¹³C signatures of the soil-respired CO₂. At the beginning of the 2007 growing season we applied 0.7 kg m⁻² of ¹³C-depleted or undepleted biomass (Lolium perenne and Trifolium repens mixtures from a free air CO₂ enrichment study²⁷ with isotopic difference ~10‰) on the plots (6 plots per treatment) in order to partition soil respiration. During the whole experiment the rings were covered with net material (25 × 25 cm, mesh size 4 mm) to avoid the removal of any litter or the introduction of new litter and to allow free soil air diffusion. The rings were spatially distributed in an area of approximately 30 × 30 m. We made biweekly measurements of the soil CO₂ efflux over the whole growing season in 2007 (April–October), using an absolute, non-dispersive IRGA (LI-8100; Li-Cor Inc., Lincoln, NE, USA). The IRGA was calibrated regularly with N₂ and two CO₂-in-air standards with [CO₂] 340 and 986 ppm (±1%). Oztech, Safford, AZ, USA. We collected air samples directly from the chamber (V = 4823.9 cm³). The chamber was modified with five exchangeable rubber septa (maximal use = 5 times) for sampling the soil air with syringes while simultaneously making the soil CO₂ efflux measurement. For the measurement of ¹³CO₂ from the closed chambers we sampled ~15 mL of air five times during a 15 min period with a 20 mL syringe equipped with a 0.4 × 25 mm needle (Plastipak syringe and 27G × 1” needle; Becton Dickinson, Fraga, Spain). The air sample was injected into a previously evacuated special glass vial (12 mL exetainer gas testing vials, cappied with airtight rubber septa, cat. #738W; Labco Ltd., High Wycombe, UK). We used the dry air [CO₂] values obtained by the IRGA as our reference; the non-dispersive, infrared gas analysers produce highly reliable data sets for analysis and the LI-8100 is especially suitable for field work.²⁸,²⁹

Vial preparation

The vials were prepared in the lab by different methods (as detailed below) in order to avoid contamination of the samples by ambient air and to prolong their useful storage life before the analysis. All vials were evacuated to 2 × 10⁻² hPa, flushed with N₂ for ~30 s and evacuated again. They were now ready for use (i.e. the taking of a ~15 mL of air sample). To investigate the possible effects of different vial preparations we conducted an experiment on the above prepared vials using three different methods:

M1: With this method we added ~14 mL N₂ just before leaving for the field campaign (i.e. before the air samples were injected into the vials); this was also done with the standard vials.

M2: For the second method the sample vials were treated as in M1, but the standard vials were only pre-evacuated before the CO₂-in-air standards were injected (to demonstrate the importance of identical preparation of the standard vials).

M3: For the third method we used only pre-evacuated vials without N₂-filling for all standard and sample vials. The last evacuation step was done directly before the sample injection in the field and before the CO₂-in-air standards were injected.

With M1 and M3 the standards were left with overpressure before the analysis (M1: ~15 mL standard gas + 14 mL N₂ and M3: ~15 mL standard gas); with M2 the standard vials were depressurised with a needle before analysis. The [CO₂] in our CO₂-in-air standards was 340 and 986 ppm (±1%).

Storage and isotope analysis

The air samples were analysed within 0–4 days after sampling. If we could not analyse the samples immediately, they were stored in a desiccator under a pure N₂ atmosphere (desiccator was flushed with N₂ for ~2 min after placement of the samples). We found no significant effect of storage time for up to 120 h, in agreement with the findings of Laughlin and Stevens and Werner et al.³⁰,³¹ (data not shown). The air samples were collected without further pre-treatment, e.g. water removal. In order to test whether water vapour in our samples affected the measured [CO₂], we added 0.1 mL of water to vials filled with CO₂-in-air standards (400 and 999 ppm; n = 3). This produced a saturated water vapour atmosphere in the vials. We determined the peak areas of dry and moist CO₂-in-air standards that were prepared according to M3 (see above). For all IRMS analyses the CO₂ of the gas samples was purified by an automated online preparation and introduction system (Gasbench II; ThermoFinnigan, Bremen, Germany) for the isotope ratio mass spectrometer. The system consists of an autosampler for 12 mL glass vials (CombiPAL; CTC Analytics, Zwingen, Switzerland), a diffusion tube for water removal (Nafton™), a loop injection system, a liquid nitrogen trap for cryofocusing, a gas chromatograph and an open split transfer interface (ThermoFinnigan). The gas sampling system includes a two-port needle which adds a flow of He into the sample vial, thus diluting and displacing sample gas. After concentrating the CO₂ in the liquid nitrogen trap, the δ¹³C is determined with a Delta Plus continuous-flow isotope ratio mass spectrometer (ThermoFinnigan). The [CO₂] of every analysed sample was calculated from the calibration curve prepared with CO₂-in-air standards (340 and 986 ppm); these standards were measured at least three times each during an analysis (before the first sample, after the first half of the samples, and at the end of the analysis). The area under the voltage signal peak of the isotope ratio mass spectrometer

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for CO₂ (in units of Vs) was determined for masses 44, 45 and 46. The sum of these areas was used as a measure of the [CO₂].²² While most of the concentration is due to mass 44, the contribution of the minor isotopes was also considered and all calculations were based on the sum of the peak areas 44, 45 and 46. The [CO₂] calculation from the mass spectrometer signal needs careful calibration because the peak size depends on measurement parameters, such as freezing time, flow rates and pressure in the sample vial. We used several CO₂-in-air standards from Oztech for calibration of the isotopic values versus V-PDB and performed comparisons with other laboratories for verification. The $\delta^{13}$C values of CO₂ are reported in the delta notation and referenced to the international V-PDB standard.

Keeling plots
Keeling plots were made by regressing ($\delta^{13}$C versus 1/ [CO₂]), ideally, 15 gas samples taken within three 15-min sampling periods. The y-intercept gives the $\delta^{13}$C of the respiratory CO₂ source. We used least squares regression (LSR) to perform the Keeling plots, as our results always gave $R^2 > 0.95$ and previous studies have demonstrated that, in this case it is not necessary to use geometrical mean regression (GMR), as recommended by Pataki et al.,¹² if $R^2 < 0.95$.²³

RESULTS AND DISCUSSION
Measurement precision
Figure 1 shows a typical calibration curve with the sum of the peak areas (44, 45, 46) obtained by IRMS by injecting the CO₂-in-air standards with 340 and 986 ppm [CO₂] (n = 5). In this example the average area for the 340 ppm standard is 4.13 ± 0.04 Vs (mean ± standard deviation), while the area is $12.71 ± 0.17$ Vs for the 986 ppm standard (mean ± standard deviation). Therefore the [CO₂] can be determined with a precision of ±1–1.3% (3.46–13.14 ppm) within the concentration range of about 300–1000 ppm. We calculated the accuracy of the [CO₂] measurement for M1 as the difference between the IRMS and IRGA analyses (n = 54) according to Schauer et al.,¹⁶ and found it to be 4.86 ppm. Schauer et al.,¹⁶ obtained a precision of 0.12% (0.48 ppm) and an accuracy of −0.26 ppm when IRGA and IRMS analyses were performed on the same sample using 100 mL vials. In our study we compared data obtained by in situ field IRGA measurements with IRMS measurements of 12 mL samples made in the lab. Our results show that despite several potential sources of error (e.g. small variations in sampling volume, differences of sample air from chamber air, etc.) relatively good agreement between the IRMS and IRGA measurements can be obtained.

A comparison of peak areas determined for dry versus moist CO₂-in-air standards showed that the water vapour had no significant influence on the results (Table 1).

Comparison of [CO₂] from soil air samples measured with IRMS and the IRGA
An important observation is that soil air samples taken with our standard vial preparation method M2 (Fig. 2) and

![Figure 1. Typical example of a calibration curve for [CO₂]. CO₂ peak areas (Σ (44, 45, 46)) of two CO₂-in-air standards (340 and 986 ppm; n = 5) determined by IRMS. The corresponding regression curve is used for the calculation of the [CO₂] of the samples.](image)

<table>
<thead>
<tr>
<th>Standard gas</th>
<th>F-value</th>
<th>p-value</th>
<th>dry</th>
<th>moist</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 ppm</td>
<td>2.713</td>
<td>0.175</td>
<td>4.77 (0.07)</td>
<td>5.38 (0.36)</td>
</tr>
<tr>
<td>999 ppm</td>
<td>0.007</td>
<td>0.939</td>
<td>13.88 (0.37)</td>
<td>13.81 (0.68)</td>
</tr>
</tbody>
</table>

![Figure 2. [CO₂] samples taken in the field and measured by IRMS versus direct measurement of the [CO₂] by the IRGA in the field (y = 0.705x + 65.321; R² = 0.91; p < 0.0001; n = 637).](image)
measured by IRMS always gave lower values of [CO₂] than when measured using the IRGA. The comparison of [CO₂] measurements showed that the IRMS results were, on average, 20% lower than those by the IRGA. This underestimation is obviously due to the different vial preparations for sample and standard vials. The sample air is diluted and the standard gas is not.

Comparison of different vial preparations for field sampling and for lab standards
We tested with a one-way analysis of variance (ANOVA) if the three different methods of vial preparation (M1–M3) had an effect on the accuracy of the [CO₂] measurement by IRMS (accuracy defined as above) and found that the methods significantly influenced the accuracy \((p < 0.0001)\). From the three different methods, vial preparation method M1 (where the sample air and CO₂-in-air standards were injected into vials which were filled with 14 mL N₂) gave the best agreement between the IRMS and IRGA data (Fig. 3). Our results show that the M2 vial pre-treatment yielded very different results from the other two methods whereas methods M1 and M3 differed only slightly from each other. Vial preparation method M2 always resulted in an underestimation of [CO₂] when measured by IRMS. It seems evident that the sample and standard vials must receive an identical pre-treatment to obtain accurate results. Another one-way ANOVA comparing only M1 and M3 still showed a significant difference in accuracy of the two methods \((p < 0.0001)\). Our data indicate that the M1 preparation procedure yields a better correspondence between the IRMS and IRGA [CO₂] determinations than M3; thus, it seems appropriate to fill the vials with N₂ and have an overpressure in them. The comparison showed that N₂-filled and overpressurised vials led to a better [CO₂] quantification by IRMS than with only pre-evacuated vials. The most likely reason for this is the lower contamination from ambient air that is unable to enter the vial through the punctured septa.

Comparison of \(\delta^{13}C\) values measured with different vial preparations (M1 and M3)
For vial preparation methods M1 and M3 we investigated their effect on the \(\delta^{13}C\) measurement by IRMS. Because of the natural temporal variability of [CO₂] (sampling done with ~15 min time lags) we took into account only sample pairs that did not differ by more than 15 ppm (as the \(\delta^{13}C\) values systematically decrease with increasing [CO₂]). When we plotted the \(\delta^{13}C\) values obtained from the two preparation methods (M1 and M3) against each other, the data matched the 1:1 line quite well (Fig. 4). We found no significant effect from the preparation procedures M1 and M3 on the \(\delta^{13}C\) values (paired t-test: \(p = 0.61\)). M1 showed an advantage regarding the [CO₂] measurement (see above) making it the best method for obtaining reliable concentrations and isotope values; therefore, we recommend it for field applications.

Comparison of the Keeling plot intercepts obtained with IRMS and the IRGA
To support the previous recommendation we compared the Keeling plot intercepts (\(\delta^{13}C\) of the respired CO₂) obtained from the three different vial preparation procedures. We found, once again, that the data from M1 came closest to the 1:1 line, especially when the measurement uncertainty is taken into account (Fig. 5). The mean standard error of the Keeling plot intercepts (\(n = 4\)) was 0.67‰ for the IRGA (range: 0.44–0.80‰) and 1.00‰ for IRMS (range: 0.67–1.34‰).

By comparing this dataset with previous measurements made during the growing season we found that large

![Figure 3](image-url)  
**Figure 3.** The effect of the three different vial preparations on the [CO₂] as measured by IRMS and the IRGA. M1 (x-symbols): \(y = 0.973x + 8.437; \ R^2 = 0.95; \ p < 0.0001; \ n = 54\). M2 (solid triangles): \(y = 0.683x + 91.789; \ R^2 = 0.99; \ p < 0.0001; \ n = 60\). M3 (open circles): \(y = 0.725x + 101.717; \ R^2 = 0.94; \ p < 0.0001; \ n = 59\).

![Figure 4](image-url)  
**Figure 4.** Resulting \(\delta^{13}C\) values when the vials are prepared in different ways: M1 (N₂-filled vials) and M3 (pre-evacuated vials): \(y = 0.911x - 1.141; \ R^2 = 0.94; \ p < 0.0001; \ n = 37\).
CO₂ concentrations determined from small samples by IRMS. Pataki et al.\textsuperscript{12} showed at a lower scale for ecosystem respiration. During the vial preparation experiment the CO₂ ranges were always relatively low and lay between 73 and 474 ppm. A regression (standard error of the Keeling plot intercept = \( a + b/\text{CO}_2 \) range) testing the effect of CO₂ range on the standard error of the intercept showed that the CO₂ range significantly influenced the standard error of the intercept in both cases (for the IRGA and IRMS: \( p < 0.0001 \)). The data in Fig. 6 suggest that, for soil respiration, to maintain a standard error in \(^{13}\text{C} \) estimation with Keeling plots below 0.5\%, a CO₂ range of approximately 300 ppm should be obtained. The low fluxes could be explained by dry (during summertime) or cold conditions (late autumn). In addition to the low fluxes the spatial and temporal heterogeneity of the soil respiration rate and of the isotopic source contribute to the uncertainty in ecosystem studies. Together with increasing standard errors of the intercepts the accuracy also decreased (the difference between IRMS and IRGA [CO₂] measurements increased).

CONCLUSIONS

A reliable estimation of the [CO₂] with IRMS measurement simplifies the field work and lowers the costs. It also enables the application of the Keeling plot approach in situations of limited air volume (e.g. when using soil tubes) and where measurements with an IRGA are not possible. The proposed procedures also ensure that isotopic values and [CO₂] are obtained from exactly the same sample. Our results show that this is possible, provided that the following points are considered:

1. The vials for the sample air and the standard gas must be identically pre-treated (i.e. filled with N₂).
2. When collecting air samples identical volumes must be injected for sample air and standard gas such that an overpressure is created. The overpressurisation ensures improved results, probably because backdiffusion of ambient air during sample storage is minimised.

3. Sampling design in the field should ensure a [CO₂] range of at least 300 ppm.

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