Minimization of sample requirement for $\delta^{18}$O in benzoic acid

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The measurement of the oxygen stable isotope content in organic compounds has applications in many fields, ranging from paleoclimate reconstruction to forensics. Conventional High-Temperature Conversion (HTC) techniques require >20 µg of O for a single $\delta^{18}$O measurement. Here we describe a system that converts the CO produced by HTC into CO$_2$ via reduction within a Ni-furnace. This CO$_2$ is then concentrated cryogenically, and ‘focused’ into the isotope ratio mass spectrometry (IRMS) source using a low-flow He carrier gas (6–8 mL/min). We report analyses of benzoic acid (C$_7$H$_6$O$_2$) reference materials that yielded precise $\delta^{18}$O measurement down to 1.3 µg of O, suggesting that our system could be used to decrease sample requirement for $\delta^{18}$O by more than an order of magnitude.

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The measurement of the oxygen stable isotope composition of organic compounds such as cellulose, chitin, sucrose and keratin is of great utility for several fields (Table 1). Numerous recent studies have reported the $\delta^{18}$O of organic compounds and discussed implications for paleoclimate.$^{1–8}$ plant-water relationships,$^{1,4,5,9}$ wildlife migration,$^{10}$ human provenancing,$^{11–13}$ geolocation,$^{14,15}$ sourcing of explosives,$^{15,16}$ and food authentication.$^{17–19}$

Offline techniques for converting organic oxygen into CO$_2$, for subsequent introduction into an isotope ratio mass spectrometer via a dual-inlet system included sample oxidation with mercuric chloride at 500–550°C to produce CO, CO$_2$, and HCl.$^{20–22}$ The HCl is then removed via reaction with quinoline,$^{20}$ ZnCl$_2$,$^{23}$ or absorption by Amberlyst IRA-93, a macroreticular resin (Sigma-Aldrich, St. Louis, MO, USA).$^{24}$ The CO is converted into CO$_2$ either by a high-voltage discharge$^{21,22}$ or by disproportionation using a nickel catalyst.$^{25}$ An alternative offline method involved thermal decomposition of the sample between 950 and 1125°C within a nickel pyrolysis tube,$^{26,27}$ producing CO, CO$_2$ and H$_2$. The H$_2$ fraction is removed via diffusion through the nickel tube, and a high-voltage discharge converts CO into CO$_2$. These techniques were time-consuming and required large quantities of sample material per analysis (3 to 15 mg).

Within the last 15 years, automated continuous flow methods featuring High-Temperature Conversion (HTC) have become routine for the $\delta^{18}$O analysis of organics.$^{28–30}$ HTC methods are rapid, cost-effective, require smaller sample sizes, and attain precision comparable with those of the traditional offline methods.$^{30}$ During HTC, the sample is decomposed at 1080–1450°C in a reduction furnace. The resulting mixture of CO, H$_2$ and N$_2$ is carried by a stream of helium for separation in a gas chromatography (GC) column. Oxygen enters the isotope ratio mass spectrometer as CO and the $\delta^{18}$O value is determined by measuring the signal intensities of the CO$^+$ ions at m/z 28 and 30. The amount of sample required for these online analyses varies between 0.05 and 1.5 mg, or $\geq$20 µg of oxygen per sample, depending on substrate composition and instrument sensitivity.

Reduction of sample size to below current HTC requirements would be useful for a wide range of $\delta^{18}$O applications. At present, the amount of alpha-cellulose required for $\delta^{18}$O analysis precludes all but the largest ring-width fossil wood.$^6$ Thus a reduction in sample size requirements would enable the resolution of seasonal variations within a ring, potentially including the sub-millimeter thick rings commonly seen in modern bristlecone pine.$^{31}$ In addition, a wide range of forensic applications would benefit from reduced sample size requirements for $\delta^{18}$O in organics, such as fine-scale sampling along a single strand of hair for human geolocation and travel history.$^{11,14}$ Trace evidence such as pollen,$^3$ explosives$^{15,16}$ and anthrax spores$^{32}$ may be best sampled with swab collection, which results in very small samples available for isotopic analysis. Here we report on our efforts to decrease the current minimum sample requirement by more than one order of magnitude for the online analysis of $\delta^{18}$O in benzoic acid. We performed this by catalytically converting HTC-produced CO into CO$_2$, cryofocusing it, and introducing it as CO$_2$ into the isotope ratio mass spectrometer (Fig. 1).

THE CRYONIC SYSTEM

In order to reduce required sample amounts, it is necessary to increase the amount of oxygen-containing sample gas that reaches the isotope ratio mass spectrometry (IRMS) source. Conventional HTC produces CO, which is then carried directly to the IRMS instrument. If the CO that is produced is instead converted into CO$_2$, condensed, and then introduced into the IRMS ion source using a low flow of helium, the ultimate proportion of CO$_2$ to carrier gas entering the source...
is greatly increased. The disproportionation of CO to CO$_2$ is catalyzed by reduced Ni at 600°C.$^{33}$ The emerging CO$_2$ is condensed and thus concentrated using a liquid nitrogen (LN$_2$) cryotrap, also known as ‘cryofocusing’.$^{34,35}$ Therefore, we have dubbed the entire system ‘CryoNiCat’ (Fig. 2).

General operating principles for the HTC process have been described previously.$^{28,29}$ For this study, a Thermo Chemical Elemental Analyzer (TCEA) was used as the HTC unit, and the stable isotope measurements were made with a DeltaV Advantage stable isotope ratio mass spectrometer (both from Thermo Fisher, Bremen, Germany). The glassy carbon reactor tube was contained within a ceramic (Al$_2$O$_3$) outer tube, with glassy carbon chips filled to the hot zone and set to 1370°C. A graphite crucible collected the molten silver capsules and ash. The helium carrier gas (99.999%) entered the HTC unit at a flow rate of 90 mL/min. A Zero-Blank autosampler (Costech Analytical, Valencia, CA, USA) was used to minimize atmospheric blank contributions. The factory-installed TCEA GC column (5 Å molecular sieve, 0.6 m long) was used to separate H$_2$ from CO gas at 100°C. A Carboxsorb (Elementar Americas, Mt. Laurel, NJ, USA) and magnesium perchlorate trap was installed between the glassy carbon reactor and the gas chromatograph to remove trace amounts of CO$_2$ and H$_2$O generated during thermal decomposition.

The Ni-reactor was based on the design of Loader and Buhay;$^{33}$ the reactor consisted of a 40 cm long, 9 mm o.d. piece of quartz tubing (7 mm i.d.) with Ni powder (stock #10255; Alpha Aesar, Ward Hill, MA, USA) packed lightly to comprise an 8 cm length in the center of the tube. Quartz chips made up the remainder of the reactor volume with quartz wool plugs at each end. The reactor was enclosed in a standalone furnace (Applied Test Systems Inc., Butler, PA, USA) with a variable autotransformer used to control the temperature. The temperature was maintained at 600°C, according to the recommendation of Loader and Buhay.$^{33}$ Prior to using the nickel reactor, it was necessary to condition the nickel by flowing H$_2$ (99.999%) over it while at operating temperature to remove surface oxides via reduction. A three-way valve was installed upstream so that a flow of either He or H$_2$ gas could be flushed through the reactor. The outflow from the nickel furnace was diverted to a vented gas cabinet.

![Figure 1](Image)

**Figure 1.** Fate of oxygen in benzoic acid (C$_7$H$_6$O$_2$) within the CryoNiCat system.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Application</th>
<th>Routine sample size</th>
<th>Advantage of reduced sample size capability (e.g., reduction to ~1 µg O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-cellulose</td>
<td>paleoenvironment reconstruction</td>
<td>50 µg O$^6$</td>
<td>intra-ring analyses for tree rings; fossils with low cellulose content</td>
</tr>
<tr>
<td>explosives</td>
<td>source determination; production pathway</td>
<td>80–260 µg O$^{15,16}$</td>
<td>trace amounts on swabs</td>
</tr>
<tr>
<td>fly larvae</td>
<td>paleoenvironment reconstruction</td>
<td>&gt;20 µg O (~50–120 head pieces)$^2$</td>
<td>individual insects; approaches</td>
</tr>
<tr>
<td>hair</td>
<td>geolocation; provenancing</td>
<td>20–60 µg O$^{11,14}$</td>
<td>1 head piece</td>
</tr>
<tr>
<td>pollen</td>
<td>paleoenvironment reconstruction</td>
<td>not reported$^3$</td>
<td>~2 days of hair growth</td>
</tr>
</tbody>
</table>

Figure 2. Schematic of the ‘CryoNiCat’ system. Carbon monoxide (CO) exits the conventional HTC system within a He carrier gas at a flow rate of 90 mL/min. The CO is converted into CO$_2$ via catalytic disproportionation in the Ni reactor, then frozen under LN$_2$ and ‘focused’ into the mass spectrometer, carried by a He flow rate of only ~8 mL/min. Thus, the initial 10:1 split for sample gas that is normally present has been eliminated such that ~10× more sample gas arrives at the mass spectrometer ion source, allowing for precise determination of $\delta^{18}$O in small samples.

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RESULTS OF BENZOIC ACID (C₇H₅O₂) ANALYSIS

We sought to gain precise and correct δ¹⁸O values for benzoic acid (C₇H₅O₂) to illustrate the utility of the CryoNiCat system. We used two benzoic acid reference materials: IAEA-601 benzoic acid with δ¹⁸OVsmow = 23.14‰ and Jahren Lab benzoic acid (‘JLB’), an in-lab reference material with δ¹⁸OVsmow = 20.68‰, as determined by conventional HTC. We chose these materials because their δ¹⁸O values bracket the mean δ¹⁸O value seen in alpha-cellulose from Eocene wood, our major application of interest.⁵,⁷,³⁶ There exists a second IAEA-consensus benzoic acid (IAEA-602 with δ¹⁸OVsmow = 71.28‰), but it has been shown to exhibit isotopic heterogeneity, probably as a result of the equilibration and re-crystallization process necessary to generate an enriched isotopic value.⁶ Our goal was the successful analysis of a 5 µg size solid benzoic acid sample, which is barely enough to be seen on the tip of a fine-scale weighing spatula. Because we could not be sure of the homogeneity of IAEA-602 at this level, we chose to exclude it from our study. We also excluded IAEA-600 caffeine (C₇H₁₄O₄) because it yielded mixed results in the inter-laboratory comparison, presumably due to the confounding effects of NO (mass 30) generated in the ionization source of the mass spectrometer.⁵⁰,³⁶,³⁸ Later in this paper we discuss the larger implications of both heterogeneity and N-content in samples for the implementation of CryoNiCat.

Two benzoic acid reference materials (IAEA-601 and JLB) were analyzed in size-series, which varied from 5 to 71 µg (=1.3 to 18.6 µg of O). The benzoic acid samples were weighed into 3.3 × 5 mm silver capsules (IVA Analysetechnik, Düsseldorf, Germany) using an M2P microbalance (Sartorius Mechatronics, Goettingen, Germany). Empty silver capsules were incorporated every 3 samples for blank assessment and subtraction; the sample size was randomly distributed within the lineup. The tray of weighed capsules was desiccated under vacuum for 5 days prior to transfer to the Costech Zero-Blank autosampler. Once loaded, the autosampler was purged for 10 min with He through a vent valve to remove atmospheric gases. After purging, the vent valve was closed, and the autosampler opened to the HTC He carrier gas flow. In preparation for the analysis sequence, the source tuning was optimized for δ¹⁸O linearity to obtain less than 0.06‰ total variation within the ion current intensity range of 0.5 to 4 V. The analysis sequence occurs as the following: (1) at 0 s sample drops into glassy carbon reactor; (2) at 105 s the cryofocusing loop is immersed in LN₂ (−196 °C); (3) after ~115 s the CO₂ exits the Ni reactor; (4) at 180 s the four-way valves (A and B within Fig. 2) are switched to divert the HTC flow towards vent, and a low flow of He carrier (6–8 mL/min) through the cryotrap and on to the IRMS ion source; and (5) at 240 s, after pressure and flow stabilization, the cryotrapping loop is thawed.

The ion current intensity for each analysis ranged between 0.5 and 3.1 V, within the pre-determined linearity range of the instrument. However, at the lowest sample sizes, the blank accounted for between 45 and 55% of the peak areas. This ‘blank’ CO₂ originates from four potential sources: (1) impurities from the silver capsule, such as surface oxides; (2) CO produced from the reaction of C with Al₂O₃ within the HTC instrument;³⁶,⁴⁰ (3) atmospheric oxygen diffusing through the porous Al₂O₃ reactor tube and reacting with C; and (4) trace amounts of CO₂ in the He carrier gas.

For these reasons it was important to perform a blank correction using the stable isotope composition of the blank as determined from the two end-member mixing model described by Gelwicks and Hayes.⁴¹,⁴² The measured stable isotope value (δ₀) is a combination of the procedural blank delta value (δₐ) and the true sample value (δₛ), weighted by the integrated area of the peak signal (A) of each contribution (M, B and S subscripts, respectively):

\[ A_Mδ_M = A_Bδ_B + A_Sδ_S \] (1)

Within the above, AM and AB can be determined by direct measurement. Because the peak areas are additive, it follows that:

\[ A_S = A_M - A_B \] (2)

thus allowing us to determine AS. The only remaining unknowns in Eqn. (1) are δₐ and δₛ.

Substitution of Eqn. (2) for AS within Eqn. (1) gives:

\[ δ_M = (A_B(δ_B - δ_S)/A_M) + δ_S \] (3)

We then performed a regression of δₐ on 1/Aₐ using multiple analyses (i.e. a size-series) to determine the slope (m = A_B(δ_B - δ_S)/A_M) and intercept (y = δ_S). A value for δₐ can now be determined, which can then be used for blank corrections on single unknown samples. Our results showed that A_B varied between different batch runs, probably due to variability in the amount of contact between the glassy carbon reactor and the Al₂O₃ reactor, which is affected by warping of reactor tubes during each cooling and heating cycle, that is necessary for reactor maintenance. Within a single batch-run the blank contribution was extremely consistent.

Figures 3 and 4 show the relationship between the sample size and signal intensity, and the δ¹⁸O of benzoic acid for IAEA-601 and JLB, respectively. Both graphs show excellent reproducibility of the δ¹⁸O value between peak areas of 10 and 60 V·s, after correction for the blank using the methods described above. This corresponded to a minimum of 1.3 µg of O required for analysis. For IAEA-601, the mean (raw) value of 9 samples ranging in size from 1.3 to 16.5 µg O was 22.71‰ with a standard deviation of 0.14‰; this was only a −0.43‰ offset from our calibrated CO₂ reference gas. For JLB, the mean value of 7 samples ranging in size from 1.3 to
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operate the system at a lower temperature (e.g., 1080 °C), thus reducing diffusion of atmospheric oxygen through the $\text{Al}_2\text{O}_3$ reactor tube, and also decreasing the rate at which $C$ reacts with the $\text{Al}_2\text{O}_3$ reactor tube. However, this other approach must be applied with caution, due to the potential for incomplete reduction of sample $O$ to $CO$ at lower reaction temperatures.

As with all stable isotope techniques, it is important to optimize a particular analysis protocol in order to adhere to the principle of identical treatment, and CryoNiCat is no exception. As an example, the hygroscopicity of cellulose and the potential for equilibration with environmental air makes it critical to use similar reference compounds. In addition, as the sample size decreases, ensuring the homogeneity of each capsule’s content becomes important, and handling issues such as static electricity can cause difficulty.

The CryoNiCat system might be an optimal method for determining the $\delta^{18}$O value of nitrogen-containing organic compounds. Currently, use of conventional HTC is complicated by isobaric interferences at $m/z$ 30 from NO produced in the IRMS source. Since the CryoNiCat system determines $\delta^{18}$O using $\text{CO}_2$ ($m/z$ 44 and 46), as opposed to $\text{CO}$ ($m/z$ 28 and 30), isobaric interference at $m/z$ 30 is not an issue. In addition, $N_2$ does not react with $O$ when in contact with Ni at elevated temperatures, thus preventing the production of $\text{NO}_2$ ($m/z$ 46) and/or fractionation due to oxygen reactions. CryoNiCat may also be extendable to the measurement of $\delta^{18}$O in inorganic substances (e.g., nitrates, phosphates, etc.). The very low sample size afforded by CryoNiCat makes possible the application of $\delta^{18}$O analysis to new substrates and/or scientific questions. More experiments with different compounds are needed to confirm the possibility of wide application of the technique.

Acknowledgements

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REFERENCES


