RCM

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 ($C_7H_6O_2$) 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. Copyright © 2010 John Wiley & Sons, Ltd.

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,[11,14] 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 CRYONICAT 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

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**Table 1.** Potential advantages of sample reduction size for existing applications

Sample	Application	Routine sample size	Advantage of reduced sample size capability (e.g., reduction to ~1 µg O)
alpha-cellulose	paleoenvironment reconstruction	50 µg O[6]	intra-ring analyses for tree rings; fossils with low cellulose content
explosives	source determination; production pathway	80–260 µg O[15,16]	trace amounts on swabs
fly larvae	paleoenvironment reconstruction	>20 µg O (~ 50–120 head pieces)[2]	individual insects; approaches 1 head piece
hair	geolocation; provenancing	20–60 µg O[11,14]	~2 days of hair growth
pollen	paleoenvironment reconstruction	not reported[3]	~100 pollen grains per analysis

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_2O_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 *Carbosorb* (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_2O 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
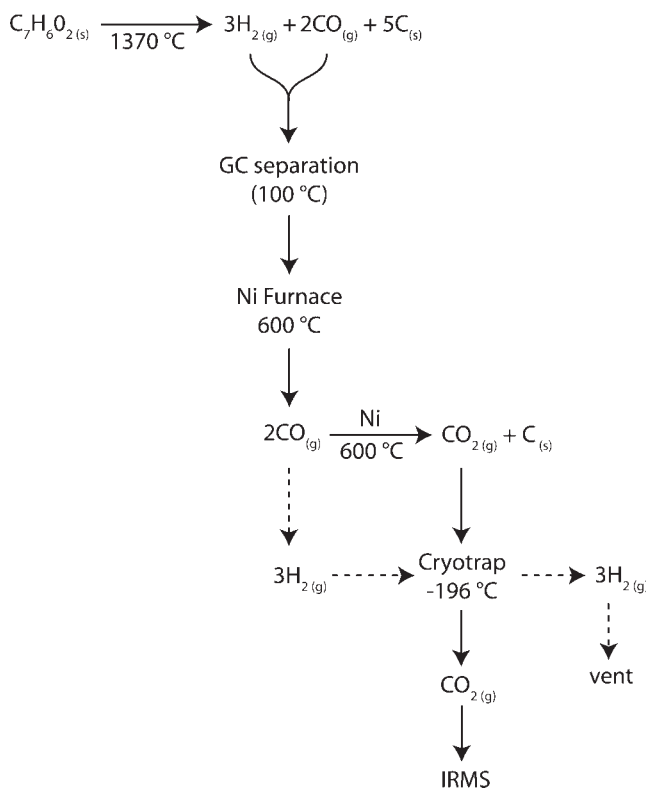
Figure 1. Fate of oxygen in benzoic acid ($C_7H_6O_2$) within the CryoNiCat system.
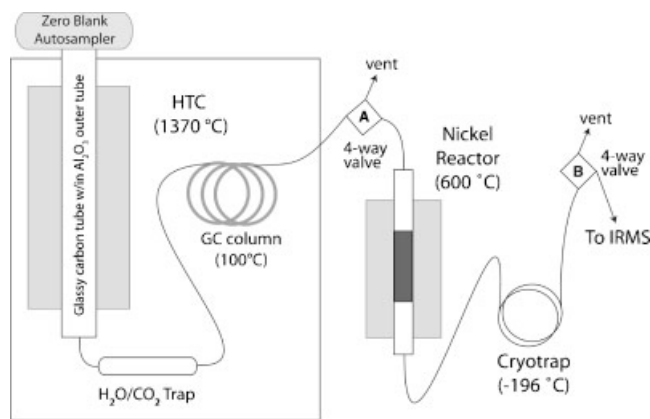
Figure 2. Schematic of the 'CryoNiCat' system. Carbon monoxide (CO) exits the conventional HTC system within a He carrier gas at a flow rate of 90 mL/min. The CO is converted into CO_2 via catalytic disproportionation in the Ni reactor, then frozen under LN_2 and 'focused' into the mass spectrometer, 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.

for safety. The H_2 flow could then be switched to He, thus maintaining an oxygen-free environment at all times.

The cryotrap was configured inline at the outflow of the Ni reactor, and consisted of 0.6 m of 1/16'' stainless steel capillary tubing (0.8 mm i.d.) coiled into 5 cm diameter loops that can be immersed in a Dewar of liquid nitrogen (LN_2). Two manually operated four-way valves (Swagelok, Solon, OH, USA: catalog #B-43YF2) were placed inline (positions A and B within Fig. 2) in order to switch between the high-flow output of the HTC system (90 mL/min) and a low-flow He option (6–8 mL/min). This eliminated the need for the initial 10:1 sample split at the HF1 input at the ConfloIV (Thermo Fisher) interface.

RESULTS OF BENZOIC ACID ($C_7H_6O_2$) ANALYSIS

We sought to gain precise and correct $\delta^{18}O$ values for benzoic acid ($C_7H_6O_2$) to illustrate the utility of the CryoNiCat system. We used two benzoic acid reference materials: IAEA-601 benzoic acid with $\delta^{18}O_{VSMOW} = 23.14\permil$[36] and Jahren Lab benzoic acid ('JLB'), an in-lab reference material with $\delta^{18}O_{VSMOW} = 20.68\permil$, as determined by conventional HTC. We chose these materials because their $\delta^{18}O$ values bracket the mean $\delta^{18}O$ value seen in alpha-cellulose from Eocene wood, our major application of interest.[6,7,37] There exists a second IAEA-consensus benzoic acid (IAEA-602 with $\delta^{18}O_{VSMOW} = 71.28\permil$), 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.[36] 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 ($C_8H_{10}N_4O_2$) 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.[30,36,38] 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 Analysentechnik, 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 $\delta^{18}O$ 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 LN_2 (−196°C); (3) after ∼115 s the CO_2 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 cryotrap 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' CO_2 originates from four potential sources: (1) impurities from the silver capsule, such as surface oxides; (2) CO produced from the reaction of C with Al_2O_3 within the HTC instrument;[39,40] (3) atmospheric oxygen diffusing through the porous Al_2O_3 reactor tube and reacting with C; and (4) trace amounts of CO_2 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 (δ_M) is a combination of the procedural blank delta value (δ_B) and the true sample value (δ_S), weighted by the integrated area of the peak signal (A) of each contribution (M, B and S subscripts, respectively):

$$A_M\delta_M = A_B\delta_B + A_S\delta_S \tag{1}$$

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

$$A_S = A_M - A_B \tag{2}$$

thus allowing us to determine A_S. The only remaining unknowns in Eqn. (1) are δ_B and δ_S.

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

$$\delta_M = (A_B(\delta_B - \delta_S)/A_M) + \delta_S \tag{3}$$

We then performed a regression of δ_M on $1/A_M$ using multiple analyses (i.e. a size-series) to determine the slope ($m = A_B(\delta_B–\delta_S)$) and intercept ($y = \delta_S$). A value for δ_B 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 Al_2O_3 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 $\delta^{18}O$ of benzoic acid for IAEA-601 and JLB, respectively. Both graphs show excellent reproducibility of the $\delta^{18}O$ 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 CO_2 reference gas. For JLB, the mean value of 7 samples ranging in size from 1.3 to
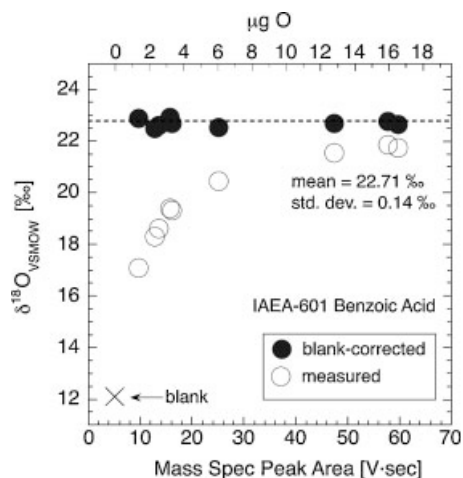
Figure 3. Oxygen isotope composition of IAEA-601 benzoic acid ($C_7H_6O_2$) plotted against sample size. Average blank-corrected $\delta^{18}O_{VSMOW} = 22.71‰$; standard deviation = 0.14‰; consensus value = 23.14‰.

18.6 µg O was 20.33‰ with a standard deviation of 0.14‰; this was only a −0.35‰ offset from our calibrated CO_2 reference gas. The consistent, and small, offset between the δ_S determined using CryoNiCat and the accepted values suggests that the CryoNiCat system can successfully analyze ~1 µg of O – an order-of-magnitude reduction in the sample sizes commonly analyzed by online HTC techniques.

CONSIDERATIONS FOR APPLICATION OF CRYONICAT TO ORGANIC 'UNKNOWNS'

Because the blank represented a large proportion of the signal (45–55%) at the lowest sample sizes, it is necessary to perform a size-series on a known reference material during each run. However, alternative approaches may lower this blank signal, such as the use of a molybdenum foil liner to block interaction between C and Al_2O_3 within the reduction furnace of the HTC system.[39] Another approach would be to
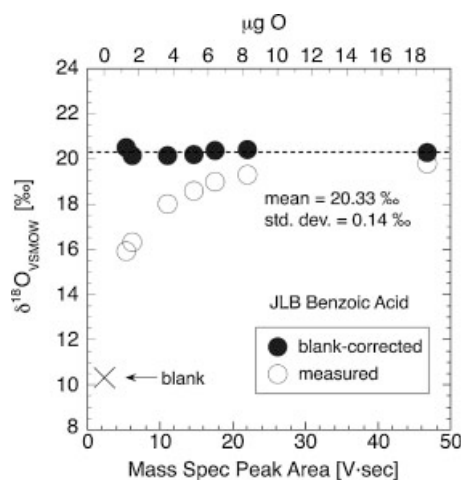

Figure 4. Oxygen isotope composition of JLB benzoic acid ($C_7H_6O_2$) plotted against sample size; average blank-corrected $\delta^{18}O_{VSMOW} = 20.33‰$; standard deviation = 0.14‰; conventional-HTC value = 20.68‰.

operate the system at a lower temperature (e.g., 1080°C), thus reducing diffusion of atmospheric oxygen through the Al_2O_3 reactor tube, and also decreasing the rate at which C reacts with the Al_2O_3 reactor tube.[43] 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.[44]

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,[45] 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.[46] 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.[30,36,38] Since the CryoNiCat system determines $\delta^{18}O$ using CO_2 (m/z 44 and 46), as opposed to 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,[25] thus preventing the production of NO_2 (mass 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.

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