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A High-Throughput Method for the Conversion of CO_2 Obtained from Biochemical Samples to Graphite in Septa-Sealed Vials for Quantification of ^{14}C via Accelerator Mass Spectrometry

Ted J. Ognibene,* Graham Bench, and John S. Vogel

Center for Accelerator Mass Spectrometry, Lawrence Livermore National Laboratory, Livermore, California 94551

Graham F. Peaslee

Department of Chemistry, Hope College, Holland, Michigan 49423

Steve Murov

Science, Math and Engineering Division, Modesto Junior College, Modesto, California 95350

The growth of accelerator mass spectrometry as a tool for quantitative isotope ratio analysis in the biosciences necessitates high-throughput sample preparation. A method has been developed to convert CO_2 obtained from carbonaceous samples to solid graphite for highly sensitive and precise ^{14}C quantification. Septa-sealed vials are used along with commercially available disposable materials, eliminating sample cross contamination, minimizing complex handling, and keeping per sample costs low. Samples containing between 0.25 and 10 mg of total carbon can be reduced to graphite in ∼4 h in routine operation. Approximately 150 samples per 8-h day can be prepared by a single technician.

Accelerator mass spectrometry (AMS) is a highly sensitive isotope ratio spectrometry that uses a high-voltage accelerator to destroy interfering molecular isobars.[1,2] Attomole (10^{-18}) amounts of ^{14}C can be quantified, with high precision, in milligram-sized biochemical samples (e.g., 25 μL of blood). Small sample sizes permit expanded experimental resolution through frequent blood draws, extensive chemical fractionation, or selective biological dissection. This sensitivity allows the detailed tracing of nutrients, toxins, and therapeutics in humans and animals with less than microgram per kilogram chemical doses containing nanocurie (nCi) exposures to ^{14}C.[3,4] Hundreds of samples whose ^{14}C levels can vary over several orders of magnitude can be generated. Methods for preparing samples for measurement must be high-throughput processes in which cross contamination is eliminated through careful design protocols. Per sample costs should be kept low and human intervention minimized. Additionally, the method must be able to treat the wide variety of biological matrixes equally to ensure the chemical and physical equivalence for all carbon atoms in the samples. For ^{14}C-AMS, solid graphite is used most often as the sample material for quantification.

Graphite samples for AMS quantification are most commonly prepared by the reduction of carbon dioxide by hydrogen onto a catalytic iron or cobalt surface at temperatures around 500 °C.[5−9] Reduction of CO_2 to a fullerene "graphite" proceeds rapidly, and yields of >95% are routinely obtained. Samples containing as little as 20 μg of carbon can be converted to graphite. The graphite quickly produces intense, long-lasting negative ion beams upon introduction to the cesium sputter ion source. Isotopic and mass fractionation are extremely small. Since inception in the mid-1980s, many AMS facilities have studied this process as part of the development of their sample preparation laboratories; presenting details of their procedures in the AMS[10−15] and Radiocarbon[16−21]

* Corresponding author: (phone) 925-424-6266; (fax) 925-423-7884; (e-mail) ognibene1@llnl.gov.

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conference proceedings.

A method for the rapid production of graphite from carbonaceous biochemical samples was developed by Vogel in 1992.[8] It uses disposable gas manifolds to transfer combustion products to a borosilicate tube that is sealed by a torch for subsequent reduction of the CO_2 to graphite. Conversion yield is ~80%, with bulk isotopic fractionation in the finished graphite less than 0.5%. With this method, 300 samples/week per technician can be processed and our laboratory has prepared over 60 000 samples. The use of disposable materials minimizes sample cross contamination. However, the torch-sealing of the transferred combustion products necessitates dexterous handling, is somewhat time-consuming, and uses custom-made components. It would be desirable to replace this step with a more time-efficient technique that minimizes human involvement and the use of a torch.

This paper describes a method for preparing filamentous graphite from CO_2 gas in septa-sealed vials. This method increases sample preparation throughput while maintaining our success in graphite preparation. This new method also reduces the amount and complexity of handling required with each sample, which leads to lower process backgrounds and allows for the preparation of smaller sized samples. The use of septa-sealed vials will form the basis for an integrated automated system in which biochemical samples can be converted to graphite for ^{14}C quantification via AMS.

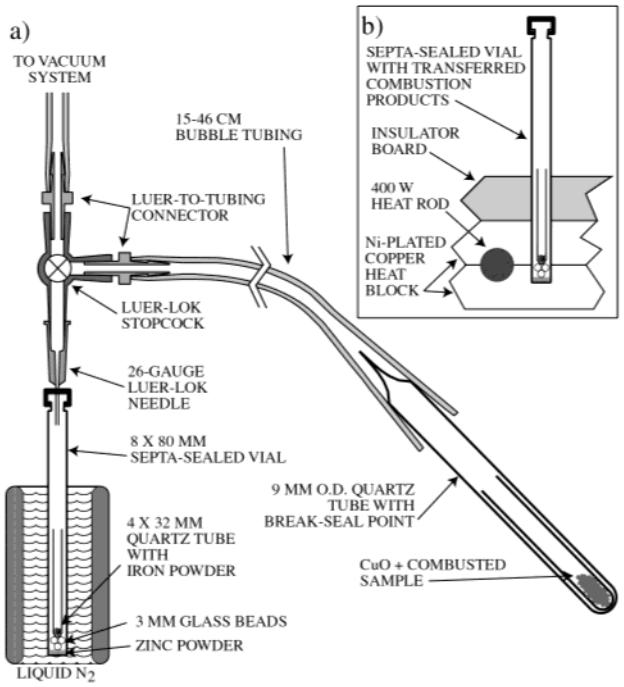

Figure 1. Schematic representation of (a) the transfer system and (b) the block heater for the preparation of biochemical samples for ^{14}C-AMS measurements.

EXPERIMENTAL SECTION

Procedures. The biochemical sample is dried in a vacuum centrifuge in a new 6 mm × 50 mm quartz glass culture tube. A few tens of milligrams of wire-form cupric oxide are added to the dried sample, and the culture tube is placed inside a 9 mm × 150 mm quartz combustion tube, which has a break-seal point. The tubes are flamed-sealed under vacuum and placed in a 900 °C furnace for 2 h. Individual racks isolate tubes from each other in the event of overpressurization causing a tube to burst. In any event, postcombustion tubes should be handled with caution as they may contain gases with up to 4 atm pressure. After heating, the cooled tube is connected to a Luer-Lok stopcock with $^1/_2$-in.-i.d. bubble tubing (Oxford Labware), as depicted in Figure 1a. A small amount of silicone grease produces a vacuum-tight seal between the glass tube and the plastic tubing. Attached to one end of the stopcock is a 26-gauge needle that is inserted into the 8 × 80 mm septa-sealed borosilicate glass vial containing a small amount of zinc dust (Sigma-Aldrich; 20,998-8) and a 3 mm i.d. x 30 mm long borosilicate glass tube (Scientific Instrument Services, Ringoes, NJ; SPV5000). Inside the tube is 2−3 mg of −400-mesh iron powder (Sigma-Aldrich; 25,563-7). Separating the glass tube and the zinc are several 3-mm borosilicate glass beads. After evacuation, the valve is closed to isolate the system from the vacuum pump and the tip of the combustion tube is broken. More than 95% of the condensable combustion products (mainly, CO_2 and H_2O) are cryogenically trapped with liquid nitrogen into the septa-sealed vial in less than 20 s. Noncondensing gases, such as N_2, are then removed by the vacuum pump. The vial is pulled off the needle, and the lower 3 cm is inserted into a block heater held at 500 °C.

A cross-sectional view of the reduction heater is shown in Figure 1b. The vial sits in holes drilled into two 1.3-cm-thick copper blocks that have been plated with 0.13-mm nickel. Approximately 5 cm of vial remains above the reduction oven surface to prevent heating of the septa. At this distance, without additional cooling, the septa remain only slightly warm to the touch, while the bottom 3 cm of the vial is heated to 500 °C. The copper blocks are insulated on the sides and bottom by 6−10 cm of machinable ceramic (Cotronics, Brooklyn, NY; RESCOR 310) and on the top by a 13-mm-thick ceramic foam board (Cotronics, Brooklyn, NY; RESCOR 360). Heat is supplied by 400-W (at 120 VAC) 9.5-mm-diameter Firerod cartridge heaters (Watlow, St. Louis, MO). Temperature regulation is through a K-type thermocouple inserted into one of the holes normally occupied by a vial. The output of the thermocouple serves as the input to a temperature controller (Watlow, St. Louis, MO; Series 935), which drives a 120 VAC solid-state relay to supply up to 1200 W of power to the heating elements. Although only one heater and one vial is shown, each 12-in.-long heat rod can accommodate multiple vials. Our oven, which consists of two heat rods to heat 67 samples, reaches its normal operating temperature of 500 °C in 30 min.

The 8 × 80 mm long septa-sealed glass vials were produced from our design by Kimble Kontes Scientific Glassware (Vineland, NJ). The vials are overcrimped and PTFE/Silicon septa are used to minimize losses due to leakage. Under these conditions, the vials will hold up to 10 atm of carbon dioxide gas (~11 mg in the 2.25-mL volume at room temperature) with a very low leak rate (typically a few percent per hour at the highest pressures). While this much CO_2 could potentially pressurize the vials to almost 400 psi at 500 °C, we found that excessive gas leaked through needle

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punctures in the septum, rather than the catastrophic explosion of the vial. Reduction temperatures are kept below 600 °C to prevent gas pressure from causing extreme deformation of the glass vials, which impedes their removal from the oven.

All materials shown in Figure 1 that come into direct contact with the sample are disposable and commercially available with a total cost of approximately $5.50 U.S. per sample; $3 U.S. of which is due to the quartz glass combustion tubes. Heretofore, the materials cost were approximately $9 U.S. per sample.

Graphitization of the sample is >95% complete in less than 4 h. The graphite/iron mixture is then pounded into a 2.5-mm-deep hole drilled into a 9.5 mm × 29 mm long aluminum target with a fresh No. 55 drill stem. The $^{14}C/C$ isotope ratio is quantified by accelerator mass spectrometry. Details of our spectrometer are presented elsewhere.[22] In general, three to seven replicate measurements of either 10 000 ^{14}C counts or 30-s analysis time are conducted to obtain at least 3% precision. As AMS measures isotope ratios, all measurements are normalized to similarly prepared and measured standards of known carbon isotope ratios. The biosciences AMS program at LLNL uses graphitized ANU (Australian National University) sucrose, with an accepted $^{14}C/C$ ratio of 1.5081 modern[23] as the primary standard. The unit modern[24] is a well-defined number equal to a $^{14}C/C$ isotope ratio of 1.180×10^{-12}, which is close to the naturally occurring ^{14}C concentration in the biosphere. This unit is also equivalent to 97.8 amol of ^{14}C/mg of C and 226 μBq of ^{14}C/mg of C.

Experiments. While the basic chemistry of the reduction process remains the same, we developed modifications to the previous preparation procedures. Hydrogen increases throughput by shortening the reduction time in the vial, but excess hydrogen can lead to methane production and loss of quantitative conversion. Vogel used 10−40 mg of TiH_2/mg of carbon as a hydrogen gas source while carefully removing all water from the combusted sample.[8]

We rely on the hydrogen present in carbonaceous biochemical samples. For example, dried biological tissue can be roughly approximated by the chemical formula $C_5H_9O_2N$. Upon combustion, the hydrogen is oxidized to H_2O. Zinc metal is used to reduce the hydrogen to H_2 gas. The samples are dried before combustion, removing any excess of hydrogen from wet tissues. In practice, it is easier to transfer most of the water from the combustion tube than to prevent most of it from transferring. Retention of the water for use as a reductant worked well for this application, but it may not be compatible with tissue samples that are not dried before combustion or when conducting a ^{14}C, ^{3}H dual-labeling experiment. In the case of the former, there might be too much hydrogen present, while in the latter, the water is separately trapped for tritium-AMS quantification.[25]

We determined the range of carbon masses that can be routinely prepared in septa-sealed vials. Additionally, we verified that the graphite produced by this method continues to yield the high-precision and high-accuracy measurement capabilities of AMS.

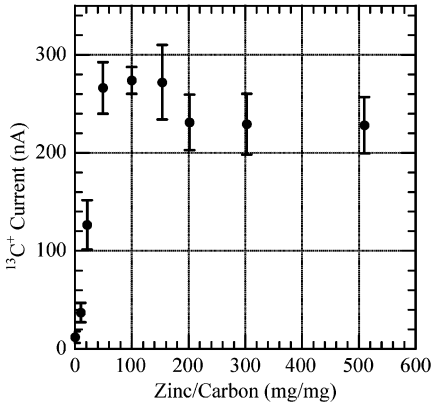

Figure 2. Average $^{13}C^+$ current with respect to the amount of zinc powder added to the septa-sealed vials. Error bars are the $\pm 1\sigma$ standard deviation on the average of three replicate samples.

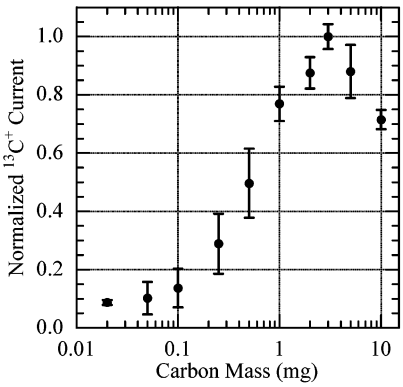

Figure 3. Analyzed $^{13}C^+$ current with respect to precombusted carbon mass of sucrose samples. Error bars are the $\pm 1\sigma$ standard deviation on the average of at least three replicate samples.

RESULTS AND DISCUSSION

Reductant Quantities. Figure 2 shows a plot of analyzed $^{13}C^+$ current for graphitized sucrose (Sigma-Aldrich, S-9378) containing 0.5 mg of carbon as the amount of zinc metal powder was varied. All other conditions were kept constant. Approximately 75−150 mg of zinc/mg of carbon maximizes satisfactory graphite production. A band of zinc metal condenses at the cooler regions of the septa-sealed vials near the top of the foam insulator sheet, extending 1−2 cm downward. Excessive quantities of zinc increased the thickness of this band to the extent that some of the zinc would be incorporated into the graphite/iron mixture at the bottom of the inner glass tube. The presence of zinc in the graphite decreases sputtering yields in the ion source. In some cases, the inner vial would be trapped in the resolidified zinc metal, preventing recovery of the graphite sample. The addition of the 3-mm glass beads elevates and separates the glass vial from the zinc metal.

Carbon Mass Range. Figure 3 shows a plot of analyzed $^{13}C^+$ current of graphite with respect to precombusted carbon mass of sucrose (Sigma-Aldrich, S-9378) samples with all other conditions (i.e., amount of zinc, reduction time, and temperature) kept fixed. For large sample masses, geometrical factors of the cathode and the cesium sputter ion beam prevent an ever-increasing output current as the amount of graphite increases. It is also possible that there is insufficient zinc in the reduction vial to maximize graphite yield. Dilution of the iron with graphite reduces the

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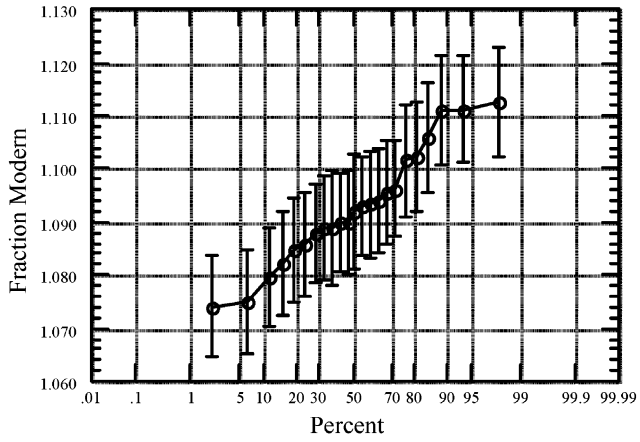

Figure 4. Cumulative probability plot of fraction modern of 23 samples of mouse liver tissue. Error bars are the $\pm 1\sigma$ standard deviation on the average of the three to seven replicate measurements.

thermal and electrical conductivities of the iron/graphite mixture, limiting the effectiveness of the sputtering process.[6] As the amount of carbon in the sample decreases, the ion current goes to a maximum as the optimal iron-to-graphite ratio is obtained. After this maximum, the analyzed current drops as dilution of the graphite with excess iron limits sputtering efficiency. This trend, however, does not continue and another mechanism must be invoked to explain the results seen for the smallest samples. There is no pretreatment of the iron, zinc, or glass to remove dissolved or absorbed atmospheric CO_2. This background carbon dioxide, albeit at small levels, may be reduced along with the carbon dioxide from the combusted sucrose sample, effectively raising the graphite yield. The measured carbon isotope ratios of this graphite are not significantly different from that of the larger mass samples, as both sources have nearly contemporary levels of ^{14}C. Brown and Southon quantified such incorporation.[26]

The data indicate that a routine procedure in the preparation of samples containing at least 250 μg of carbon can produce graphite in sufficient yields for ^{14}C quantification via AMS. Samples containing more than 10 mg of carbon risk loss through explosive overpressurization of the 9 × 150 mm sealed combustion tubes or excessive leakage of the septa-sealed vials. While graphite has been produced from samples containing <250 μg of carbon; the ion currents were sufficiently low that high measurement throughput would be compromised. Special techniques are required to maximize yield and ion source output. These might include the use of smaller septa-sealed vials, a corresponding lesser amount of reactants, and pretreatment of the glass and reductants.

Reproducibility and Reliability. Twenty-three individual samples of mouse livers, each containing between 2 and 10 mg of carbon were combusted and subsequently converted to graphite. Figure 4 shows a probability plot of the fraction modern determined from the measured $^{14}C/C$ isotope ratios. The data fits a normal Gaussian distribution with a high correlation coefficient ($r = 0.99$), indicating that the data set represents a single distribution. That distribution has a mean of 1.0928 modern and a standard deviation of 0.0107 or 0.98%. This width is slighty less than the average of the $\pm 1\sigma$ error bars on the individual data points. These data confirm that the new technique is able to produce graphite from individual identical biological samples reliably and with a high degree of reproducibility.

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Table 1. Fraction Modern Values and Percent Differences for a Group of Samples Prepared through Torch-Sealed Tubes and Septa-Sealed Vials[a]

	average fraction modern		
sample	torch-sealed	septa-sealed	difference (%)
tributyrin	0.0891 ± 0.0010	0.0854 ± 0.0018	4.2
sucrose	1.1316 ± 0.0052	1.1367 ± 0.0075	−0.5
ANU sucrose	1.5179 ± 0.0074	1.5105 ± 0.0011	0.5
plant extract	9.1748 ± 0.1530	9.3784 ± 0.0935	−2.2

[a] See text for details.

Comparisons of Carbonaceous Material. Six samples of tributyrin (ICN Pharmaceuticals, Inc.; 10311), sucrose (Sigma-Aldrich; S-9378), ANU sucrose, and ^{14}C-elevated plant extract, each containing between 2 and 10 mg of carbon each were combusted in sealed quartz tubes. Three of each were transferred to and reduced to graphite in septa-sealed vials while graphite from three of each was prepared in torch-sealed tubes via the method of ref 8. The $^{14}C/C$ isotope ratios of all samples were measured by AMS. These four samples were chosen as their ^{14}C levels span the range of the majority of biochemical samples typically measured by AMS. The results of this comparison are presented in Table 1. The last column shows the percent difference in the averages for each method with a positive value indicating that the torch-sealed method gave a higher fraction modern. The torch-sealing method gave relatively high values for the fraction modern values compared to the septa-sealed vials for the tributyrin sample. A plausible explanation for this is that less background contamination is present in the septa-sealed vials. The vials have less surface area than the tubes used for torch-sealing, which could lower the amount of ambient CO_2 that gets absorbed onto the glass. This CO_2, which would have contemporary levels of ^{14}C, would be desorbed at high temperatures and reduced to graphite during the reduction.[26] However, a more detailed experiment, with finer control of the carbon inventory and the environmental conditions would need to be conducted in order to confirm this hypothesis.

CONCLUSIONS

A method has been developed in which CO_2 from biochemical samples is transferred to and reduced to graphite in septa-sealed vials for ^{14}C measurement by accelerator mass spectrometry. A single recipe can be employed to prepare samples with a range of carbon between 0.25 and 10 mg and that span a wide range of ^{14}C levels. Sample cross contamination is eliminated through the extensive use of disposable Luer accessories and tubing with relatively low costs per sample. The system is amenable to high throughput as over 150 samples per 8-h day can be processed by a single technician. Septa-sealed vials will form the basis for an automated sample preparation system in which combustion products from a commercial carbon analyzer will be trapped and subsequently reduced to graphite. Such automated processes should greatly improve throughput and reduce the amount of

sample handling leading to the routine preparation of samples with as little as 20 μg of carbon.

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