Protocol P-13-42

Diatom Cleaning by Nitric Acid Digestion with a Microwave Apparatus

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1. PURPOSE

1.1. To identify and enumerate diatoms accurately at the species and variety levels, it is necessary to remove both extracellular and intracellular organic matter from the siliceous frustules of diatoms and other material in the sample. Removing the organic matter is necessary so that all details of diatom structures necessary for taxonomic identification are clearly visible. This protocol describes a method for removing organic material from a sample by digesting it with nitric acid in a microwave apparatus.

1.2. Traditional nitric acid digestion methods utilize a hotplate to heat samples and acid in open beakers. The procedure described herein takes advantage of a microwave apparatus to heat the acid/sample mixture in closed containers. This procedure produces a cleaner sample (more complete digestion) with less contamination, and is safer and more convenient (much less use of acid) (Acker et al. 1993).

2. SCOPE

2.1. This procedure is applicable for cleaning diatoms from a wide variety of samples including, but not limited to, periphyton samples from diatometer slides or other artificial substrates, collections from natural substrates, surface sediment or sediment core samples, and net or whole water collections of phytoplankton. Material to be cleaned may be in a moist, dry or preserved state. If material to be processed contains preservatives or other chemical substances, refer to the cautionary notes in sections *5* and *7.14*.

2.2. This procedure applies to personnel responsible for preparing diatom slides for taxonomic or community analysis purposes.

2.3. This procedure applies to the use of Advanced Composite Vessels (CEM Corporation).

2.4. The procedure for diatom sample digestion using nitric acid and a microwave apparatus has been employed by the ANSP since 1992. In 1998, Lined Digestion Vessels were replaced by Advanced Composite Vessels because of their durability. The procedures described above evolved from nitric acid/hotplate digestions performed at the ANSP for over 40 years. The acid/hotplate procedures are described in ANSP Protocol No. P-13-02 and may be used occasionally for single samples.

2.5. If samples are essentially free from organic detritus, and critical taxonomic work is not required (e.g., multiple phytoplankton samples where the taxonomy is well known), this nitric acid digestion method may not be necessary. A hydrogen peroxide method (not described here) or burn mount procedure may be considered in these cases. However, for the majority of applications, the nitric acid digestion method is recommended.

3. REFERENCES

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3.2. American Public Health Association, American Water Works Association, Water Environment Federation (APHA, AWWA, WEF). 1992. Standard Methods for the Examination of Water and Wastewater. 18th ed.

3.3. J.T. Baker Chemical Company. Saf-T-Training Manual and Tests.

3.4. CEM Corporation. Instructions for Use of Lined Digestion Vessels (P/N 323000 Rev. 0).

3.5. CEM Corporation. Instructions for Use of Advanced Composite Vessels (P/N600214 Rev. 1).

3.6. Patrick, R. and C. Reimer. 1967. Diatoms of the United States. Vol 1. Monograph No. 13, Academy of Natural Sciences of Philadelphia. 688 pp.

3.7. PCER, ANSP. 2002. Analysis of Diatoms in USGS NAWQA Program Quantitative Targeted-Habitat (RTH and DTH) Samples. Protocol No. P-13-39.

3.8. PCER, ANSP. 2002. Log-In Procedures for USGS NAWQA Program Algal Samples. Protocol No. P-13-47.

3.9. PCER, ANSP. 2002. Subsampling Procedures for USGS NAWQA Program Periphyton Samples. Protocol No. P-13-48.

3.10. PCER, ANSP. 1988. Diatom Cleaning by Nitric Acid Digestion. Protocol No. P-13-02.

3.11. PCER, ANSP. 1994. Subsampling and Determination of Wet and Dry Weights of Lake Sediment Samples. Protocol No. P-13-43.

3.12. PCER, ANSP. 1990. Laboratory Safety Manual.

4. DEFINITIONS

4.1. **Digestion** in this procedure refers to the solubilization of organic material by strong acid oxidation.

4.2. Diatom cells, called **frustules**, are composed of two **valves**. They have a siliceous structure, the features of which are used for taxonomic identification.

5. APPARATUS/EQUIPMENT

5.1. Positive-draw fume hood.

5.2. Safety glasses.

5.3. Acid-impervious hand protection.

5.4. Laboratory coat or apron, acid resistant.

5.5. Microwave apparatus (CEM Model MDS-2100):

5.5.1. 0-950 watts (1% intervals).

5.5.2. Controlled and monitored temperature (fiber optics) and pressure.

5.5.3. Programmability for different cycles of temperature, pressure, and time at various temperature and pressure combinations.

5.5.4. Rotating turntable.

5.6. Closed microwave digestion vessel [see Figure 1 "Standard Advanced Composite Vessel (Cross Section)"]:

5.6.1. Vessel liner (Teflon PFA®).

5.6.2. Vessel liner cover (Teflon PFA®).

5.6.3. Vessel cap (reinforced polyetherimide, microwave "invisible").

5.6.4. Thread ring (reinforced polyetherimide, microwave "invisible").

5.6.5. Sleeve (advanced composite material, microwave "invisible").

5.6.6. Rupture membrane (Teflon PFA®).

5.6.7. Vent tube (Teflon®).

5.6.8. Vent fitting (Teflon PFA®).

5.6.9. Ferrule nut (Teflon PFA®).

5.7. Digestion vessel for temperature and pressure control and monitoring; see Figure 2 "Advanced Composite Vessel for Pressure and Temperature Control." Vessel liner, vessel cap, thread ring, sleeve, rupture membrane, vent fitting and ferrule nuts as in sections *5.6.1*, *5.6.3*, *5.6.4*, *5.6.5*, *5.6.6*, *5.6.7* and *5.6.8*, respectively.

5.7.1. Vessel liner cover (Teflon PFA®) with exhaust, temperature and pressure ports.

5.7.2. Thermowell (Pyrex with Teflon coating).

5.8. Reagent grade nitric acid (~70%).

5.9. Potassium dichromate (crystal form).

5.10. 100-ml tall glass beakers.

5.11. Reverse osmosis water (RO) or distilled water (DW).

5.12. Faucet siphon apparatus.

5.13. 20-ml glass vials; caps with coned liners.

5.14. Vial labels.

5.15. Diamond scribe.

5.16. Wash bottle for RO or DW.

5.17. Single-edged razor blades.

5.18. pH indicator paper.

6. SAFETY PRECAUTIONS

6.1. Nitric acid is an extremely hazardous reagent. As a strong acid oxidizer it can cause severe burning of exposed skin and clothing. At room temperature, concentrated nitric acid produces intense fumes when exposed to open air.

6.2. Any concentrated nitric acid containers open to the air must be contained within a positive-draw fume hood at all times. There are no exceptions to this rule.

6.3. Personnel are required to wear safety glasses, protective gloves and lab coats at all times when handling concentrated nitric acid. This is especially important when handling/venting the digestion vessels.

6.4. When samples are delivered to the Diatom Preparation Laboratory, personnel who will work with the samples must be informed if any preservatives have been used in the samples (e.g., formaldehyde, Lugol's solution, glutaraldehyde, etc.). This information should be on the shipping forms included with the samples or affixed to the shipping container. Consult the Material Safety Data Sheets (MSDS) located on the 2nd floor of ANSP near the Diatom Preparation Laboratory for information on any of the above preservatives and how to handle them properly. Unexpected, violent and/or noxious reactions can occur during the cleaning procedure if nitric acid is mixed with other chemical substances. **Samples are to be rejected by laboratory personnel if their collection history and content are not fully known.**

6.5. **Never** use an Advanced Composite Vessel without a composite sleeve.

6.6. **Never** install more than one rupture membrane in the vessel cover.

6.7. Prior to use, all vessel components must be dry and free of particulate matter. Drops of liquid or particles will absorb microwave energy, causing localized heating which may char and damage vessel components, possibly leading to vessel failure.

7. METHODS

Algal material that requires processing for diatom analysis comes to the preparation lab in several forms, depending on the collection protocol. Diatom collections may come from glass periphytometer slides, tile or other artificial substrates, phytoplankton suspensions, culture material, dried herbarium material and lake sediments. To produce a diatom slide, regardless of the kind of collection, the lab technician must remove all organic materials from the sample so that diatom frustules can be identified. This may require a preliminary examination of the raw sample with a microscope to determine the proper amount to digest. Proper cell densities for diatom slide analysis are described in Protocol No. P-13-39. Procedures for other sample types are discussed below, including samples with preservatives (sections *5* and *7.14*), and samples with carbonates (section *7.14*). This procedure should be started only after all samples are logged in (refer to Protocol No. P-13-47).

Record sample digestion data (Beaker #, Microwave Vessel #, notes) on the appropriate "Diatom Slide Preparation Form" (see, for example, Figure 2 in ANSP Protocol No. P-13-48). For most types of samples this form will have been generated during the process of preparing subsamples, and placed in the "Diatom Analysis" folder. Sample digestion data are not added to a computer database. If a "Diatom Slide Preparation Form" has not been generated, it can be printed using a report in the PHYCLGY database. There are different reports for different types of samples and

projects (e.g., diatometer, survey, NAWQA, sediment), all of which contain "Diatom Prep Form" as the first part of their name. Confirm that all samples to be processed are recorded on the form before proceeding.

7.1. **Glass slides with attached periphyton (e.g., diatometer slides).** Based on the thickness of attached growth, choose one or more slides from the same sampling station or site for the cleaning process (set aside those not used in the cleaning process for eventual curation as "uncleaned material"). Place the chosen slides from individual sites in a single, numbered 100-ml beaker. Record the number of slides to be processed and the respective beaker number on the "Diatom Slide Preparation Form". Fill each beaker with distilled water so as to completely immerse the slides, and allow the slides to soak for a minimum of 12 hours. After the soaking period, use a disposable single-edged razor blade to scrape algae from the slides (including the slide edges) into the beaker. Then dip the slides and razor blade in the water-filled beaker to transfer remaining scraped material from the slides. Using a wash bottle filled with distilled water, carefully rinse any material still adhering to the slides or the razor blade back into the beaker. At this point, cross check the etched label of the slide with the information on the "Diatom Slide Preparation Form" to ensure that the slides have been transferred from the proper field sites and that the date of installation is correct. If at this stage there are any discrepancies, they should be noted on the "Diatom Slide Preparation Form" and resolved. Record the number of sides scraped for each sample.

Allow the material in the beakers to settle for a period of at least 8 hours without being disturbed. Then siphon off the supernatant liquid without disturbing any of the material that has settled to the bottom. In the siphoning process, the tip of the siphon should be placed just beneath the water's meniscus, and moved slowly down as the water level drops, to prevent loss of material through water column turbulence and contamination of the siphon tip. As much water as possible should be removed without disturbing the sedimented material. Proceed to section *7.5*.

7.2. **Water suspended samples (e.g., phytoplankton samples, culture material).** If necessary, concentrate samples by siphoning water off undisturbed samples as described in section *7.1* until the sample contains approximately 20 ml of water and associated material. If a quantitative analysis is required, the original volume of the sample must be recorded. If the sample is too large or too dense to clean in its entirety, a subsample should be taken and processed. Volumes of the subsample and the original sample must be recorded on a "Sample Volume/Subsample" form (e.g., See Protocol P-13-48). Then transfer the sample to 100-ml beakers as in section *7.1*. Proceed to section *7.5*.

7.3. **Sediments (e.g., bottom substrate samples or sediment core samples).** Transfer approximately 0.5 to 1.0 cc of either moist or dry sediment to a 100-ml beaker. If dry, a small amount of water may be added to the sample (approximately 10 ml) to hasten disaggregation. If the sample is to be analyzed quantitatively, record the wet weight, volume, or dry weight of the sample to be processed, according to the specifications of the study protocol. Unless otherwise specified, use procedures and forms in Protocol No. P-13-43. Proceed to section *7.5*.

7.4. **Other.** Samples from other sources (e.g., natural rock substrates; soft sediments from rivers) may also be digested using this procedure. As in the above cases, it is important to begin with a concentrated sample to which nitric acid can be added safely in order to process a large enough sample to yield a sufficient concentration of cleaned diatoms for the required analyses. Care should always be taken to ensure that violent reactions will not

take place upon the addition of acid. For quantitative analyses, the weight, area or volume of the original sample should be noted as required by the protocol.

7.5. **Addition of nitric acid. WARNING. THE FOLLOWING PROCEDURE IS TO BE PERFORMED ONLY IN A POSITIVE-DRAW FUME HOOD. TECHNICIANS ARE REQUIRED TO WEAR SAFETY GLASSES AND PROTECTIVE GLOVES!**

Assemble part of the digestion vessel before adding acid (sections *7.6.1* and *7.6.2*). Place sample into the vessel liner portion of a microwave digestion vessel. Add concentrated nitric acid to the sample in the vessel liner; add an amount of acid equal to the amount of sample. If the sample contains a very high amount of organic material, more acid can be added (routinely the sample is in a 10-ml water matrix requiring the addition of 10-ml nitric acid). Initially, add acid very slowly and with great caution, anticipating that an unexpected reaction may take place. After determining that there is no possibility of a violent reaction, slowly and cautiously add the remainder of the acid to the samples.

7.6. **Preparation, assembly of digestion vessels and connecting to the microwave apparatus.**

7.6.1. Before the assembly of the digestion vessels, new rupture membranes must be seated in the vessel liner covers as illustrated in Figure 3, "Installation and removal of rupture membrane." Make sure that there is only one rupture membrane seated in each vessel liner cover.

7.6.2. Once the rupture membrane is seated, turn the vent stem back into the vessel liner cover until hand tight. Do not apply excess pressure or use a wrench to tighten the vent stem! Sections *7.6.1* and *7.6.2* should be completed prior to the acid addition (section *7.5*).

7.6.3. Place the vessel liner cover (with rupture membrane and vent stem installed) on top of the vessel liner.

7.6.4. Thread the vessel cap onto the thread ring (by turning in a clockwise direction) until hand tight. As with the vent stem assembly, do not apply excess pressure or use a wrench to tighten the vessel cap!

7.6.5. Insert the vent tube into the vessel by threading through the ferrule nut into the vent fitting (located on top of the vessel cover extending above the vessel cap).

7.6.6. Place the complete vessel assembly into a turntable, orienting the vent tube towards and into the collection vessel at the center of the turntable. Record position of vessel (in turntable) on the Diatom Slide Preparation Form.

7.6.7. Complete the vessel assembly by placing an advanced composite sleeve over the liner and under the thread ring.

7.6.8. For the remaining vessels repeat steps *7.6.1* to *7.6.7*. Note the differences for the vessel with the temperature and pressure controls (assembly is similar but with a different type of vessel liner cap and cover); see Figure 4, "Installation of rupture membrane in vent fitting of Advanced Composite Vessel with Temperature/Pressure control cover."

7.6.9. Place turntable with vessels into the microwave apparatus cavity on its drive lug. Turn on microwave apparatus and rotate the turntable. After confirming the operation

of the turntable, rotate so that the vessel with the temperature and pressure sensors is at 12:00 (as looking into the microwave cavity).

7.6.10. Bleed and connect pressure sensing line:

7.6.10.1. Using keypad controls and menu system, rotate turntable to 9:00.

7.6.10.2. Turn pressure valve (outside left of microwave cavity) to **open**.

7.6.10.3. Tap pressure sensing line to get air bubbles to connection end.

7.6.10.4. With the syringe filler (outside left of microwave cavity), flush air bubbles out of the pressure sensing line.

7.6.10.5. Connect pressure sensing line to the pressure port on the temperature and pressure vessel liner cover (use only hand pressure!) and place pressure sensing line in center post of the turntable; see Figure 5, "Routing of pressure sensing line and fiber optic temperature probe."

7.6.10.6. Turn pressure valve to neutral.

7.6.11. **Connect fiber optic temperature probe:** Without bending, place fiber optic probe carefully (note: it is glass!) into the thermowell of the temperature and pressure vessel liner cover and connect (use only hand pressure for the connection!). Thread probe into center of the turntable, similar to the pressure sensing tube; see Figure 5, "Routing of pressure sensing line and fiber optic temperature probe."

7.7. **Configuring the microwave apparatus.**

7.7.1. From the main menu, choose either to load a preexisting program (current program "Diatom New Vessel") or to enter new operating conditions (and new programs) from the keypad.

7.7.2. In the view mode, use the arrow keys and numeric keypad to set the following operating conditions:

Cycle 1 - 25% power, 20 PSI, 5 min @ pressure;

Cycle 2 - 80% power, 60 PSI, 5 min @ pressure;

Cycle 3 - 90% power, 100 PSI, 20 min @ pressure;

Adjust power settings for number of vessels by reducing 3% for each vessel less than a full tray (12 vessels);

Record additional information in the "Sample Information" screen.

7.7.3. Make sure printer is connected and on and print out operating conditions and sample information.

7.8. Start the microwave apparatus; the program will take about 60 min.

7.9. It is crucial that the operator monitor the temperature and pressure controls (readings printed every 30 sec) during the course of the digestion. In stages the temperature will rise from room temperature to about 170°C (up to 90°C in Cycle 1; up to 140°C in Cycle 2; up to 170°C in Cycle 3) and pressure will go from 0 psi to 100 psi (0-25 psi in Cycle 1, 25 to 70 psi in Cycle 2; 70-100 psi in Cycle 3). In the first two cycles, the rise will be even for 5-10 min and then hold near the maximum for 5 min; in Cycle 3 the rise will be fast (within a minute or two) and then hold for 20 min (@ 90-100 psi and 160-170°C). Deviations from

this controlled rise in temperature and pressure could indicate a break in a vessel or problem sample reaction. The digestion should be stopped immediately if either the temperature or pressure does not rise evenly through the cycles.

7.10. Remove samples from the microwave apparatus (after temperature and pressure conditions are back to normal levels; ~30-45 min):

7.10.1. Place the vessel with the temperature and pressure controls at 9:00.

7.10.2. Vent the vessel with the temperature and pressure controls by turning the vent fitting counter- clockwise. Note: gloves, lab coat and eye protection should be worn during venting of vessels.

7.10.3. Carefully take out the fiber optic probe and place in one of the holes near the top of the microwave cavity.

7.10.4. Remove pressure sensing line and place away from the turntable.

7.10.5. Remove turntable from microwave cavity and place in fume hood; carefully vent each of the vessels by slowly turning the vent fitting counter-clockwise.

7.11. Remove vessel caps, vessel liner covers and transfer samples to tall, 100-ml beakers, washing liner with DW or RO from wash bottle. Fill beaker with DW; be sure to check beaker numbers on "Diatom Slide Preparation Form."

7.12. **Decanting procedure.** After 8 hours, siphon off the supernatant in a manner similar to that described in section *7.1.* DW is again added to beakers. Repeat this settling and siphoning procedure at least five more times or until the pH is similar to that of the DW or RO (above 6.5). Note date of each decant on the "Diatom Slide Preparation Form."

7.13. **Transfer procedure.** Carefully swirl the cleaned material remaining in the bottom of each beaker after the final siphoning and then pour it into a 20-ml glass vial which has been previously labeled with Sample ID and SubSample ID. (Labels should be made on the side of the vial using a diamond scribe and on the cap using an indelible marker). Using a wash bottle containing DW or RO water, wash any remaining material adhering to the beaker sides into the vial, and bring the volume of each vial to exactly 20 ml. Cap the vial and store with others until ready to make slides. Initial and date the "Diatom Slide Preparation Form," put it in the "Diatom Analysis" folder, and keep the folder with the samples.

7.14. **Precautions.** Samples containing preservatives or other chemicals should be clearly identified at the time of submission to the diatom laboratory. If chemical additives are suspected, but not indicated on the preparation form, technicians should request confirmation from the Principal Investigator or the Project Leader as to the contents of the samples. If the samples are known to contain formaldehyde, Lugol's solution, glutaraldehyde or any other noxious material, a dilution step should be performed by the addition and siphoning of DW or RO similar to that described in section *7.12* (excluding the consideration of pH) until it is safe to assume that the added chemical or chemicals have been significantly diluted. Samples containing significant amounts of carbonate will tend to bubble and sputter on the initial addition of acid. In this case, acid addition should be suspended immediately until all evidence of a reaction has ceased.

8. QUALITY ASSURANCE/QUALITY CONTROL

8.1. Observed loss of material at any stage of this procedure compromises quantitative samples. Samples which experience such loss should be discarded immediately if sufficient material has been retained to allow for a fresh sample. If the compromised sample is the only material available from the original sample, the procedure should be completed, and the nature of spillage or loss should be documented clearly on the "Diatom Slide Preparation Form."

8.2. Diatom frustules are microscopic, generally falling in the fine silt size range; therefore, there is a possibility that samples can be contaminated. Laboratory rooms where raw or processed samples are handled should be kept as clean as possible. Lab bench surfaces should be kept clean and free of debris. Techniques similar to those used for sterile experiments (bacteriological plating, etc.) should be followed to minimize the risk of cross contamination of samples. Where feasible, disposable pipettes, stirrers, etc. should be used. Where they cannot, they should be rinsed in DW or RO water after each sample, and stored dry.

8.3. Samples with live algae should be refrigerated and kept in the dark (live diatoms are capable of continued growth as long as they are receiving light).

8.4. New glassware and digestion vessel liners should be washed and/or rinsed prior to use. Used glassware should be vigorously scrubbed, washed with a detergent, and rinsed at least three times with DW or RO to prevent contamination. Previously used digestion vessel liners should be washed with a detergent and soft brush (beware of abrasives, however) and rinsed at least three times with DW or RO. (Explanatory note: at times tap water, because of algal blooms and use of diatomaceous earth filters, may contain diatoms.) All equipment should be stored dry to prevent growth of algae or fungi.

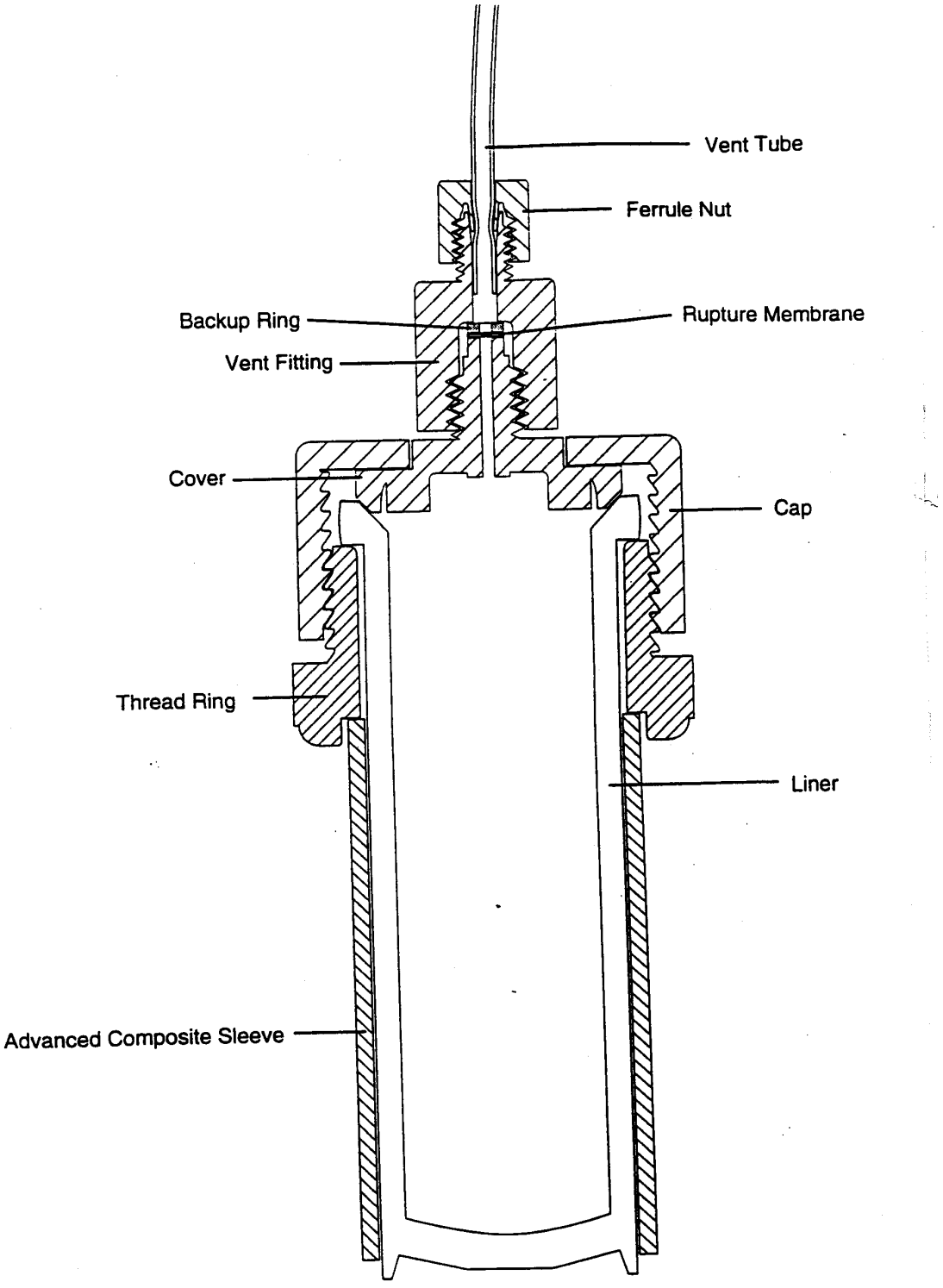

Figure 1. Standard advanced composite vessel (cross section).

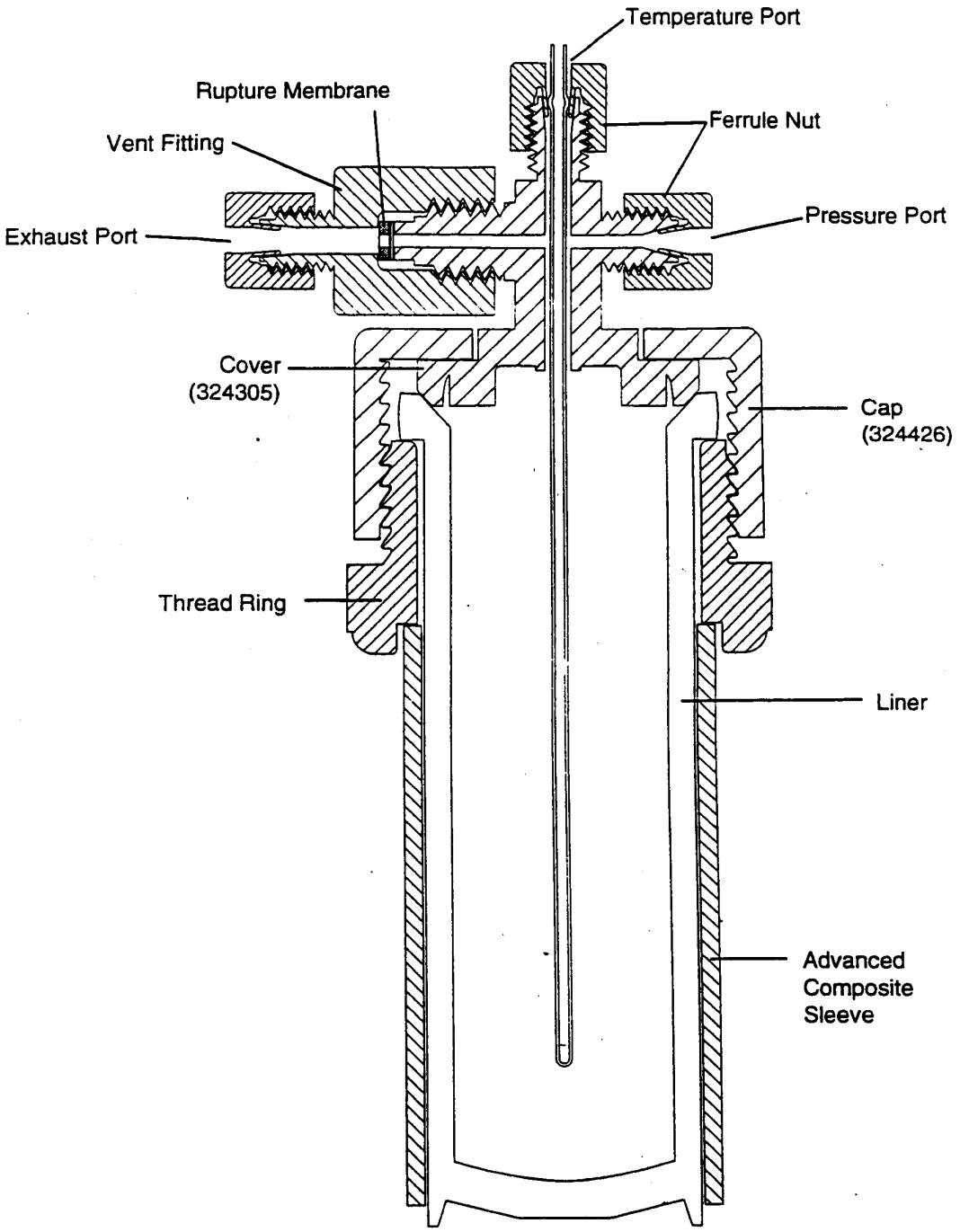

Figure 2. Advanced composite vessel for pressure and temperature control.

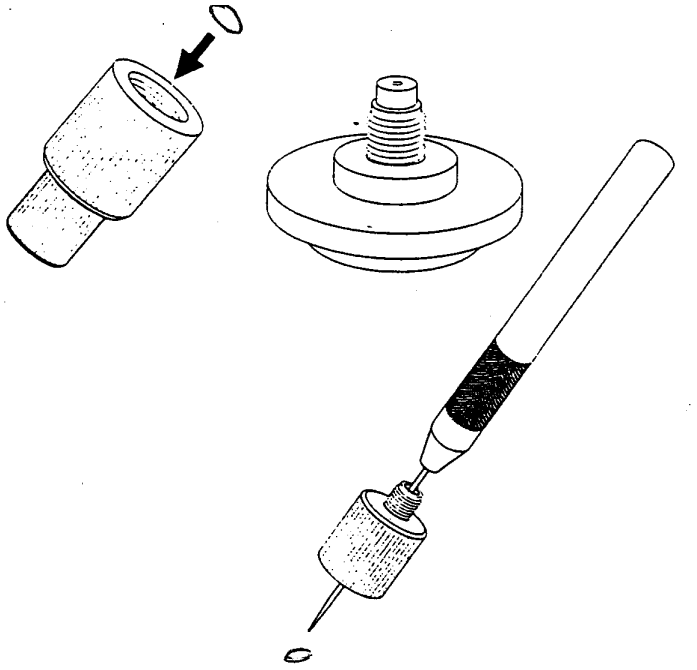

Figure 3. Installation and removal of rupture membrane.

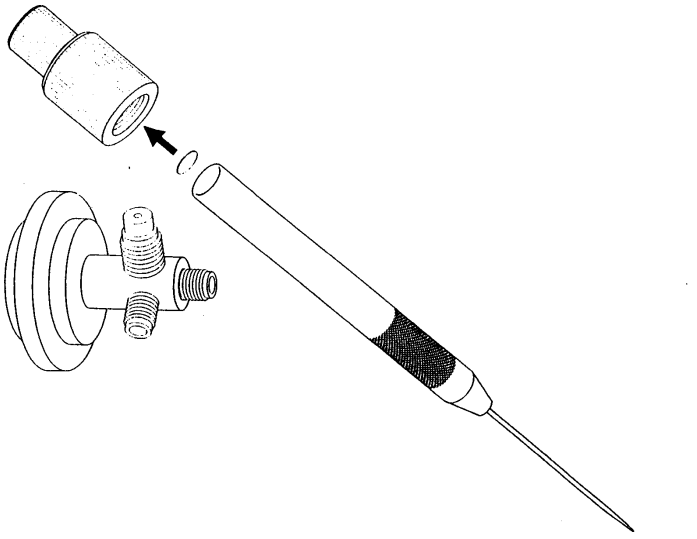

Figure 4. Installation of rupture membrane in vent fitting of advanced composite vessel with temperature/pressure control cover.

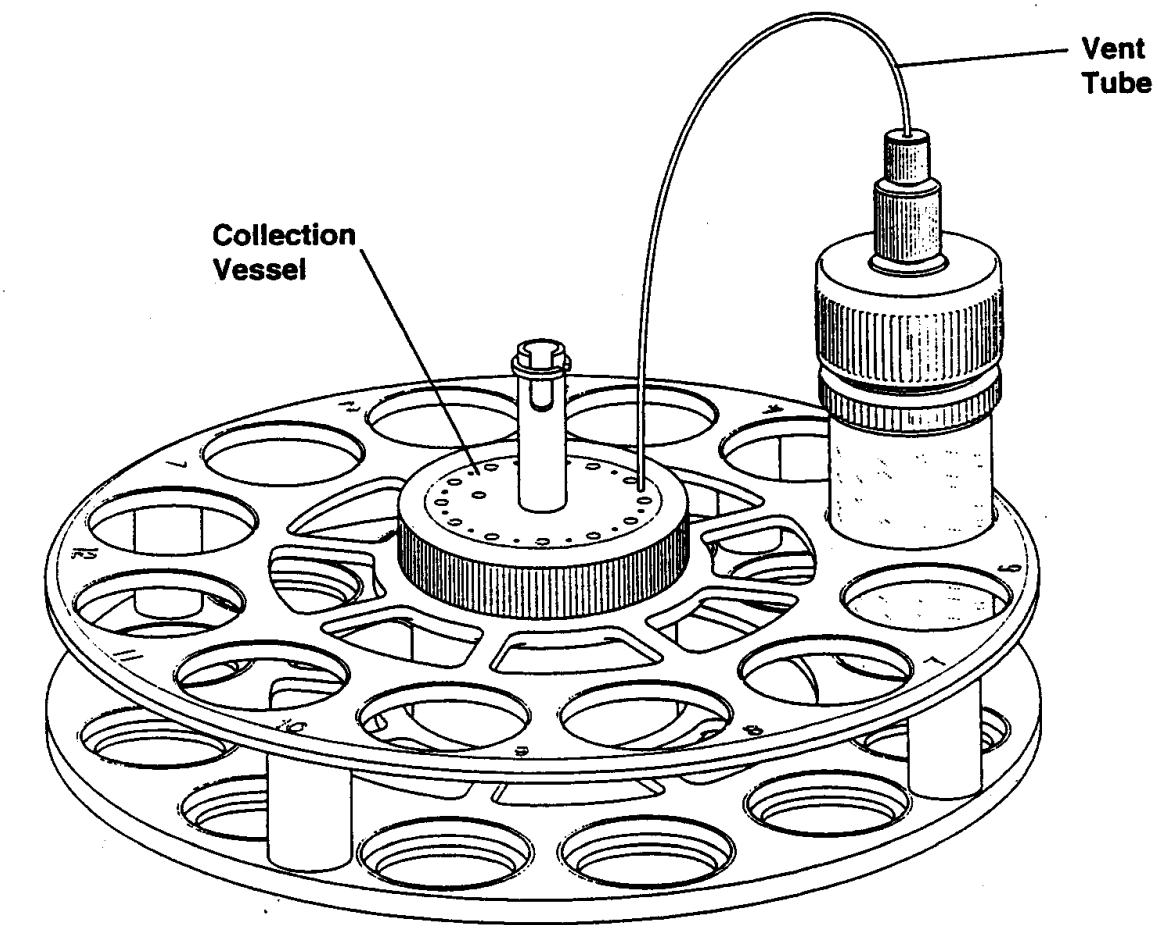

Figure 5. Routing of pressure sensing line and fiber optic temperature probe.