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Standard Guide for Preparation of Biological Samples for Inorganic Chemical Analysis¹

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1. Scope

1.1 This guide describes procedures for the preparation of test samples collected from such locations as streams, rivers, ponds, lakes, estuaries, oceans, and toxicity tests and is applicable to such organisms as plankton, mollusks, fish, and plants.

1.2 The procedures are applicable to the determination of volatile, semivolatile, and nonvolatile inorganic constituents of biological materials. Analyses may be carried out or reported on either a dry or wet basis.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For a specific hazard statement, see 9.3.3.

2. Referenced Documents

2.1 ASTM Standards:

- D 1129 Terminology Relating to Water²
- D 1193 Specification for Reagent Water²

3. Terminology

3.1 *Definitions*—For definitions of terms used in this guide, refer to Terminology D 1129.

4. Summary of Guide

4.1 Samples are collected, where possible, with nonmetallic or TFE-fluorocarbon-coated sampling equipment to prevent contamination, stored in plastic containers, and kept either at 4°C or frozen until returned to an adequate facility for analysis.

4.2 Before analysis, samples are allowed to return to room temperature. Large foreign objects are mechanically removed from the samples based upon visual examination; smaller foreign objects are also removed mechanically, with the aid of a low-power microscope.

4.3 Wet samples of small organisms such as plankton, are mixed for preliminary homogenization, then allowed to settle,

to remove most of the occluded water. Larger organisms, such as fish, should be patted dry, using paper towels.

4.4 Where less than a whole organism is to be analyzed, tissue excisions are made with nonmetallic tools such as plastic knives or TFE-fluorocarbon-coated scalpels.

4.5 Moisture determinations are made on separate samples from those analyzed for volatile or semivolatile constituents.

4.6 Analyses for volatile constituents are made using wet samples from which supernatant liquid or occluded water has been removed (see 4.3). The results may be calculated to the dry, original-sample basis, using the results of a moisture determination carried out on a separate sample.

4.7 Analyses for semivolatile constituents are made on wet samples or samples previously dried at a temperature (dependent on constituents of interest), or using a procedure, found to be adequate for the purpose, and specified in the corresponding analytical procedure.

4.8 Analyses for nonvolatile constituents are made on samples previously dried at a temperature (dependent on constituents of interest), or using a procedure found to be adequate for the purpose, and specified in the corresponding analytical procedure.

4.9 Digest the samples according to the procedures outlined in Section 9.

4.10 A flow diagram outlining typical procedures is shown in Fig. 1.

5. Significance and Use

5.1 The chemical analysis of biological material, collected from such locations as streams, rivers, lakes, and oceans can provide information of environmental significance. The chemical analysis of biological material used in toxicity tests may be useful to better interpret the toxicological results.

5.2 Many aquatic biological samples, either as a result of their size, or their method of collection, are inherently heterogeneous in that they may contain occluded water in varying and unpredictable amounts and may contain foreign objects or material (for example, sediment) not ordinarily intended for analysis, the inclusion of which would result in inaccurate analysis.

5.3 Standard methods for separating foreign objects, to facilitate homogenization, will minimize errors due to poor mixing and inclusion of extraneous material.

5.4 Standardized procedures for drying provide a means for

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² *Annual Book of ASTM Standards*, Vol 11.01.

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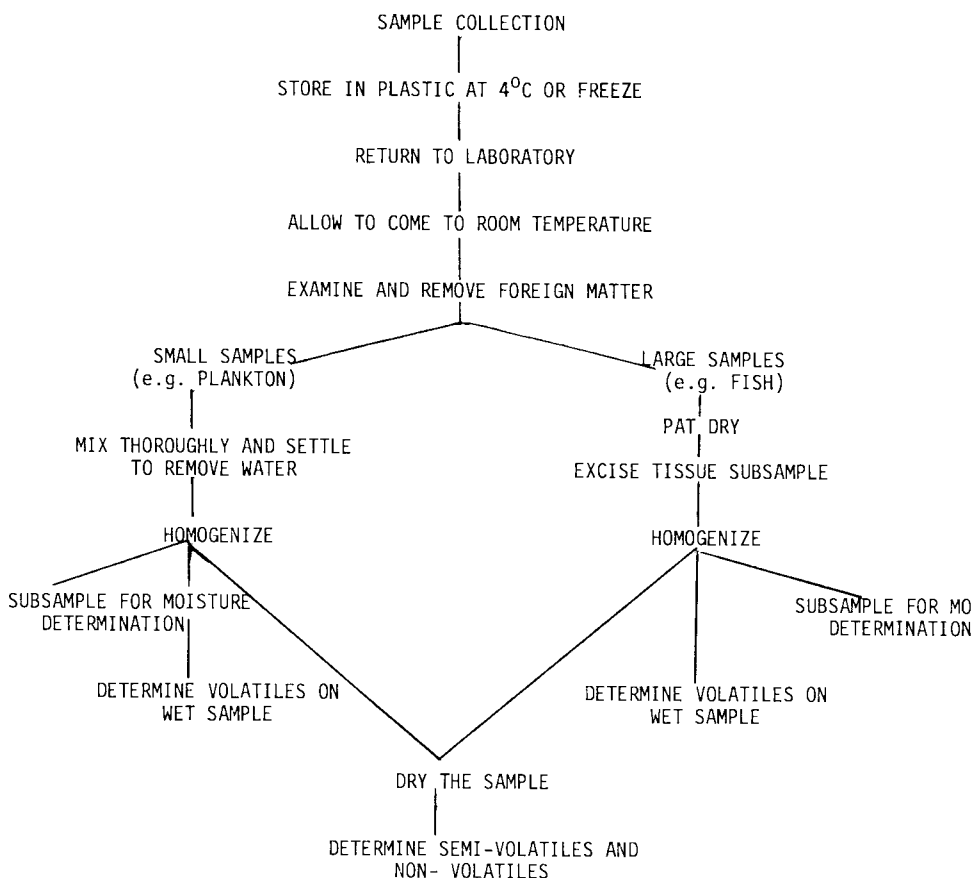


FIG. 1 Flow Diagram for the Preparation of Biological Samples for Inorganic Analysis

reporting analytical values to a common dry weight basis, if desired. Analyses may also be carried out or reported on a wet weight basis.

6. Preliminary Treatment of Samples

6.1 Treat small heterogeneous samples, such as plankton, as follows:

6.1.1 Allow for the sample to return to room temperature.

6.1.2 Remove foreign objects, such as leaves and twigs, mechanically, using nonmetallic instruments. Use a low-power microscope to facilitate removal of smaller foreign objects such as paint chips.

6.1.3 Transfer the sample to a beaker and thoroughly mix it with a glass stirring rod or equivalent, and allow it to settle so that most or all of the occluded water can be decanted.

6.1.4 If chemical analyses are to be carried out on a wet sample, and a large amount of material is available, remove a number of small portions (at least 5) from random locations in the beaker, and composite them to obtain a representative sample of a size sufficient for chemical analysis and a separate moisture determination. Using a tissue disrupter, blender, or equivalent, homogenize the sample or composite (to ensure lack of contamination, carry a standard or blank, or both, through this procedure). Remove a subsample for moisture determination and proceed to Section 7. Retain the remainder and proceed to Section 9.

6.1.5 If chemical analyses are to be carried out on a dry sample, and a large amount of material is available, remove a

number of small portions (at least 5) from random locations in the beaker, and composite them to obtain a representative sample of a size sufficient for the analysis. Using a tissue disrupter, blender, or equivalent, homogenize the sample, or composite (to ensure lack of contamination, carry a standard or blank, or both, through this procedure), and proceed to Section 7.

6.2 Treat large samples such as fish as follows:

6.2.1 Allow the sample to return to room temperature.

6.2.2 Pat the sample dry with paper toweling to remove as much water as possible.

6.2.3 Transfer the sample to a nonmetallic surface, such as a flat glass plate, and excise a sufficient quantity of material, or specific organs, to obtain sufficient material for analysis. Make excisions with plastic knives or TFE-fluorocarbon-coated scalpels.

6.2.4 If chemical analyses are to be carried out on a wet sample, use a tissue disrupter, blender, or equivalent, to homogenize the material (to ensure lack of contamination, carry a standard or blank, or both, through this procedure). Remove a subsample for moisture determination and proceed to Section 7. Retain the remainder and proceed to Section 9.

6.2.5 If chemical analyses are to be carried out on a dry sample, use a tissue disrupter, blender, or equivalent, to homogenize the material (to ensure lack of contamination, carry a standard or blank, or both, through this procedure) and proceed to Section 7.

7. Drying Procedures

7.1 Use a sample or subsample prepared in accordance with the directions given in Section 6.

7.2 Treat subsamples from biological materials which are to undergo chemical analysis without drying for moisture determinations as follows:

7.2.1 Accurately weigh 5 to 10 g \pm 1 mg or 10 to 25 g \pm 10 mg of material into a nonmetallic container which has been previously tared, and weighed with the same accuracy.

7.2.2 When a limited amount of material is available, determine the moisture on a 1 to 2-g sample, and weigh with an accuracy of \pm 0.1 mg. The use of samples smaller than 1 g is not recommended for moisture determination.

7.3 When an entire sample is to be dried prior to chemical analysis, a moisture determination is also required. Transfer the accurately weighed material (1 to 2 g \pm 0.1 mg, 5 to 10 g \pm 1 mg, >10 g \pm 10 mg) into a dry nonmetallic container which has been previously tared, and weigh with the same accuracy.

7.4 If a moisture determination (or sample drying) is to be made using an oven, treat as follows:

7.4.1 Transfer the containers holding the material to an oven and dry for 2 h at one of the following temperatures:

7.4.1.1 For the determination of semivolatile constituents, use the temperature specified in the analytical procedure for the constituents(s).

7.4.1.2 For determination of nonvolatile constituents use 105 \pm 2°C.

7.4.2 Cool in a desiccator, then weigh the dried samples with the same accuracy as the wet samples.

NOTE 1—Biological materials tend to be very hygroscopic. Keep weighing times to a minimum.

7.4.3 Repeat drying at hourly intervals, to attain a constant weight.

7.5 If a moisture determination (or sample drying) is to be made at room temperature, treat as follows:

7.5.1 If drying is to be done in a desiccator, ensure that the desiccant in the bottom is fresh, and some means is available to indicate when the desiccant loses its drying capacity (for example, color change). A vacuum desiccator may also be used.

NOTE 2—If a vacuum desiccator is used, bear in mind that this may cause the loss of volatile or semivolatile inorganics such as mercury, if the dried sample is to be subjected to chemical analysis.

7.5.1.1 Transfer the containers holding the material to a desiccator.

7.5.1.2 Leave the material in the desiccator for 48 h, then weigh the dried samples with the same accuracy as the wet sample.

7.5.1.3 Repeat weighings at 4-h intervals, to attain a constant weight (see Note 1).

7.5.2 Alternatively, sample drying or moisture determinations may be carried out in a laminar flow hood; treat as follows:

7.5.2.1 Transfer the containers holding the material to an appropriate hood and turn it on.

7.5.2.2 Leave the material in the hood for 48 h, then weigh the dried samples with the same accuracy as the wet sample.

7.5.2.3 Repeat weighings at 4-h intervals, to attain a constant weight (see Note 1).

NOTE 3—Air-drying in the open is strongly discouraged unless it is carried out in a clean room, where possible contamination from airborne particulates can be controlled.

7.6 If a moisture determination (or sample drying) is to be made using a freeze dryer, treat the determination as follows:

7.6.1 Transfer the containers holding the material to the freeze dryer.

7.6.2 Follow the manufacturer's instructions for the particular unit in use. Make certain that a trap is placed between the vacuum pump and the drying chamber to prevent pump oil fumes from possibly contaminating the sample. Drying is usually complete when the internal pressure in the drying chamber reaches 50 millitorrs or less.

7.6.3 Transfer the freeze-dried samples to a desiccator for storage, and weigh them with the same accuracy as the wet samples (see Note 1).

NOTE 4—Because freeze drying occurs under vacuum, this may cause the loss of volatile or semivolatile inorganics such as mercury, or both, if the dried sample is to be subjected to chemical analysis.

7.7 The possibility of loss of volatile constituents dictates the drying procedure to be used, prior to chemical analysis. Determine volatile constituents using undried samples. Determine semivolatile constituents using samples dried at a temperature at which no significant losses occur.

7.8 Analytical data reported on a dry weight basis should include percent moisture so that wet weight values can be obtained. Likewise, wet weight analytical data should include percent moisture to permit recalculation to a dry weight basis.

7.9 Use the following equations to calculate percent moisture and to correct analytical results from samples analyzed when wet.

7.9.1 Calculate percent moisture as follows:

$$\text{moisture, \%} = (W_w/W_d)100 \quad (1)$$

where:

W_w = wet weight, g, and

W_d = dry weight, g

7.9.2 To calculate concentrations on a dry weight basis, when determinations have been made on an undried sample, use the following equation:

$$C_d = \frac{C_w(100)}{100 - \% \text{ moisture}} \quad (2)$$

where:

C_d = concentration on a dry weight basis, and

C_w = concentration on a wet weight basis.

8. Reagents

8.1 *Purity of Reagents*—Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society where

such specifications are available.³ Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

8.2 Purity of Water— Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D 1193, Type I. Other reagent water types may be used, provided it is first ascertained that the water is of sufficiently high purity to permit its use without adversely affecting the bias and precision of the test method. Type II water was specified at the time of round robin testing of this method.

8.3 All of the following reagents may not be required for a particular procedure. Check the digestion procedure(s) of interest (Section 9) prior to preparing any reagents.

8.3.1 Amyl Alcohol.

8.3.2 Hydrochloric Acid (1 + 1)—Mix 1 volume of hydrochloric acid (HCl, sp gr 1.19) with 1 volume of water.

8.3.3 Hydrogen Peroxide Solution (30 % H₂O₂ w/v)—Commercially available.

8.3.4 Magnesium Nitrate Solution (7 g/L)—Dissolve 7 g of magnesium nitrate Mg(NO₃)₂ · 6H₂O in water and dilute to 1000 mL.

8.3.5 Nitric Acid (sp gr 1.42)—Concentrated ultra-pure nitric acid (HNO₃).

8.3.6 Nitric Acid (1 + 9)—Mix 1 volume of nitric acid (HNO₃, sp gr 1.42) with 9 volumes of water.

8.3.7 Nitric-Perchloric Acid Solution (3 + 1)—Mix 3 volumes of ultra-pure concentrated nitric acid (HNO₃, sp gr 1.42) with 1 volume of ultrapure concentrated perchloric acid (HClO₄, sp gr 1.67).

8.3.8 Sulfuric Acid (sp gr 1.84)—Concentrated ultra-pure sulfuric acid (H₂SO₄).

8.3.9 Sulfuric Acid (1 + 9)—Mix 1 volume of sulfuric acid (H₂SO₄, sp gr 1.84) with 9 volumes of water.

9. Digestion Procedures

9.1 Many procedures are available for the destruction of biological material prior to inorganic analysis, but almost all the methods fall into one of two main classes: dry ashing and wet digestion. Each of these classes have advantages and disadvantages, as do the individual procedures which fall under them. Before choosing a particular method, the user should consult the pertinent literature to determine the utility and applicability of any method, prior to a final selection. Even then, experience with a particular sample type or digestion apparatus, or both, may have to be the final arbiter in method selection.

NOTE 5—Contradictory reports, regarding recoveries for various procedures, can be found in the following literature.^{4,5,6,7}

³ *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmaceutical Convention, Inc. (USPC), Rockville, MD.

⁴ Middleton, G., and Stuckey, R., "The Preparation of Biological Material for the Determination of Trace Metals, Part II," *Analyst*, Vol 79, 1954, pp. 138–142.

9.2 Dry ashing entails procedures in which organic matter is oxidized by reaction with gaseous oxygen, generally with the application of energy in some form. Included in this general term are the methods in which the sample is heated to a relatively high temperature in a stream of air or oxygen and the related low-temperature technique where excited oxygen is used.

9.2.1 For high temperature ashing, digest as follows:

9.2.1.1 Place a weighed 2 to 10-g sample, prepared according to the procedures outlined in Section 7, into an acid-washed 250-mL borosilicate beaker. Spread the sample evenly, and over as wide an area as possible. If a wet sample is used, as in the case of volatile or semivolatile constituents, first dry the sample at low temperature. This may be accomplished with an infrared lamp, on a low-temperature hot plate, or at a low setting in a muffle furnace.

9.2.1.2 Add an ashing aid such as sulfuric or nitric acid, or magnesium nitrate, if appropriate.

NOTE 6—Opinions vary as to the most opportune time to add an ashing aid if it is used at all. However, it should be added as early as possible to avoid the possibility of loss during preliminary ashing. Ashing aids are normally added as solutions so that the entire sample is wetted. Typical concentrations are 10 mL of sulfuric acid (1 + 9), or of nitric acid (1 + 9), or 10 mL of Mg(NO₃)₂ solution per 5 g of sample. After addition, evaporate to dryness as outlined in 9.2.1.1.

9.2.1.3 Place the beaker in a muffle furnace, and raise the temperature to 500 ± 10°C in 50°C increments every 30 min.

9.2.1.4 After 500°C has been achieved, ash the sample for an additional 4 h.

9.2.1.5 If unoxidized organic matter is left in the beaker, wet the residue with water or nitric acid (1 + 9), evaporate to dryness (9.2.1.1) and return to the furnace for an additional period of time.

9.2.1.6 When a suitable ash has been obtained, cool the beaker and moisten its contents with a small quantity of water. Carefully add 10 mL of hydrochloric acid (1 + 1) and gently heat the resulting solution to completely dissolve any residue (if analyses are to be carried out by graphite furnace, another acid, such as nitric acid, should be substituted to reduce interferences).

9.2.1.7 If necessary, filter the sample through an acid-resistant filter paper, and then take up the solution in a suitable solvent, to a volume commensurate with the analytical procedure to be employed.

NOTE 7—Simple ashing may lead to the loss of significant quantities of As, Ag, Cu, Cd, and Sr. When nitric acid is used as an ashing aid, losses of Ag, As, Cd, and Sb are possible. If magnesium nitrate is used, Cd and Cr may be lost.

9.2.2 For low temperature ashing, digest as follows:

⁵ Gorsuch, T. T., "Radiochemical Investigations on the Recovery for Analysis of Trace Elements in Organic and Biological Materials," *Analyst*, Vol 84, 1959, pp. 135–173.

⁶ Prasad, M., and Spiers, M., "Comparative Study of Ashing Techniques for the Digestion of Horticultural Plant Samples," *Journal of Agricultural Food Chemistry*, Vol 26, 1978, pp. 824–827.

⁷ Knight, M. J., "A Comparison of Four Digestion Procedures not Requiring Perchloric Acid for the Trace Element Analysis of Plant Material," *Argonne National Laboratory Report ANL/LRP-TM18*, 1980, p. 27.

9.2.2.1 Low temperature ashers employ electronically excited oxygen produced by subjecting air to a high frequency electrodeless discharge at low pressures (about 1 torr). An oxygen-bearing plasma oxidizes organic matter. Temperatures normally do not exceed 150°C.

9.2.2.2 Because several low-temperature ashers are currently available, no specific instructions are provided herein; follow the procedures supplied by the instrument manufacturer.

NOTE 8—Keep in mind that low temperature ashing is carried out under vacuum and as such, could lead to the loss of certain volatile constituents.

9.3 *The majority of wet digestions entail the use of one, or any combination of four reagents: sulfuric acid, nitric acid, perchloric acid, and hydrogen peroxide.* Various glassware configurations have been employed to carry out the digestion process. The particular configuration is not limited to that outlined below; the ideal apparatus to be employed for a particular material or constituent(s) may well have to be found through experimentation.

9.3.1 Perform a nitric-sulfuric acid digestion as follows:

9.3.1.1 Place a weighed 1 to 5-g sample, prepared according to the procedures outlined in Section 7, into an acid-washed 500-mL round-bottomed flask. Add 15 mL of cold HNO₃(sp gr 1.42) and 10 mL of cold H₂SO₄(sp gr 1.84) per 2 g of sample to the flask, and swirl thoroughly.

9.3.1.2 Connect a 30.5-cm water-jacketed reflux condenser to the flask and allow the sample mixture to react at room temperature for 30 min.

9.3.1.3 Place the flask and condenser in a heating mantle and increase the temperature slowly over a 1-h period to bring the mixture to boiling. Allow the mixture to reflux for 2.5 h. Digestion is complete when the rust-colored mixture becomes light yellow. If the mixture is not light yellow, continue refluxing until it turns this color. Additional digestion reagent may be required to complete the color change.

9.3.1.4 If necessary, filter the sample through an acid-resistant filter paper, and then take up the solution, in a suitable solvent, to a volume commensurate with the analytical procedure to be employed.

NOTE 9—Due to poor recoveries, this method may not be suitable when there are high concentrations of Ca, or for As, Fe, Mn, Mo, Ni, Pb, Se, and Sr.

9.3.2 Perform a repeated nitric acid digestion as follows:

9.3.2.1 Place a weighed 2 to 5-g sample, prepared according to the procedures outlined in Section 7, into an acid-washed 1-L borosilicate glass beaker. Add 10 to 20 mL of water sufficient to wet the sample and 5 mL of HNO₃(sp gr 1.42) per gram of sample.

9.3.2.2 Place the beaker on a hot plate set to maintain a temperature of 125°C and evaporate the mixture. If frothing occurs during the evaporation, remove the beaker from the heat, allow it to cool, and add several drops of amyl alcohol.

9.3.2.3 After evaporation is complete, leave the beaker on the hot plate until no further color changes occur, remove from the hot plate and allow it to cool. When cool, add 5 mL of HNO₃(sp gr 1.42) per gram of sample, cover the beaker with a watchglass, return it to the hotplate, and evaporate to dryness. Ignition may occur but does not adversely affect the digestion.

9.3.2.4 Allow the beaker to remain on the hotplate for 15

min after the residue dries, then remove it and allow it to cool.

9.3.2.5 Repeat 8.3.2.3 and 8.3.2.4 until the residue turns white. This may take from 5 to 9 repetitions.

9.3.2.6 Dissolve the residue in 5 mL of HNO₃ (sp gr 1.42) and heat gently.

9.3.2.7 If necessary, filter the sample through an acid-resistant filter paper, and then take up the solution, in a suitable solvent, to a volume commensurate with the analytical procedure to be employed.

NOTE 10—Due to poor recoveries, this method may not be suitable for As, Fe, Hg, Ni, Pb, Se, Sr, and Zn.

9.3.3 Perform a nitric-perchloric acid digestion as follows, but take the requisite precautions necessary when working with perchloric acid:

9.3.3.1 Place a weighed 2 to 5-g sample prepared according to the procedures outlined in Section 7, into an acid-washed 500-mL tall form beaker and add a premixed solution of concentrated nitric and perchloric acids (3 + 1) at the rate of 20 mL per gram of sample. If sulfuric acid is to be used as well, add 2.5 mL of sulfuric acid (sp gr 1.84) per gram of sample. Swirl the beaker and allow it to stand for 30 min.

9.3.3.2 Cover the beaker with an acid-washed watchglass, place it on a hot plate, and gradually raise the temperature until the mixture is boiling. Permit the boiling to continue until evaporation has occurred and perchloric fumes are evolved. Charring must not be allowed to occur, if it starts, remove the beaker from the hot plate, allow it to cool, add several drops of HNO₃(sp gr 1.42) and restart the heating and evaporation processes. Do not let the flask boil dry.

9.3.3.3 Repeat 9.3.3.2 to achieve a complete oxidation. This may take 2 or 3 repetitions.

9.3.3.4 Allow the beaker to thoroughly cool, then dissolve the residue by the addition of 5 mL of HNO₃(sp gr 1.42) and gentle heating.

9.3.3.5 If necessary, filter the sample through an acid-resistant filter paper, and then take up the solution in a suitable solvent, to a volume commensurate with the analytical procedure to be employed.

NOTE 11—Due to poor recoveries, the nitric-perchloric digestion may not be suitable for Hg. Due to poor recoveries, as a result of insoluble sulfide formations, the nitric-perchloric-sulfuric acid digestion may be unsuitable for As, Hg, Fe, Mn, Mo, Ni, Pb, Sr, and Zn.

9.3.4 Carry out a nitric acid-peroxide digestion as follows:

9.3.4.1 Place a weighed 2 to 5-g sample, prepared according to the procedures outlined in Section 7, into an acid-washed 250-mL beaker and add HNO₃(sp gr 1.42) at the rate of 5 mL per gram of sample. Swirl the beaker, cover with an acid-washed watchglass, and allow it to stand for 1 to 2 h to allow the initial frothing to subside.

9.3.4.2 Place the beaker on a hot plate at a low setting and watch for possible frothing. If it occurs, remove the beaker from the heat and tap the bottom of the beaker to break up the large bubbles. Gradually raise the temperature until the solution begins to reflux, and continue for 30 min.

9.3.4.3 Remove the watchglass and evaporate to dryness, reducing the heat near the end to prevent spattering. When smoking ceases, raise the temperature slowly to 340 ± 10°C; care should be taken to prevent ignition.

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9.3.4.4 Cool the sample and add 5 mL of HNO₃ (sp gr 1.42), cover the beaker with a watchglass, and heat the beaker at low temperature until the residue dissolves. Then add 30 % H₂O₂ dropwise until the solution becomes clear and pale yellow in color.

9.3.4.5 Remove the watchglass, and evaporate the solution to approximately 3 mL. If the solution begins to darken, add additional 30 % H₂O₂.

9.3.4.6 If necessary, filter the sample through an acid-

resistant filter paper, and then take up the solution, in a suitable solvent, to a volume commensurate with the analytical procedure to be employed.

NOTE 12—Due to poor recoveries, the nitric-peroxide digestion may not be suitable for As and Hg.

10. Keywords

10.1 digestion; tissue homogenation

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