VIII. APPENDIX II.

.DITHIZONE METHOD OF ANALYSIS OF LEAD IN AIR AND BIOLOGIC SAMPLES*

The following directions for analysis of lead are taken from the first part of the report, "The 'USPHS' Method for Determining Lead in Air and in Biological Materials" by Keenan, Byers, Saltzman, and Hyslop.[72] Additional information on the reproducibility and accuracy of the method is given in other portions of the report.

REAGENTS

Analytical grade reagents are used. Purification is essential when analyzing biological tissues and fluids because of the very low levels of lead in these materials; purification of reagents may not be required for air samples containing quantities of lead sufficiently greater than that present in the reagent blank. A reagent blank sample is carried through the entire procedure with each set of unknown samples (air, biological, or other type) and its analyzed lead content is subtracted from each analytical result to calculate the net quantity of lead in each unknown sample.

A boiling rod is used to prevent bumping in the flasks when distilling reagents. This is prepared by cutting 3 or 4 mm O.D. glass tubing to a length which is one cm greater than the height of the flask. The tubing is sealed at a spot about one cm above the bottom end which is fire-polished but left open. Before each use, the liquid is shaken out of

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*Alternite methods for analyzing for lead in air (method 5341) and in blood (method P & CAm 262) are given in the NIOSH manual of analytical methods, 2nd Edition 1977 DHEW (NIOSH) Publications No-77-157A and 157B. Available from the Government Printing office, Washington, D.C.

the bottom section and the rod inserted in the flask. As the flask is heated a steady stream of air and vapor bubbles issues from the open space, thus providing nuclei for smooth boiling.

Double-distilled Water - To distilled water in an all borosilicate-glass still add a crystal each of potassium permanganate and barium hydroxide and redistill. Use for reagent and biological sample solutions unless tests indicate that single-distilled water is satisfactory; single-distilled water is usually adequate for determinations on air samples.

Nitric Acid, Concentrated - Redistill in an all borosilicate-glass still the ACS reagent grade acid, 69.0% minimum, specific gravity 1.42. Use an electric heating jacket on the boiling flask to minimize danger of its breakage, and a boiling rod to prevent bumping, which otherwise would be severe. Discard the first 50 ml of distillate; this may be combined with the acid allowed to remain in the flask at the end of the distillation and used for washing glassware. The reagent is conveniently dispensed from a small automatic burette. No grease should be used on the stopcock.

Nitric Acid, 1:99 - Dilute 10 ml of the redistilled, concentrated acid to one liter with doubled-distilled water.

Ammonium Hydroxide, Concentrated - Distill in an all borosilicate-glass still 3 liters of the ACS reagent grade, 28.0% minimum specific gravity 0.8957 at 60 F, into 1.5 liters of double-distilled water,

contained in a 2-liter reagent bottle which is chilled in an ice bath. Continue the distillation until the bottle is filled up to the previously marked 2-liter level. Submerge the condenser tube deeply in the water in the receiver, but withdraw it before discontinuing the heat to avoid siphoning back of distillate. This reagent may be prepared more conveniently from tank ammonia, using a small wash bottle to scrub the gas and a sintered glass delivery tube which extends to the bottom of the reagent bottle. The ammonia gas is absorbed in double-distilled water until the solution reaches the desired specific gravity.

Chloroform - Use a brand with a statement on the label that the chloroform passes the American Chemical Society test for suitability for use in dithizone procedures. In addition, each batch of chloroform should be purchased in glass containers only and should be tested as follows in the laboratory to make sure that it is satisfactory for preparing the dithizone solutions: add a minute quantity of dithizone to a portion of the chloroform in a test tube, shake gently, then stopper with a cork. The faint green color should be stable for one day. Our experience has indicated that the procedures for reclaiming used chloroform are tedious, time-consuming, sometimes unsuccessful, and no longer warranted in view of the commercial availability of acceptable reagent grades.

Extraction Dithizone - Dissolve 16 mg of diphenylthiocarbazone (dithizone), Eastman Kodak Co. No. 3092, or equivalent, in one liter of chloroform. Store in a brown bottle in the refrigerator.

Standard Dithizone - Dissolve 8 mg of diphenythiocarbazone in one liter of chloroform. Store in a brown bottle in the refrigerator but allow to warm to room temperature before using. Age for at least one day, then standardize as described in the procedure. Restandardize every few months.

Sodium Citrate - Dissolve 125 g of the 2 $Na_3C_6H_5O_7 \cdot 11\ H_2O$ salt in sufficient distilled water to provide a solution nearly 500 ml in volume. Adjust the pH to 9-10, using a very small quantity of phenol red indicator solution (strong red color) and fresh, pHydrion test paper to check the pH. Extract in a large separatory funnel with a 100 mg per liter solution of dithizone and finally with the extraction dithizone reagent until a green extract is obtained with the latter reagent. Add a small volume of lead-free citric acid until an orange color (pH 7) appears. Extract the excess dithizone repeatedly with chloroform until a colorless extract is obtained. Remove the last traces of chloroform.

Hydroxylamine Hydrochloride - Dissolve 20 g of the salt in distilled water to provide a volume of 65 ml. Add a few drops of m-cresol purple indicator, then add ammonia until the indicator turns yellow (pH 3). Add a sufficient quantity of a 4% solution of sodium diethyldithiocarbamate to combine with metallic impurities, then mix. After a few minutes extract repeatedly with chloroform until the excess carbamate reagent has been removed, as indicated by the absence of a yellow color in the final chloroform extract tested with a dilute copper solution.

To the aqueous solution of the hydroxylamine hydrochloride add redistilled, 6N hydrochloric acid until the indicator turns pink, and adjust the volume to 100 ml with double-distilled water.

Potassium Cyanide - (Danger! Highly poisonous!!) To 50 g of potassium cyanide in a beaker, add sufficient distilled water to make a sludge. Transfer the sludge to a separatory funnel previously marked to show 100-ml volume. Add a small amount of distilled water to the beaker and warm. (Potassium cyanide cools the solution as it dissolves, thus retarding the solution process.) Add this warm water to the separatory funnel but do not permit contents to exceed the 100-ml mark. Shake, then let stand until the contents come to room temperature. A practically saturated solution results.

Extract the lead by shaking repeatedly with portions of the extraction dithizone solution until the lead has been removed. Part of the dithizone dissolves in the aqueous phase but enough remains in the chloroform to color it. A green extract indicates that all the lead has been completely extracted. Most of the dithizone in the aqueous phase is then removed by repeated extractions with pure chloroform. Dilute the concentrated solution of potassium cyanide with double-distilled water to 500 ml. It should not be necessary to filter the solution, if the directions are followed precisely. Extraction is carried out before dilution because the higher pH of the dilute solution is less favorable.

(NOTE: A colorless solution usually results if above directions are followed. Occasionally aging results in a brown color or precipitate due to polymerization of hydrogen cyanide. This does not interfere with use of the reagent if it is carefully decanted. Old potassium cyanide reagent may lose enough strength to cause insufficient complexing of large amounts of zinc.)

Ammonia-cyanide Mixture - Mix 200 ml of the purified 10% potassium cyanide reagent with 150 ml of distilled ammonium hydroxide (specific gravity 0.9, corresponding to 28.4% NH_3) and dilute to one liter with double-distilled water. If the measured specific gravity of the ammonia is not 0.9, use the equivalent volume as calculated from a table of specific gravity vs. percentage ammonia.

Standard Lead Solution - Dissolve 1.5984 g of pure lead nitrate in one liter of 1:99 nitric acid to provide a strong stock solution containing one mg Pb per ml. Pipet exactly 20 ml into a 500-ml volumetric flask and make to mark with 1:99 nitric acid to give a dilute stock solution containing 40 µg Pb per ml. (A standard lead solution, 10 µg Pb/ml, was stable in 1:99 nitric acid for three years.) Prepare a working solution, containing 2 µg Pb per ml, just before it is needed by pipetting 5 ml of the dilute stock solution into a 100-ml volumetric flask and making to mark with 1:99 nitric acid.

Phenol Red - 0.1% aqueous solution.

Ashing Aid Acid - Dissolve 25 g potassium sulfate in sufficient redistilled concentrated nitric acid to make 100 ml.

White Petrolatum - Supplied in a glass jar, for greasing stopcocks. To check on the purity, put a pinch of this petrolatum in a beaker, add a few milliliters of the standard dithizone and swirl. If the dithizone is no longer green after a few minutes, the material is unsatisfactory for greasing stopcocks.

APPARATUS

A Beckman Model DU Spectrophotometer has been used in this laboratory since this instrument became available in the 1940s. However, the Beckman Model B and the Bausch and Lomb Spectronic 20 have been shown to give comparable results for blood lead determinations, provided that appropriate standardizations are conducted with each instrument. Other laboratories, whose results are reported in this paper, have presumably used a diversity of available photometers and spectrophotometers. In our laboratory, 22 x 175 mm matched test tubes are used in most spectrophotometric procedures employing the Model DU, which is fitted with a tube holder which does not interfere with the use of the instrument with regular cells. These same tubes are used in the Model B fitted with a test tube adaptor. A 3/4-inch tube, supplied by the manufacturer, is used in the Bausch and Lomb Spectronic 20.

Borosilicate glassware is used throughout the procedures (except for vacutainers used for blood sampling). Ashing is performed in 125- or 250-ml Phillips beakers. Automatic burettes are used for the addition of most reagents. The extractions are conducted in the Squibb-type, 125-ml separatory funnels supported in electrically operated shakers provided with timer switches. The stopcocks of the separatory funnels

are greased with white petrolatum (purchased in a glass jar rather than in a metal can or tube) unless Teflon stopcocks, which require no grease, are used. All glassware should be reserved for trace analysis only to avoid possible gross contamination.

Soak all ashing beakers in a detergent solution (Alconox or Duponol is suitable) immediately after each usage to prevent any material from drying on the surfaces. Rinse 8-10 times with hot water and store in a dust-proof drawer or cabinet until needed. Use the following acid cleaning, lead-freeing techniques immediately before the next use of the glassware: Rinse the ashing beakers with a saturated solution of sodium dichromate in concentrated sulfuric acid. Leave a 1-2 ml portion in the beaker or flask (proportionally less in a small volumetric flask!). Add about 5-10 ml of warm tap water and allow the hot solution to flow over all inner surfaces to remove the last traces of grease. Rinse with three or four portions of cold tap water. Rinse with one portion of either concentrated or 1:1 nitric acid, as preferred. (This wash nitric acid may be used repeatedly until it loses its strength.) Then rinse successively with three or four portions each of tap water, distilled and double-distilled water. Set the beakers upright on the bench and cover with a clean dust-case or a large piece of filter paper (or otherwise protect from dust). Under no circumstances is glassware turned upside down to drain on a towel or cheesecloth placed on a laboratory bench. Use an oven operating at 105 C if dry glassware is required.

Separatory funnels are rinsed with tap water immediately after use. If a high lead sample was present or if a visible precipitate remains on the inside, it is rinsed with a small portion of 1:1 wash nitric acid (which is discarded), followed by tap water. The stoppered funnels are stored in double-deck racks. Immediately before use, stopcocks are regreased if necessary. Then the funnels are rinsed with wash acid, four times with tap water, and four times with distilled water. Each rinse is accomplished by shaking with the stopper, then draining through the stopcock with two or three turns.

Spectrophotometer tubes are rinsed four times each with tap and distilled water immediately after use. They are placed upright in a large beaker and dried in an oven at 105 C, then stored under a dust-cover. Occasionally they are cleaned with dichromate-sulfuric acid and nitric acid as described above.

(NOTE: With this method of cleaning glassware we have never encountered cross-contamination from chromium, lead, or from any other trace element being determined routinely in this laboratory.)

ANALYTICAL PROCEDURE

1. Warm the sample ash (prepared as described in the following sections) with 2 ml of concentrated nitric acid for a few minutes, then add 25 ml of distilled water, heating on the hotplate until a clear solution is obtained.

2. Cool to room temperature. Add to the solution in the beaker one ml of hydroxylamine hydrochloride, 4 ml of sodium citrate (10 ml is required for a urine sample), one drop of phenol red indicator, and titrate to a strong red color with concentrated ammonia reagent. Add a few drops excess of ammonia to make sure that the pH is between 9 and 10, using fresh pHydrion test paper to check the pH.

(NOTE: Phenol red has a weak orange-red color in strong acid, yellow in weak acid, and a red color in alkaline solution. Do not mistake the

first color for that produced in alkaline medium!)

3. Transfer the sample quantitatively with double-distilled water rinsings to a 125-ml Squibb separatory funnel containing 5 ml of the potassium cyanide reagent.

4. Add 5 ml of the extraction dithizone and shake two minutes, after releasing the initial pressure by momentarily opening the stopcock of the inverted separatory funnel. Allow the chloroform layer to settle.

5. Draw off most of the extraction dithizone into a second funnel containing exactly 30 ml of 1:99 nitric acid.

6. Add a second 5-ml portion of extraction dithizone to the first funnel and shake as before. Allow the layers to separate and combine the extracts in the second funnel. Continue this process with fresh portions of extraction dithizone until the reagent remains green. A rough estimate of the lead present in the sample may be made on the basis of 20 μg for each cherry-red 5-ml extract portion.

7. Shake the second funnel for two minutes to transfer the lead to the 1:99 nitric acid layer. Allow the layers to separate. Discard the chloroform layer.

8. Shake the nitric acid solution with approximately 5 ml of reagent chloroform and let settle. Drain the settled chloroform through the stopcock bore as completely as possible without loss of the aqueous layer. Evaporate the last drop of chloroform clinging to the upper surface of the liquid.

(NOTE 1: Start a zero lead standard at the beginning of this step by placing 30 ml of 1:99 nitric acid in a separatory funnel. This zero lead standard will be used to set the spectrophotometer at zero absorbance for

each series of samples being analyzed.)

(NOTE 2: If the quantity of lead estimated for any sample exceeds the 25 µg range of the colorimetric determination, pipet an appropriate aliquot of the nitric acid solution at the end of step 7 into a clean separatory funnel containing 5 ml 1:99 nitric acid to minimize errors caused by possible leakage of the stopcock, add sufficient additional 1:99 nitric acid to make 30 ml total volume, and continue with step 8.)

(NOTE 3: Start lead standards at this point if required. Add 5-ml portions of 1:99 nitric acid to each of four separatory funnels, then 2.5, 5.0, 7.5, and 12.5 ml of dilute standard lead solution (2 µg Pb/ml) from a burette, respectively to the separatory funnels, finally add the proper quantity of 1:99 nitric acid to make total volume 30 ml in each. Continue with step 8.)

9. Add 6.0 ml of the ammonia-cyanide mixture, exactly 15.0 ml of the standard dithizone, and shake two minutes. Allow the layers to separate. Drain the chloroform layer containing the lead dithizonate into a clean, dry test tube, and cork the tube immediately.

10. Decant this solution carefully into a dry photometer tube leaving the water behind. If any water spots are visible in the optical light path, transfer again to another photometer tube.

11. Set the spectrophotometer at a wavelength of 510 nm.

12. Set the instrument at zero absorbance using the zero lead standard solution.

13. Read the absorbances of the samples and of the reagent blank.

14. Calculate the lead content of each by multiplying its absorbance by the standardization factor (which is the slope of the standardization plot in micrograms of lead per unit of absorbance.) Subtract the blank value from the gross lead content of each sample to obtain the net amount of lead expressed in micrograms.

SPECIAL MATERIALS FOR BLOOD SAMPLING

1. Vacutainers, Becton-Dickinson, No. 3208, 20-ml or 10-ml, complete with stoppers are used for blood sampling. The vacutainers are used repeatedly and are lead-freed by the technique described previously. Blood is removed from the vacutainers and the stoppers, after each use, by soaking in cold tap water. When no further visible trace of blood remains on these items, they are soaked overnight in the detergent solution. They are then rinsed repeatedly with hot tap water to remove alkaline materials. The vacutainers are then subjected to the chromic and nitric acid cleaning procedures. The stoppers are soaked for 20- to 30-minute periods, three times, with single distilled water and finally three times with double distilled water. The lead-freed vacutainers are dried at 105 C, fitted with clean stoppers, and stored in a drawer reserved for them. Layers of cheesecloth are placed between the separate layers of vacutainers and the drawer is sealed with masking tape to prevent the admittance of any dust. They are evacuated just before shipment to the field. A vacuum tester is used both in the laboratory and field to test for loss of vacuum, which usually will not occur until stoppers have been used several times.

2. Vacuum Tester, High Frequency, Fisher Cat. No. 1-179, or equivalent.

3. Needles, Becton-Dickinson, Gauge 20, one and one-half inches in length, stainless steel, B-D No. 3200 N. As these needles are used repeatedly, check the tips for burrs by drawing them across the thumb nail. When burrs develop either discard the needles or file off the burrs. After filing, they must be recleaned. Vacutainer needles are soaked in a dilute detergent solution. A Becton-Dickinson Needle Cleaner, No. 3200 C, is used to force detergent solution and subsequent rinse water through the needles. Needles are subjected to thorough rinsing with distilled water. They are then placed in steritubes and either autoclaved or heated for two hours in a drying oven operating at 180 C. The steritubes are then fitted with rubber caps.

4. Steritubes, Becton-Dickinson, No. 3200 D, with rubber caps.

5. Stillets for No. 3200 N needles, 20 Gauge, two and seven-eighths inches long.

(These BD items are available from the Becton-Dickinson Company, Rutherford, New Jersey.)

COLLECTING AND ASHING BLOOD SAMPLES

Collect a 10-ml sample of whole blood using a lead-free vacutainer and a sterilized, stainless steel needle. In the laboratory, transfer the sample to a weighed, lead-free, 125-ml borosilicate Phillips beaker. No aliquoting of the blood is permissible, as most of the lead is present in the clot. Determine the weight of the blood sample to the nearest 0.01 gram, weighing rapidly to minimize evaporation. Add 2 ml of ashing aid acid reagent. Add 7 ml of concentrated nitric acid. (This ashing system

permits the analyst to handle a large number of samples at a time as the blood clot breaks up readily and smoothly without bumping and without requiring the constant attention of the analyst.) Place the samples on a hotplate operating about 130 C and evaporate just to dryness. After the water is driven off in the initial evaporation to dryness, keep the beaker covered with a lead-free watchglass to increase the reflux action of the concentrated acid. This serves to wash solids down from the sides to the hotter zone at the bottom, and also reduces the amount of acid needed. Cool the beaker briefly and then add successive portions of the nitric acid ranging from 2 ml down to 0.5 ml as the ashing proceeds. Do not remove the watchglass at any time but merely slide it back sufficiently to facilitate each new addition of the acid. Each time, as soon as the residue becomes light colored, heat on a 400 C hotplate just long enough to blacken the residue, then remove and cool the sample. Throughout the remainder of the ashing procedure, alternately heat the sample with a few drops of nitric acid on the 130 C hotplate and bake the residue for the few minutes required to darken it on the 400 C hotplate. Finally, the residue will remain pale yellow or light brown (due to iron content) after heating for 5-10 minutes at the high temperature. Avoid excess baking at this stage as the ash will become decomposed to a difficultly soluble form. It is now ready for solution and analysis. Report results as milligrams of lead per 100 grams of whole blood.

COLLECTING AND ASHING URINE SAMPLES

Use lead-free, narrow-mouthed, reagent-type, borosilicate, 250-ml bottles provided with standard taper glass stoppers to collect grab samples of

urine. Add 2.0 ml of a 37% formalin solution as a preservative, shaking the bottle 10-12 times after the contribution of the urine to mix the specimen with the formalin thoroughly.

Alternatively, urine specimens may be collected in 125-ml polyethylene bottles containing as a preservative 100-200 mg of EDTA (acid form) per bottle. This is convenient and economical for shipping samples considerable distances.

If the urine sample is clear and only one or two days old, measure a 50 ml portion into a graduated cylinder. However, if the sample is older, much of the lead may be in a sediment or on the walls of the bottle and must be dissolved before aliquoting. Transfer the entire specimen to a glass-stoppered graduated cylinder, record the volume, rinse the sample bottle with three small portions of concentrated nitric acid and add these rinsings to the cylinder. Mix thoroughly (Caution! Old samples may foam over.) Note the total volume and remove an aliquot equivalent to 50 ml of urine for analysis. Transfer the aliquot portion to a lead-free, 250-ml borosilicate Phillips beaker and add 5 ml of redistilled concentrated nitric acid. Evaporate just to dryness on a hotplate operating at about 130 C. Cool, add sufficient nitric acid to moisten the residue and cover the beaker with a lead-free watchglass. Heat on the 130 C hotplate and then alternately bake for a few minutes and digest with minimal amounts of nitric acid (as described in the ashing method for blood) until a white residue remains after the final heating for 5-10 minutes at the high temperature. The sample is now ready for solution and analysis. Report results as milligrams of lead per liter of urine.

PROCEDURE FOR AIR SAMPLES

It is convenient to wash out samples in electrostatic precipitator tubes with redistilled ethanol, using a special policeman made with a rubber disc cut to fit the tube like a piston, and transferring the sample through a short stem funnel into a 250-ml Phillips beaker; gently evaporate just to dryness. (Ethanol is helpful in removing greasy deposits on the walls of the precipitator tube. Some chemists may prefer hot 1 to 5% nitric acid to transfer the sample.) Transfer impinger samples or membrane filter samples to Phillips beakers. If little ash is expected (usually for impinger or membrane filter samples), add 2 ml of ashing aid acid reagent. (The presence of this salt will prevent loss of lead by glazing onto the surface of the beaker during ashing.) Otherwise add 1-2 ml nitric acid. Evaporate to dryness. Continue ashing with nitric acid at a moderate heat until organics are destroyed.

Dissolve the ash in 2 ml of concentrated nitric acid and distilled water and then transfer quantitatively to a 100-ml volumetric flask and make to mark. Pipet a suitable aliquot into a separatory funnel, containing about 5 ml of double-distilled water, add sufficient additional double-distilled water to make the total volume about 25 ml, and apply the Analytical Procedure, starting with step 2. In step 3, as the sample is already in a separatory funnel, merely add the cyanide. The amount of lead present in the aliquot may be estimated as described in step 6. If it is less than a few micrograms, an additional aliquot may be added to the same funnel, and the pH readjusted with ammonia. The extraction is then continued, and extracts combined with those collected previously in the second funnel. If the estimated amount

of lead exceeds the range of the method (25 micrograms), take an aliquot as described in Note 2, step 8.

When calculating the results, make allowance for the total number of aliquots. If convenient, aliquot the reagent blank in the same manner so that the correction represents the same amounts of ashing and extraction reagents as are present in the sample. However, the blank correction is usually small for air samples. Report results as milligrams of lead per cubic meter of air.