examples have been devised by M. Pring (see Ohnishi[17], and Dutton *et al.*[20]), with other ones given by Denis[21] and Hendler *et al.*[22]

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[20] Dutton, P. L., Erecinska, M., Sato, N., Mukai, Y., Pring, M., and Wilson, D. F. (1972). *Biochim. Biophys. Acta* **267**, 15–24.

[21] Denis, M. (1973). Ph.D. Thesis, University of Marseilles.

[22] Hendler, R. W., Town, D. W., and Shrager, R. L. (1975). *Biochim. Biophys. Acta* **376**, 42–62.

[24] Micro Methods for the Quantitative Determination of Iron and Copper in Biological Material

By HELMUT BEINERT

Introduction

There are many different methods available for the determination of iron and copper, chemical and spectroscopic; and for the chemical determination a great number of reagents have been proposed. No single investigator can be expected to have experience with all these methods, and it is reasonable that an investigator use the method that he has set up and working and in which he has gained confidence. The description that follows is therefore aimed at those who do not have a method set up and reliably working in their laboratory and who prefer to resort to chemical determinations rather than purely spectroscopic ones. However, irrespective of the final determination of the metal, which can be carried out by various means, the preparation of the samples for the final determination shares common features in most instances. In this respect also the precautions to be taken with the two metals, iron and copper, are very similar, since they are both ubiquitous contaminants. The main problem can be stated as this: The difficulty does not lie in finding the metal that is there, but rather in not finding metal that does not belong there.

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The essential features of the method to be described[1] are wet ashing, evaporation of excess acid, reduction, neutralization with excess sodium acetate, development of color with a suitable bathophenanthroline, and extraction with a small quantity of organic solvent followed by spectrophotometric determination.

For Iron

Reagents

(1) Iron standard in dilute H_2SO_4
(2) H_2SO_4, concentrated
(3) HNO_3, concentrated
(4) $HClO_4$, 60%
(5) HCl, 2 *N*
(6) Mercaptoacetic acid, 1% (w/v) solution
(7) Sodium acetate, saturated solution
(8) 3-Methyl-1-butanol
(9) 4,7-Diphenyl-1,10-phenanthroline solution in 3-methyl-1-butanol

Comments to Procurement and Preparation of Reagents

(1) A concentrated primary standard, containing approximately 1 mg-atom Fe/100 ml, is prepared first. From this a working standard of 0.1 μg-atom/ml is derived by dilution with dilute H_2SO_4. The use of HCl is avoided, as iron chloride is volatile. Analytical-grade iron wire (56 mg) is degreased, placed in a 100-ml volumetric flask, and dissolved in a solution containing 2 ml concentrated HCl and 2 ml concentrated HNO_3 in 10–15 ml. When all the iron is dissolved the flask is made to volume with water. This standard is stable indefinitely. Iron(II)-ethylenediammonium sulfate is also a suitable stable primary standard.

(2–5) Ultrapure acids of low metal content are available from various sources (J. T. Baker Chemical Co.; EM Laboratories, Inc.; British Drug Houses, Ltd.).

(6) Also called thioglycolic acid. The aqueous solution has to be prepared approximately once a month since oxidation destroys it. The original sample, once opened, is also not stable for more than a few months. Disulfide formation can generally be recognized by the appearance of a precipitate. Low color yield in the reaction with phenanthroline is generally due to deterioration of this reagent.

(7) Sodium acetate may be obtained as ultrapure reagent. However,

[1] M. Van De Bogart and H. Beinert, *Anal. Biochem.* **20**, 325–334 (1967).

even this reagent, when used in the quantity needed here, contributes to the blank color. Its use in the required quantities also becomes very costly. Commercial reagent-grade acetate must be purified before use, and it is also advisable to apply the following procedure to the ultrapure reagent. A saturated solution of sodium acetate is prepared. It is advantageous to let this solution sit undisturbed for a few days, as some ferric hydroxide will settle out which then does not have to be removed by the laborious extraction procedure described below. However, extraction has to be carried out in any event, since some iron always remains in solution. After standing, the solution is filtered and collected in an Fe-free bottle. (The filter paper is prepared by washing with one of the Versenes and thereafter with sufficient water to completely remove Versene. The complete removal of Versene is essential or interference with reduction and binding of Fe by the phenanthroline may occur.) To the filtered solution a few milliliters of glacial acetic acid are added (about 6 ml/liter) to lower the pH to about 6, and some undiluted thioglycolic acid (1 ml/liter acetate) is added to reduce the contaminating iron left in solution. Iron is not reduced by thioglycolic acid at a pH higher than 6. Each liter of acetate solution is then shaken out with several 50-ml portions of phenanthroline/isoamyl alcohol reagent. The pink color of the isoamyl alcohol layer increases when the two phases are left standing together for several hours after each extraction. When after several extractions and standing periods only a faint pink color appears in the organic phase, the acetate solution is extracted twice with isoamyl alcohol to remove any phenanthroline from the acetate.

(8) Also called isoamyl alcohol; some lots of the reagent-grade alcohol were found satisfactory, while others contained iron. Isoamyl alcohol specially purified for iron analysis can be obtained commercially (G. Frederick Smith Company, Columbus, Ohio), or the ordinary reagent can be redistilled.

(9) Bathophenanthroline (G. Frederick Smith Company, Columbus, Ohio) (molecular weight 332), approximately 200 mg dissolved in 250 ml isoamyl alcohol. The reagent dissolves slowly and should preferably be left standing overnight. Warming the suspension in a water bath also speeds dissolution. This reagent slowly develops a yellowish color, which adds to the blank color. It is, therefore, advisable to prepare it in small lots fresh.

Glassware

Conical 12-ml graduated centrifuge tubes with ground-joint stoppers (e.g., Pyrex #8144) were found most suitable for ashing and extraction, as the approximate fluid volume can be continuously monitored. Micropi-

pettes are used for all additions under 1 ml. In most cases absolute accuracy of these additions is not required, merely repeatability. The pipettes are stored in tubes covered by larger tubes and all bottles are covered, since laboratory dust usually contains appreciable amounts of iron or copper. Glassware must be carefully cleaned and water of low metal content must be available. Procedures for achieving this have been published.[2] In our laboratory we found it satisfactory to redistill via a Pyrex glass condenser previously deionized or distilled water from a large metal still. Particular attention has to be given to the cleaning of new glassware. It should be kept in mind that all glassware that has been used in the analytical procedure, although possibly not "clean" with respect to other uses, is probably still the cleanest for reuse in the same procedure. Thus, the tubes used for ashing are flushed with water, ethanol, and again water and briefly boiled with 2 *N* HCl. After rinsing with water and draining but not necessarily drying, they are ready for reuse. The tubes are touched only at the lower end and care is taken not to contaminate the upper rim since on ashing, through refluxing, as well as during extraction, liquid does wet the upper parts of the tubes. We found it most convenient and safest to store the tubes upside down between analyses, on a rack provided with appropriate clamps (e.g., commercial broom-clips). After the tubes are dry, they are capped with Parafilm. After storage for several weeks the tubes have to be cleaned with acid, and after even longer storage it is advisable to carry them through a blank ashing procedure with the reagents used in the procedure. This is absolutely necessary for new tubes or tubes used for other work. The stoppers are degreased, if necessary, boiled in 2 *N* HCl, flushed with water, and stored in a suitable container. It is recommended that all containers used for storage of stoppers, pipettes, or reagents have covers with lids such as Petri dishes which prevent settling out of dust or dirt on the rim (e.g., a beaker covered with a watch glass is unsatisfactory). Pipettes are cleaned with chromic or hot nitric acid, flushed, and dried before being used in the analytical procedure. When stored for more than a few days after this treatment they should be flushed before use with 2 *N* HCl, followed with water and then with the reagent to be pipetted. Two samples of reagent are drawn up and expelled into a waste container. After this treatment a pipette is usually used to make additions to a whole series of samples. If the addition of the reagent is made carefully and in order of increasing iron content of the samples, no significant contamination can result. After use, pipettes used for aqueous solutions not containing protein are again flushed with 2 *N* HCl and water. Pipettes used for pipetting organic solvents are flushed with ethanol immediately before and after use

[2] R. E. Thiers, *Methods Biochem. Anal.* **5**, 301 (1957).

and otherwise with 2 *N* HCl and water as above. Pipettes used for protein solutions are first cleaned with detergents and thereafter as just described. To prevent contamination of the stock reagents it is necessary to have a second set of smaller reagent containers (e.g., glass-stoppered tubes), in which the reagent is frequently renewed. All pipetting is done from this second set of containers.

Apparatus

One adjustable microburner for ashing is sufficient. Occasionally a second burner for expelling condensed water at the upper end of the tube is helpful. The time required to set tubes up properly in a digestion rack, and the chance that spattering may occur when the tubes are unattended, probably outweigh the inconvenience of the labor involved when the contents of each tube are ashed by hand. With the small quantities of material this requires only 5–10 min/tube. A shaking or swirling device is required for extraction. A buzzer according to Bessey *et al.*[3] is satisfactory or a mixer of the type called "Vortex." Brief, low-speed centrifugation is necessary for separating the phases after extraction. Spectrophotometry may be performed in any instrument that permits the use of volumes of the order of 0.3 ml.

Procedure

See also "Comments to Procedure" below.

(1) Align the tubes in a rack according to increasing iron content and cap with Parafilm. Remove caps only when additions are made, but do not place Parafilm on hot tubes.

(2) Add "blanks," standards, and samples to appropriate tubes.

(3) Add 50 μl concentrated H_2SO_4 (reagent 2).

(4) Add 50 μl concentrated HNO_3 (reagent 3).

(5) Ash over microburner for approximately 6 min.

(6) Add 25 μl $HClO_4$ (reagent 4) to tubes after contents have cooled down.

(7) Ash over microburner an additional 3 min, but avoid boiling.

(8) Add, after cooling, 250 μl of water.

(9) Add 125 μl 1% thioglycolic acid (reagent 6).

(10) Add 750 μl sodium acetate (reagent 7).

(11) Add 500 μl bathophenanthroline solution (reagent 9).

(12) Stopper tubes with glass stoppers.

(13) Extract by buzzing or swirling.

[3] O. A. Bessey, O. H. Lowry, M. J. Brock, and J. A. Lopez, *J. Biol. Chem.* **166,** 177 (1946).

(14) Centrifuge to separate layers.

(15) Withdraw ~0.3 ml of the organic phase and place in dry microcuvette.

(16) Read at 535 nm against blank containing isoamyl alcohol (reagent 8).

Comments to Procedure

(2) The samples should not contain excessive amounts of interfering ions. Of metals to be expected in biological material, copper should not be present in a more than 5-fold excess over iron. Interference from molybdenum can be controlled by choosing a pH above 5.5.[4,5] Of anions to be expected in biological material, phosphate, pyrophosphate, and cyanide interfere. When the sample is dissolved in 0.1 *M* phosphate, for instance, the use of a 20-μl sample may be safe, while it is not advisable to use 0.1–0.2 ml of a 0.1 *M* phosphate solution. Tris buffers, such as Tris acetate, which decomposes on ashing, are very suitable. It is also advisable to avoid large concentrations of reagents such as sucrose or bile salts, as ashing may become very cumbersome in this case; multiple additions of $HClO_4$ may be necessary, and blanks may rise correspondingly. In general, samples must be prepared in a way suitable to remove contaminating iron. For proteins this may be accomplished by dialysis against chelating agents. After equilibrium has been reached, a sample of the dialyzate, equal to the protein sample, is used as "dialyzate" blank. If such a blank is expected or known to contain not significantly more iron than the "reagent blank" (containing all reagents only), dialysis fluid may be added to the standards as well, thus eliminating the need for running separate reagent blanks. The sample and standards should contain not more than 10 μg-atoms of iron (0.5 μg Fe) and 1 mg of organic matter, optimally about 5 μg-atoms of iron and up to 0.25 mg of organic matter. A complete standard curve need not be run, but it is advisable to run standards at two levels, above and below that expected for the sample. If different samples are run with different liquid volumes, it is also helpful to run these same volumes of "dialyzate" blanks. It may be desirable, when a set of carefully calibrated micropipettes is not available, to use the same pipette for blanks, standards, and samples, in this order, with appropriate cleaning of the pipettes (see above) between sampling.

(5) With the conical tubes used, care should be taken never to heat the tip. The tubes are held in a nearly horizontal position so that most of the

[4] W. B. Fortune and M. G. Mellon, *Ind. Eng. Chem., Anal. Ed.* **10**, 60 (1938).

[5] E. B. Sandell, "Colorimetric Determination of Traces of Metals." Wiley (Interscience), New York, 1944.

liquid is in a shallow layer at the place where the tapered and the straight portion of the tube meet. The fumes are driven up the barrel of the tube. Water should not be allowed to condense near the top of the tube, as this may cause spattering should the water run back into the hot tube. A second burner may be used to advantage to expel water condensed in the upper part of the tube. Tubes are capped again with Parafilm as soon as they have cooled down sufficiently.

(7) $HClO_4$ usually clears up the digest readily, and only when an excess of organic matter is present is the addition of more $HClO_4$ necessary. The presence of undigested organic matter is generally indicated by the persistence of a yellow-brownish color even after cooling of the digest. Toward the end of the ashing period a bright yellow color may appear, which usually disappears on cooling or further ashing. This color does not stem from organic matter. Ashing may also be effectively accomplished with H_2SO_4 alone by addition of H_2O_2. After heating with the acid and cooling, 10-μl portions of 30% H_2O_2 are added until the digest is clear and colorless; the effect of H_2O_2 is similar to that of $HClO_4$.

(10) This amount of acetate was found to buffer the amount of acid which could maximally be left over, to a pH of about 4.2. The pH should be within the range of 4–6 as otherwise reduction and color formation are not optimal. The reductant (reagent 6) should always be added before the buffer (reagent 7) in order to ensure rapid and complete reduction of iron.

(11) This solution is viscous and drains slowly. It should be slowly and uniformly delivered into all tubes, even if a quantitative delivery is not possible. Only the relative amount delivered to each individual tube is of importance, not the absolute quantity.

(13) Vigorous swirling or buzzing is necessary to extract all the iron into the organic phase. The tubes are buzzed or swirled for 2 min, then centrifuged to clear the walls, buzzed or swirled for 1 more minute, and centrifuged again. The stoppers may be lubricated very slightly with silicone grease. Poor color yield is often due to incomplete extraction.

(15) The same transfer pipette and cuvette are used for all samples. The samples are again processed in the order of increasing iron content. Between readings in the spectrophotometer the pipette and cuvette are flushed 3 times with ethanol and dried in a stream of filtered air. If difficulty is experienced in withdrawing a sufficient amount of organic phase uncontaminated by aqueous phase, it may be helpful to tilt the tube slightly so that the organic phase breaks from the wall on one side and contracts on the other side, thus providing a thicker layer to draw from.

(16) The reagent blank should give an absorbance reading not in excess of about 0.03 (1-cm light path). This blank value is mainly determined by the success in the removal of Fe from the acetate solution. Five μg-atoms of Fe

give a net absorbance value of 0.190–0.210 under the conditions described. It should be kept in mind that the small quantity of reagent soluble in isoamyl alcohol can extract only a limited amount of Fe. It is stated by Peterson[6] that 1 ml of the reagent can extract 2 μg of Fe. The absorbance should therefore never exceed a value of 0.6. Absorbance and iron content are proportional below this absorbance value. If the amount of iron present in a sample has been misjudged, so that too high an absorbance is observed, the quantity of reagent 9 added in step 11 may be increased. It is not sufficient to dilute with solvent after extraction. The amount of reagent should of course then also be increased in all other samples of the series including standards and blanks.

It often occurs that the isoamyl alcohol layer becomes turbid after transfer into the spectrophotometer cuvette. This is probably due to formation of an emulsion of water in the alcohol layer. Brief warming of the cuvette in a water bath to a few degrees above room temperature regularly clarified the solution for a time sufficiently long to take readings.

Possible Reasons for Difficulties in Procedures

(a) Erratic results with samples, blanks, and standards: contaminated glassware, random contamination during improper handling, or insufficient extraction.

(b) Erratic results in samples only: insufficient ashing, not liberating all the iron or leaving organic iron chelators behind; presence of iron complexing salts such as phosphate or pyrophosphate.

(c) Low values for standards: deteriorated mercaptoacetic acid or incomplete extraction.

(d) High but reproducible values for blanks and standards: contaminated bathophenanthroline reagent either from contaminated isoamyl alcohol or reagent itself; contaminated acetate or other reagents. The phenanthroline reagent and the acetate are the most likely sources.

(e) High values of standards only, not blanks: dilution of secondary working standard is either incorrect or this standard has increased in strength by contamination or evaporation. It is advisable to replace the secondary standard frequently. Significant contamination of the primary standard of high iron content is less likely.

Simplified Procedure for Acid-extractable Iron

In cases where it is certain or can be expected that the iron which is to be determined is released by acid (or some treatment other than ashing), the

[6] R. E. Peterson, *Anal. Chem.* **25**, 1227 (1953).

ashing procedure can be eliminated. The following procedure was found to be convenient and more precise than the procedure described, when applied, e.g., to purified iron sulfur proteins: Reagents 1, 2, 5, and 7–9 from above are used, but mercaptoacetic acid is used as a 5% solution. The iron is released with sulfuric acid since this acid can be obtained with very low iron content, so that blank readings are <0.008 absorbance units at 1-cm light path. The volume of sample plus buffer or water or of standard plus buffer or water is made up to 150 μl. Then 20 μl of 98% sulfuric acid are added and the contents are all mixed. Thereafter, 50 μl of 5% thioglycolic acid are added with renewed mixing. After 10 min 400 μl of saturated acetate are added followed by 500 μl of reagent 9. The extraction is performed as described. With the samples, which now contain precipitated protein, very thorough extraction is necessary, since the denatured protein appears to impede the exchange of the reagent between the aqueous and organic phase. However, after extraction and centrifugation, the organic solvent layer is clear. In this procedure the blank readings are as low as 0.003–0.008 and 5 ng-atoms of iron give a net absorbance of 0.215–0.220.

For Copper

The method for the determination of copper is in many ways similar or identical to that used for iron. Details will therefore be given only when differences exist; otherwise reference is made to the iron method by "(see Fe)."

Reagents

(1) Copper standard in dilute H_2SO_4
(2) (See Fe)
(3) 2 *N* HCl
(4) H_2O_2, 30%
(5) Hydroxylamine hydrochloride, 1% (w/v) aqueous solution
(6) Sodium acetate, saturated solution
(7) 1-Hexanol
(8) 2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline solution in 1-hexanol

Comments to Procurement and Preparation of Reagents

(1) Since the water content of most copper salts is not well defined, it is advisable to prepare the standard solution from metallic copper, e.g., copper shot of sufficient purity. A concentrated primary standard is prepared by dissolving 63.5 mg metal in a 100-ml volumetric flask (see Fe). The resulting standard contains 10 μg-atoms/ml. A secondary working

standard is prepared by diluting 1 ml of this standard to 100 ml with dilute H_2SO_4, so that 1 ml contains 0.1 μg-atom or 6.35 μg Cu.

(2,3) (See Fe.)

(4) Reagent-grade H_2O_2 is satisfactory.

(5) Reagent-grade hydroxylamine hydrochloride is satisfactory. This solution should be prepared fresh every 2–3 months.

(6) The acetate solution is prepared similarly to that described for Fe but the solution is shaken with approximately 1/15 its volume of a 0.01% solution of dithizone (diphenylthiocarbazone) in chloroform. The acetate solution is extracted until the purple color of the copper–dithizone complex is no longer visible in the chloroform layer. The chloroform is then shaken out with *n*-hexanol (reagent 7). Remaining *n*-hexanol, dissolved in the aqueous phase, does not interfere. In order to avoid the formation of stable emulsions shaking has to be done judiciously. Centrifugation in copper-free tubes may help in breaking emulsions when formed.

(7) *n*-Hexanol; it is advisable to redistill the commercial products. *n*-Hexanol purified for copper determination is commercially available (G. Frederick Smith Company, Columbus, Ohio).

(8) Bathocuproine (G. Frederick Smith Co.) (molecular weight 360); approximately 25 mg are dissolved in 250 ml *n*-hexanol (see Fe). This solution should be made up fresh every few weeks.

Glassware

Similar considerations apply here as for the iron method. After cleaning with acid, glassware may be treated with dithizone in chloroform (0.01%) and then rinsed with chloroform. The acid-cleaned glassware need not be dried before dithizone treatment, if an acetone rinse is included.

Procedure

(1–2) (See Fe.)
(3) Add 100 μl concentrated H_2SO_4 (reagent 2).
(4) Ash over microburner for approximately 6 min.
(5) Add 20 μl of reagent 4 to tubes after contents have cooled down.
(6) Ash over microburner an additional 3 min, but avoid boiling.
(7) Repeat step 6 if necessary.
(8) Add, after cooling, 500 μl of water.
(9) Add 0.25 ml hydroxylamine hydrochloride solution (reagent 5).
(10) Add 1.5 ml acetate solution (reagent 6).
(11) Add 0.5 ml bathocuproine solution (reagent 8).
(12–14) (See Fe.)

(15) Withdraw 0.3 ml of the organic phase and place in dry microcuvette.

(16) Read at 479 nm against blank containinq *n*-hexanol (reagent 7).

Comments to Procedure

(2) Similar considerations apply here as for the iron procedure, and only a few comments specific for the copper procedure will be made. Heavy metals in amounts expected in biological materials do not interfere. The amount of phosphate present should be kept at a minimum. When copper-binding organic anions such as EDTA are present, it is particularly important to ash the samples thoroughly so that these chelators are destroyed. Otherwise complex formation and extraction into the organic phase is interfered with.[7] The samples and standards should not contain more than 15 ng-atoms of copper ($\sim$1 μg Cu) and 2 mg of organic matter.

In the original description of the method,[1] the alternative method of ashing with H_2SO_4, HNO_3, and $HClO_4$ was also recommended. Since copper perchlorate may be volatilized under certain conditions, ashing should be carried out with H_2SO_4–H_2O_2 as described.[7]

(16) The reagent blank should give an absorbance reading not in excess of 0.02 (1-cm light path). Five ng-atoms of Cu give a net absorbance value of 0.105–0.115 under the conditions described. Since the solubility of the reagent is limited and therefore the quantity of copper that can be extracted, the total absorbance should not exceed 0.4. Absorbance and copper content are proportional below this value.

Difficulties likely to arise in the procedure for copper and their possible sources are analogous to those discussed above for iron.

[7] H. Beinert, C. R. Hartzell, B. F. van Gelder, K. Ganapathy, H. S. Mason, and D. C. Wharton, *J. Biol. Chem.* **245**, 225 (1970).