New Colorimetric Determination of Copper in Biological Matters

by Yukio MURAKAMI

(Received November 29, 1949)

Introduction

Recently the presence of minute amounts of copper in biological matter is taken up as an interesting problem. Its determination has been conducted by various colorimetric methods, but among them the method with sodium diethyldithiocarbamate has been generally applied as the most suitable and the most sensitive since it was first used by Callan and Henderson.(1) However, this reagent often requires a preliminary separation of copper from other elements for procuring good results as it does not react alone with copper. Hence, for preliminary separation of copper, hydrogen sulfide separation,(2) electrolytic separation(3) and ammonium hydroxide separation method(1)(4) were proposed by many workers, but none of these methods became widely used as an approved method.

A better preliminary separation method of copper was therefore desired. Sylvester and Lampitt(5) attempted this problem by determining copper in milk after extracting it with dithizone and chloroform. At the same time Sandell(6) determined copper by the mixed colored method or the extractive titration method with dithizone after preliminarily removing copper completely as dithizonates. This is proposed, however, for copper in silicate rocks. Nydahl(7) used the same method as Sylvester and Lampitt in determining copper in fats using carbon tetrachloride in place of chloroform as an extractant.

Thus the preliminary separation of copper had been generally improved by the application of dithizone. Yet these can not be approved to be the best and the most accurate methods in colorimetric determination owing to their own drawback. The principal reason for this defect lies in the difficulty of transferring the colored products completely into the solvent. Consequently it does not conform with the Lambert-Beer's law and the color comparison must be done at the loss of accuracy and sensitivity of the reagent as pointed out by many

---

(1) T. Callan and J. A. R. Henderson, *Analyst*, **54**, 650 (1929).

(2) L. W. Conn, A. H. Johnson, H. A. Trebler and V. Karpenko, *Ind. Eng. Chem.*, *Anal. Ed.*, **7**, 15 (1935); R. W. Thatcher, J. Am. Chem. Soc., **55**, 4524 (1933).

(3) F. Grendel, *Pharm. Weekblad.*, **67**, 913, 1343 (1930); *Z. anal. Chem.*, **97**, 47 (1934).

(4) J. H. Hamence, *Trans. Faraday Soc.*, **30**, 299 (1934).

(5) N. D. Sylvester and L. H. Lampitt, *Analyst*, **60**, 377 (1935).

(6) E. B. Sandell, *Ind. Eng. Chem.*, *Anal. Ed.*, **9**, 464 (1937).

(7) F. Nydahl, *Z. anal. Chem.*, **116**, 315 (1939).

wokers.(8)

The reaction of copper with the reagent was thoroughly studied by the author(9) and above mentioned difficulty was solved by extracting copper diethyldithiocarbamate compound with carbon tetrachloride in the presence of hydroxylamine. As already reported in a former report, colorimetric determination of copper has become more accurate and speedy. Such being the case, when it was required to investigate the copper content in biological matters, dithizone was used for preliminary separation after studying those methods mentioned above and then copper was determined colorimetrically by applying a new method, the details of which are in the following.

Experiment

Decomposition.—McFarlane(10) destroyed the biological matter in the sample by insertion in a muffle oven at 380° to 400° for 12 hours. Sylvester *et al.* and Winter(11) both recommended 500° to 550° in a silica dish with some sulfuric acid. The upper limit of temperature has not been set, but in this experiment it was performed in a silica dish at 400° to 450° in an electric furnace. The use of gas flame is undesirable owing to the greater possibilities of introducing impurities in the sample.

Nydahl recommended the wet-ashing method with nitric acid and perchloric acid in the presence of sulfuric acid after which the copper is to be determined in the resulting solution upon filtration of silica and calcium sulfate. It was noticed by the present author that the results are lower by this method especially in the presence of a large amount of silica, owing most probably to the occlusion in the silica. Such being the case, it is safer to remove the silica with hydrofluoric acid and perchloric acid and dissolve the residue in hydrochloric acid, when it is much abundant.

Isolation of Copper.—Though it cannot be said that sodium diethyldithiocarbamate is specifically reactive to copper alone comparing with other metals, it is highly sensitive to the element. It is, therefore, necessary that copper is preliminarily separated from other metals to obtain the best results. Generally there are many cases in which preliminary isolation with some reagents is undesirable as the reagent would impede the sensibility and the accuracy of the determination to follow. However such precautions are unnecessary in this case as the dithizone used to isolate copper is more sensitive than sodium diethyldithiocarbamate and moreover has spectroscopic sensitivity.

Some workers recommended isolation with hydrogen sulfide but this was found to be defective not as sensitive as had been reported: this defect was also recognized by the author. Thus dithizone was used to concentrate copper in the alkaline solution containing sodium citrate with a slight excess of ammonia (approximately pH 8.5) according to the Sandell's directions. The extraction in the alkaline solution as compared with the extraction in acid solution proposed by Hibbard(12) was purposely tried with a view of simultaneously determining zinc and lead with copper. The determination of zinc and lead are being studied(13). The presence of citrate is important in preventing the precipitation of iron hydroxide which absorbs some copper. Though some workers have denied such absortption,(14) it has been reported and was also confirmed by the author. Higher pH than the cited is not desirable, as copper may be extracted in the enol form instead of keto form, resulting in failure to separate copper from lead and zinc in the next operation. This may be avoided by not adding too much ammonia and not shaking too long in the first extraction.

Under the conditions described already, the extraction of copper is completely accomplished with Pb, Zn, Ni, Co, Bi, Cd in part and sometimes Fe and Mn. When the carbon tetrachloride layer is shaken with hydrochloric acid solution (approximately 0.01 N) copper remains virtually unchanged in the carbon tetrachloride along with the greater part of Bi, Co and Ni. But Pb, Zn and Cd transfers into the aqueous phase. This treatment has already been recommended by several workers(6),(15) as successful in separating copper from Pb and Zn.

Interfernece.—This reagent, though it has been widely used for copper determination, is not exclusively reactive with copper, but reacts with such metals as Bi, Fe, Mn, Ni, Co and Cr forming colored complexes and with Zn, Pb, Mg, Cd, Sb, Sn and Al becoming turbid. Under the conditions recommended, such metals as the majority of the elements mentioned above form dihizonates, but Sb, Sn, Ti, Al and Cr do not react with it.

Among the elements extracted as dithizonates, Zn, Pb and Cd separate perfectly into the acid solution being isolated from copper by the treatment with 0.01N hydrochloric acid. The amounts of Fe and Mn extracted are extremely small owing to the addition of citrate which prevents its reaction with dithizone.

Thus the interference due to the presence of the elements except for these above mentioned must be considered. But the interference due to Bi or Hg are practically not encountered because of their scarcity in biological matters, though there are a few exceptions. In a former report(9) the sensitivity of Mn, Co, Ni, Bi, Zn, Pb, Fe and Ti were reported by the author and permissible amounts in determination were discussed. In practi-

---

(8) Walker, *J. Assoc. Offici. Agr. Chem.*, **13**, 426 (1930); T. P. Hoar, *Analyst*, **62**, 36 (1937); D. L. Drabkin, *ibid.*, **21**, 203 (1938); O. B. Winter, *J. Assoc. Offici. Agr. Chem.*, **19**, 36 (1936); E. J. Coulson, *J. Assoc. Offici. Agr. Chem.*, **20**, 178 (1937).

(9) Y. Murakami, This Bulletin, **23**, 3 (1950).

(10) McFarlane, *Biochem. J.*, **29**, 480 (1929).

(11) O. B. Winter, *J. Assoc. Offici. Agr. Chem.*, **19**, 359 (1936).

(12) P. L. Hibbard, *Ind. Eng. Chem., Anal. Ed.*, **10**, 615 (1938).

(13) Y. Murakami, This Bulletin, in press.

(14) L. A. Haddock and E. Evers, *Analyst*, **57**, 495 (1932).

(15) Y. Morita, *J. Chem. Soc. Japan*, in press.

cal use, no serious interference were found under the desirable conditions. It is not necessary to mention here the use of dithizone as its use was studied thoroughly by many successive workers and already proved to be the best.

Reagent and Apparatus.—Practical precautions regarding apparatus and some reagents were presented in a former report. Therefore some descriptions of reagent except them will be given here.

Perchloric acid, 70%, analytical grade. Hydrofluoric acid: it is best purified by distillation with platinum apparatus. Sodium citrate, 10% solution: Add 36g. of sodium hydroxide to 63g. of citric acid (monohydrate) and dilute to 1000 ml. On the recommendation by Sandell it can be purified. Dithizone, 0.01% solution. Ammonia, saturated. Hydrochloric acid (1:1). Hydrochloric acid 0.01 N. Sodium diethyldithiocarbamate, 0.1% solution. Hydroxylamine, 4% solution. Carbon tetrachloride. Separatory funnel, about 30 ml. capacity. Silica dish.

Procedure.—Weight 0.01g. to 0.015g. ash into a silica dish and add 10 to 15 ml. of hydrochloric acid (1:1). (If the sample is particularly abundant in silica, add 0.3 ml. of 70% perchloric acid and 1 ml. of hydrofluoric acid (1:1). Maintain the temperature constant till the dish becomes dull red. Continue heating untill the solution was evaporated up and the excess of perchloric acid has been expelled). Then add 5 ml. of water to bring all soluble matter into solution by gentle heating. Repeat this procedure if necessary.

Next add 5 ml. of 10 per cent sodium citrate and add drops of saturated ammonia untill the solution becomes alkaline to the litmus paper reaction and continue to add an excess of 0.2 ml. If the solution becomes noticeably turbid on the addition of ammonia, it is well to let it stand for a while before beginning the filtration. Filter the solution through a small filter paper of medium texture: transfer any residue in the dish to the paper and wash three or four times with 1 ml. portions of cold water containing a drop of ammonia and citrate solution. Transfer the filtered solution to a separatory funnel, and 5 ml. of 0.01 per cent dithizone solution and shake for 0.5 minute: if the carbon tetrachloride is not green at the end of this time, shake for more half a minute. Allow the carbon tetrachloride to settle and draw it off into another separatory funnel leaving a few drops in it to prevent loss of aqueous solution.

It is important that the separated carbon tetrachloride be free from droplets of aqueous solution. Iron so introduced will oxidized the reagent and became the cause of difficulties in the determination. Unless the separated carbon tetrachloride is distinctly green, showing that an excess of reagent is not present, add another 5 ml. portion of 0.01 per cent reagent to the aqueous solution and shake vigorously for 30 to 40 seconds. Draw it off and continue in this manner untill the last 5 ml. of dithizone remains distinctly green after being shaken vigorously.

To separate copper add 10 ml. of 0.01 N hydrochloric acid to the combined extracts and shake vigorously for one minute. If the carbon tetrachloride remains red on shaking and does not become greenish or purple, add sufficient dithizone solution to change the red to a bluish or purplish color which recombine copper liberated on this treatment. Draw off the carbon tetrachloride layer into another separatory funnel and shake vigorously for two minutes with 10 to 12 ml. of 0.01 N hydrochloric acid. Run the carbon tetrachloride into a silica dish, discarding the aqueous solution in which zinc, lead and others may be present. Evaporate the solvent on a waterbath till dry, add 3 or 4 drops of nitric acid (1:1) to moisten the residue.

Then evaporate and ignite gently to destroy all organic matter. To the cold dish add one ml. of hydrochloric acid (1:1) and rub the interior of the dish by means of a glass rod so that all the cupric oxide will be dissolved. Add 10 ml. of water, transfer into the separatory funnel and rinse thoroughly with some water. Make the solution alkaline with ammonia to litmus paper and add the excess of 0.2 ml. ammonia solution. Then add 2 ml. of 4 per cent hydroxylamine solution and next 10 ml. of 0.1 per cent sodium diethyldithiocarbamate solution. It becomes instantly golden yellow due to the copper. Shake vigorously with 7 ml. of carbon tetrachloride and draw it off into a 25 ml. volumetric flask after leaving it to settle. Leave a few drops of the carbon tetrachloride to prevent mixing of the aqueous solution and rinse the stem of funnel with 2 ml. of carbon tetrachloride. In this extraction the stem should be perfectly dried. Then shake again in the same manner as before. Usually two shakings are sufficient to extract completely the colored matter. Then fill to the mark with carbon tetrachloride and measure the extinction with Pulfrich Photometer using S 43 Filter (434 mμ). If the final volume is reduced to less than 25 ml., then less than 5 γ of copper can be determined with fair results.

Accuracy and Reporducibility.— As reported already, the application of hydroxylamine has increased the sensitivity of the reagent because the extraction can be completely carried out and proved that it holds closely to the Lambert-Beer's law and the range of error lies within ±5% according to the amounts of copper (5 γ to 20 γ) in 25 ml. carbon tetrachlolide solution. In this report, to examine the separation and extraction with dithizone, biological matters with known amounts of copper were tested as shown in Table 1, proving that this new method can be used as a sufficiently accurate and simple method for application.

Table 1

Reproducibility

Sample		E*	Copper found, γ	Content, %
Spinach	1.0037 g	0.320[a]	7.99	0.0080
		0.321	7.96	0.0079
Burdock	0.9521 g	0.698[b]	59.22	0.0608
		0.693	57.46	0.0603
Bone dust	0.8232 g	0.245[a]	6.11	0.0074
		0.257	6.39	0.0078

* E: Extinction coefficient

(a) When the cell of 50 mm. thickeness was used (final volume 25 ml.).

(b) When the cell of 30 mm. thickeness was used (final volume 50 ml.).

Results.—The copper content of some biological matter was determined closely following the new method proposed above (Table 2).

Table 2

Determination of Copper in Biological Matter

Sample	Ash, %	Ash taken, g.	E (50 mm.)	Cu found, γ	Cu in ash, %	Remarks
Rice (Japan)	0.78	0.0328	0.064	1.58	0.0048	Saitama Prefecture (1943) uncleaned rice, 2nd class.
Rice (Burmese)	0.84	0.0444	0.426	10.60	0.0024	Basein Dist.(1943)
Spinach	16.99	0.1004	0.321	7.99	0.0080	Chiba Pref. March, 1943
Potato	1.93	0.0775	0.065	1.59	0.0021	Dehydrated, Saitama Prefecture June, 1943
Carrot	5.97	0.0121	0.294	7.30	0.0061	Dehydrated, Saitama Prefecture Autumn, 1943
Burdock	4.25	0.0952	0.693(b)	58.34	0.0606	Dehydrated, Chiba Prefecture Autumn, 1943
Soy bean	4.38	0.0915	0.190	4.73	0.0052	Manchuria
Cabbage	6.02	0.0674	1.083	26.94	0.0400	Dehydrated
Fish meal	15.43	0.1015	0.181	4.50	0.0045	
Bone dust	55.43	0.0823	0.251	6.25	0.0076	
Bone dust	57.87	0.0670	0.180	4.48	0.0066	

Though a remarkable amount of copper was found in many of the samples, the above experiments were not performed for the purpose of obtaining results form such a study. The above was conducted strictly and quantitatively to determine the use for which this method is valid.

Summary

The new method for the determination of a minute amounts of copper in biological matters by extraction with dithizone and coloration with sodium diethyldithiocarbamate has been tested. By the adaption of this method, copper can be determined in two hours or less after bringing the sample into solution. The experiments already related has proved that it is one of the most accurate methods in comparison with other methods which have been used. As recent studies are throwing light upon the biological action of copper, a more accurate method for copper determination is sought by chemists and biologist interested in this problem. This new method will fulfil its mission in being of invaluable importance in this respect.

The author wishes to take this opportunity to express his hearty thanks to Prof. Kenjiro Kimura for his kind guidance and invaluable advices throughout this study.

Chemical Institute, Faculty of Science,
the University of Tokyo,
Tokyo