THE IRON REAGENTS: BATHOPHENANTHROLINE BATHOPHENANTHROLINE-DISULFONIC ACID 2,4,6-TRIPYRIDYL-S-TRIAZINE PHENYL-2-PYRIDYL KETOXIME

2nd Edition
Revised
1965

By

HARVEY DIEHL, Ph.D.
IOWA STATE UNIVERSITY
AMES, IOWA

G. FREDERICK SMITH, Ph.D.
UNIVERSITY OF ILLINOIS
URBANA, ILLINOIS

LOREN McBRIDE and RICHARD CRYBERG
G. FREDERICK SMITH CHEMICAL COMPANY
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University of Illinois
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PREFACE TO THE SECOND EDITION

In the five years since the publication of the first edition of this monograph, the rapidly expanding use of the three reagents covered in that edition (Bathophenanthroline, Tripyridyl-s-triazine and Phenyl-2-pyridyl Ketoxime), and the introduction of a fourth (Bathophenanthrolinedisulfonic acid disodium salt), has prompted an updating of the material and the references. That the demand for such a monograph is great is evidenced by the fact that since the first edition of 14,000 copies went out of print in 1963, hundreds of requests for copies have had to be refused.

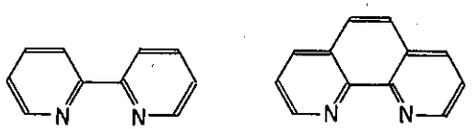
The second edition is intended to bring the reader the same information as contained in the first edition, with some clarification added, as well as a review of the more recent literature and some of the newer procedures. While it is impossible to give an exhaustive procedural review of all the literature on this subject in a booklet of this size, a complete bibliography of the recent literature is given for those who find that the information which they seek is not contained in this monograph.

The Authors

SECTION I

INTRODUCTION

In the closing years of the nineteenth century the Austrian chemist Fritz Blau synthesized 2,2′-bipyridine and 1,10-phenanthroline



and discovered that these compounds react with ferrous salts to produce soluble compounds having an intense red color. In an extensive, thorough, and imaginative study of these iron compounds, Blau isolated numerous salts of the red, positively charged ions produced in these reactions, $[Fe(bipy)_3]^{++}$ and $[Fe(1,10\text{-phen})_3]^{++}$. He showed that the bivalent iron compounds could be oxidized with strong oxidizing agents to the corresponding ferric ions, $[Fe(bipy)_3]^{+++}$ and $[Fe(1,10\text{-phen})_3]^{+++}$, intensely blue in color, and that the oxidation-reduction couples were reversible. Blau observed many other features of the chemistry of 2,2′bipyridine and 1,10-phenanthroline, some of which merit further study even now. During the late 1890's the coordination theory of Werner was in its infancy and van't Hoff, Arrhenius and Ostwald were still fighting to get the ionic dissociation theory established; even so, it is a bit surprising that Blau failed to grasp the true nature of the ionic character of his compounds and missed the ring structure formed by the union of the iron atoms with the organic molecules.

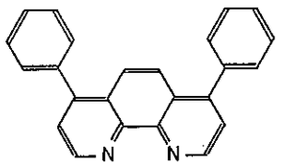
In 1912, the Swiss chemist Alfred Werner resolved the tris-(2,2′-bipyridine) ferrous ion into its optically active isomers by fractional crystallization of its tartrate and thus proved that the six nitrogen atoms of the three bipyridine molecules were attached to the iron atom at the apexes of a regular octahedron about the metal. The cyclic or ring structure so formed with the iron atom as one member of the ring is implicit in this

work. Such ring structures were later designated as *chelate rings* (after κελα, the claw of the lobster) by the English chemist G. T. Morgan.

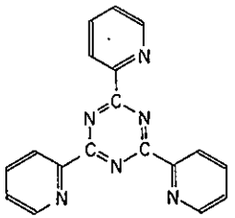
The uses of 2,2′-bipyridine and 1,10-phenanthroline in analytical chemistry began about 1930 with the application of 2,2′-bipyridine as a reagent for the colorimetric determination of iron and the use of the couple, $Fe(1,10\text{-phen})_3^{+++} + e^- = Fe(1,10\text{-phen})_3^{++}$, as a high potential oxidation-reduction indicator. The latter was proposed by G. H. Walden, Jr., L. P. Hammett and R. P. Chapman of Columbia University and was immediately appreciated and adapted to various titrations involving strong oxidizing agents. The term *ferroïin* was proposed by Karl Gleu of the University of Jena for the ferrous-1,10-phenanthroline compound which he described as the "phenolphthalein of oxidation-reduction indicators". About this time also an extensive investigation of the synthesis and numerous metal derivatives of 2,2′-bipyridine and 2,2′,2″-terpyridine was made by G. T. Morgan and F. H. Burstall and a similar investigation of the metal derivatives of 1,10-phenanthroline was made by Paul Pfeiffer of Bonn University. The use of 1,10-phenanthroline for the colorimetric determination of iron began in 1938 with the almost simultaneous introduction by G. Frederick Smith, by F. C. Hummel and H. H. Willard, and by W. B. Fortune and M. G. Mellon. Because of its superiority, the 1,10-phenanthroline method has become the preferred method for the determination of small amounts of iron.

During the 1940's and 1950's G. Frederick Smith of the University of Illinois studied the effects of substituents in the 2,2′-bipyridine and 1,10-phenanthroline molecules on the standard reduction potential of the iron couple and on the absorption spectra of the metal derivatives. In conjunction with Francis Case of Temple University, Smith studied some 150 compounds derived from or closely related to bipyridine and 1,10-phenanthroline. Out of this work came a line of oxidation-reduction indicators covering the range 0.87 to 1.33 volts in small steps and a number of colorimetric reagents of extraordinary sensitivity and specificity. In particular, there were discovered, custom built really, the reagents *bathophenanthroline*, *neocuproine*, and *bathocuproine*. Both neocuproine and bathocuproine are reagents for copper, bathocuproine being probably the greatest achievement to date in the field of deliberately tailoring an organic molecule to the purposes of analytical chemistry. The copper reagents are covered in the companion booklet to this one: "The Copper Reagents: Cuproine, Neocuproine, Bathocuproine" by Harvey Diehl and G. Frederick Smith, published by the G. Frederick Smith Chemical Company, Columbus, Ohio, 1958.

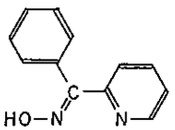
The present booklet deals with four of the newer reagents for iron. Three of these are of such extraordinary sensitivity as to make possible the determination of iron in the parts per billion range; the fourth makes possible the direct determination of iron in the presence of strong alkalies and the determination of oxidized iron in the presence of metallic iron.



4,7-Diphenyl-1,10-phenanthroline
Bathophenanthroline

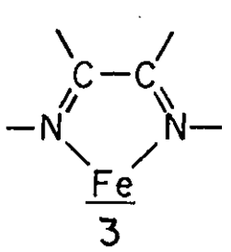


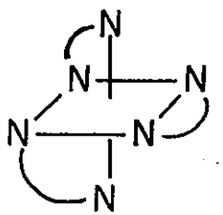
2,4,6-Tripyridyl-s-triazine
TPTZ



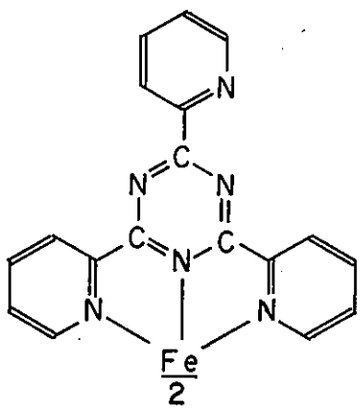
syn-Phenyl-2-pyridyl Ketoxime

Bathophenanthroline, bathophenanthrolinedisulfonic acid and phenyl-2-pyridyl ketoxime unite with ferrous iron in the ratio of three to one, each of the organic molecules uniting with the iron atom to form a five-membered ring with the whole molecule being arranged so that each nitrogen atom occupies one apex of a regular octahedron surrounding the iron atom:





2,4,6-Tripyridyl-s-triazine unites with ferrous iron in the ratio of two to one, acting to form a tridentate (three-toothed) chelate ring:



The ferrous derivatives of bathophenanthroline and tripyridyl-s-triazine carry a double positive charge, $[Fe(bathophen)_3]^{++}$ and $[Fe(TPTZ)_3]^{++}$; those of bathophenanthrolinedisulfonic acid and phenyl-2-pyridyl ketoxime probably carry negative charges, $[Fe\ (bathophenanthrolinedisulfonic\ acid)_3]^{-4}$ and $[Fe\ (phenyl\text{-}2\text{-}pyridyl\ ketoxime)_3]^{-}$.

The red, bathophenanthroline-iron compound and the violet, 2,4,6-tripyridyl-s-triazine-iron compound are formed completely only in very slightly acid solution, pH 3 to 7, and only if the iron is in the bivalent state. Provision must be made, therefore, for reduction of the iron and for pH adjustment. The reduction of trivalent iron is easily effected in acid solution, the usual reducing agent being hydroxylammonium chloride. In neutral solution the reduction proceeds more slowly and heating may be necessary. The solution is usually brought to the final acidity by the addition of sodium acetate; if a large amount of acid is present it must be neutralized first by the addition of ammonium hydroxide or sodium hydroxide. For the determination of iron in alkaline solution with phenyl-2-pyridyl ketoxime, the reduction is best effected with sodium hydrosulfite.

One of the great merits of all three unsulfonated iron reagents under discussion is the great solubility of the ferrous derivatives in organic solvents immiscible with water. The colored derivative can be extracted from the water layer into the immiscible solvent and the final spectrophotometric measurement made in the organic solvent. Two advantages accrue from this. A large sample may be used and the iron extracted into a small volume for measurement. A concentration can be achieved in this way and the sensitivity increased by a factor as great as thirty or forty. Second, the iron present as impurity in the various reagents may be extracted by a similar process and the blank thus reduced to essentially zero. The higher alcohols, isoamyl alcohol and *n*-hexyl alcohol are usually used for extracting the bathophenanthroline-ferrous compound; it is immaterial which anion be present. The only solvent for the violet, tripyridyl-s-triazine-ferrous compound is nitrobenzene and with it the extraction only occurs when perchlorate or iodide is present in the water layer. Isoamyl alcohol is the best solvent for extraction of the phenyl-2-pyridyl ketoxime-ferrous compound.

The effects of other metals on the determination of iron have been carefully studied for the bathophenanthroline, bathophenanthrolinedisulfonic acid and 2,4,6-tripyridyl-s-triazine methods. The greatest interference is caused by the presence of other transition metals, particularly copper and cobalt; fortunately the amounts of copper and cobalt which must be present to affect the result for iron is sufficiently large that no preliminary separation of the iron is necessary in the analysis of the majority of materials.

Iron is the fourth most abundant element in the crust of the earth and is widely distributed among the soils, rocks, and minerals. Iron plays a vital role in the plant and animal kingdom as one of the essential "trace elements". Because of its favorable metallurgical properties and its abundance and ease of recovery, iron is the basic material of civilization. Iron has powerful catalytic properties, particularly in oxidation-reduction reactions, and its presence in many manufactured products is objectionable. It is not surprising then that the determination of iron is the most common one the analyst is called on to make.

Fortunately, excellent methods are available for the determination of iron in all concentration ranges and in the presence of large amounts of the elements with which it is associated in natural and commercial materials. In ferrous metallurgical products a determination of iron is seldom made, for the principal interest in them is focused on the impurities and on the alloying elements present. In materials in which iron is a major component the determination is usually made by a volumetric method, the titration of bivalent iron with cerate or dichromate being most common. The gravimetric method is used to advantage in some cases and may be the only method applicable when a sequence of separations is involved as in the analysis of a silicate for other components. For small amounts of iron, 0.0001 per cent (1 part per million) to one or two per cent a colorimetric method is more appropriate, the 1,10-phenanthroline method being by far the most popular. For iron below a few parts per million (the parts per billion range) reagents of greater sensitivity must be used, bathophenan-

throline and TPTZ being among the best and being commercially available. This is the range of concentration of iron in such materials as sea water, treated municipal and industrial water, wine, blood, urine, and reagent chemicals. Certain problems are peculiar to the determination of iron in the parts per billion range; the sampling process and the storage of samples, freedom of the reagents from iron, and the loss of iron by adsorption on glassware or reduction and alloying with platinum during fusions, are all matters which require special attention. These aspects of the determination of traces of iron, as well as the chemistry and the operating procedures for the four reagents selected, are covered in the following sections.

TABLE I. DATA RELATIVE TO THE USE OF THE IRON REAGENTS

Beer-Lambert Law: $A = \varepsilon lc$ $\quad A = \log \frac{I_0}{I}$ $\quad T = 100 \log \frac{I}{I_0}$

A = Absorbancy $\quad$ T = Transmittancy $\quad$ ε = Molar extinction coefficient

l = Length of light path $\quad$ c = Concentration in moles per liter

Best working range: A = 0.7 to A = 0.1 (T=20% to T=80%)

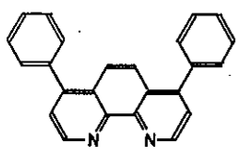
μg. = microgram (0.000,001 g.) $\quad$ 1 μg. per ml. = 1 part per million (p.p.m.)

	Bathophenanthroline-disulfonic acid	*Bathophenanthroline*	*2,4,6-Tripyridyl-s-triazine*	*Phenyl-2-pyridyl Ketoxime*
Molar extinction coefficient	22,100	22,350	24,100	15,600
Solvent	water	Isoamyl alcohol	Nitrobenzene	Isoamyl alcohol
Wave length of maximum absorption, mμ	535	533	595	550
Concentration in moles per liter corresponding to				
A=0.1 (T=80%) 1-cm. cell	4.5×10^{-6}	4.5×10^{-6}	4.1×10^{-6}	6.4×10^{-6}
A=0.7 (T=20%) 1-cm. cell	31×10^{-6}	31×10^{-6}	29×10^{-6}	45×10^{-6}
Concentration in mg. per liter (=μg. Fe per ml.) corresponding to				
A=0.1 (T=80%) 1-cm. cell	0.25	0.25	0.23	0.36
A=0.7 (T=20%) 1-cm. cell	1.8	1.7	1.6	2.5
Beer's law found to hold over the range, μg. per ml.	4.0	4.0	4.0	3.0
Recommended amounts of iron in final volume of 25.0 ml., μg.	6 to 45	6 to 42	5 to 40	9 to 65

SECTION II

BATHOPHENANTHROLINE

4,7-Diphenyl-1,10-phenanthroline



$C_{24}H_{16}N_2$

Mol. Wt.: 332.41

Molar Extinction Coefficient of $Fe(bathophenanthroline)_3^{++}$ in 70 per cent water-30 per cent ethyl alcohol: 22,143 at 543 mμ; isoamyl alcohol: 22,350 at 533 mμ; nitrobenzene: 23,300 at 538mμ

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4,7-Diphenyl-1,10-phenanthroline is an extraordinarily sensitive and highly specific reagent for iron. This substance was first prepared by Case[1] and investigated as an analytical reagent by Smith, McCurdy and Diehl.[2] The common name *Bathophenanthroline* was assigned to 4,7-diphenyl-1,10-phenanthroline because the absorption maximum of the ferrous derivative lies at a longer wave length than that of 1,10-phenanthroline, that is, 533 mμ compared with 510 mμ, a bathochromic shift.

Not only is the molar extinction coefficient of the ferrous bathophenanthroline ion (22,350) greater than that of ferrous 1,10-phenanthroline (11,100) but the new reagent can also be extracted from aqueous solutions with certain immiscible solvents, such as isoamyl alcohol and *n*-hexyl alcohol. Two important advantages are gained from this: the iron in large samples can be easily concentrated into a small volume for measurement and the solutions of the necessary reagents can be freed from iron, thus reducing the blank to zero. Whereas 1,10-phenanthroline can be used for the colorimetric determination of iron of concentrations as low as 0.1 p.p.m., bathophenanthroline can be used for as low as 0.005 p.p.m. Inasmuch as bathophenanthroline is more difficult to prepare and more expensive, its principal use is in the determination of very low concentrations of iron, such as those found in blood serum and in the finished water from water-softening plants. It can, however, be applied to larger amounts of iron and the final measurement made on the water solution as well as on the isoamyl alcohol extract.

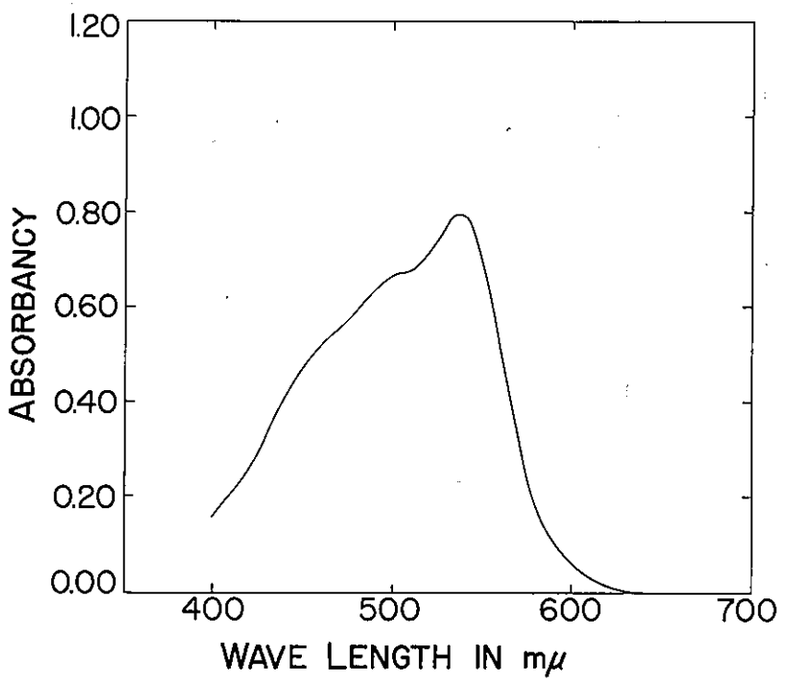

Fig. 1. Absorption spectrum of Fe(bathophenanthroline)$_3^{++}$ in isoamyl alcohol. 2.08 P.p.m. of iron. 1-cm. cell.

All the iron in the sample must be in the ferrous form. The reduction of any ferric iron is most conveniently brought about by adding hydroxylammonium chloride.

The bathophenanthroline ferrous ion is stable over the pH range 2 to 9 but forms most rapidly in slightly acid solution, pH 4. If the reaction is carried out in a slightly acid solution, one buffered with acetate, copper does not interfere.

The absorption spectrum of the ferrous-trisbathophenanthroline ion is shown in Fig. 1. At the wave length of maximum absorption, 533 mμ, the molar extinction coefficient of the isoamyl alcohol solution is 22,350. In water-ethyl alcohol solution, the wave length of maximum absorption is 543 mμ and the molar extinction coefficient is 22,143.

The color is stable for long periods of time.

The oxidation-reduction potential of the ferrous (bathophenanthroline)$_3^{++}$ couple was determined by Smith and Bannick[3] using potential buffered solutions of vanadate and vanadyl ions, present in equimolar concentrations, and dissolved in various strengths of sulfuric acid. By this method E_0 (hydrogen scale) was found to be 1.13 volts.

Specificity. Interferences. The familiar anions, chloride, sulfate, nitrate, acetate and perchlorate, do not interfere in the determination of iron with bathophenanthroline. If copper is present, certain anions such as iodide, thiocyanate, cyanide, thiosulfate, sulfide, and phosphate may cause precipitation in the aqueous solution upon addition of bathophenanthroline, but such precipitates do not interfere with the quantitative extraction of the ferrous-bathophenanthroline compound. Certain less common anions such as oxalate[4], citrate[4], lactate[5], fluoride[4,5] pyrophosphate[5] and phosphate[4] can interfere. This problem can be obviated by reducing the ferric iron in a strongly acidic solution by means of ascorbic acid, the acid reduction method, followed by the addition of bathophenanthroline and pH adjustment to the normal working range of 4 to 6 in the given order. Tartrate causes low results unless the extraction is made into chloroform in the presence of perchlorate [5,6]. Cyanide interferes by forming dicyano-bisbathophenanthroline Iron(II). Molybdates, tungstates and antimony chloride hydrolyze with formation of precipitates under the conditions of the determination; however, this problem can be solved by addition of tartrate to complex these metals, followed by a chloroform extraction of the bathophenanthroline-iron complex [5,6,7].

The following metal cations do not interfere: Li, Na, K, Be, Mg, Ca, Sr, Ba, Ce(IV), Ce (III), Pr(III) and the rare earths in general, Th, Ti, Zr, V as vanadate and vanadyl, Cr(III), W, U, Mn, Fe(III), Ru(III), Os, Ni, Pd, Pt, Ag, Zn, Cd, Hg(I), Hg(II), B, Al, Ga, Tl(I), Sn(II), Sn(IV), Pb. Tellurium and selenium are reduced to the metal by hydroxylammonium chloride. Cobalt forms a light yellow color but this is not extracted from acid solution. When sodium hydrosulfite is used as the reducing agent an extractable brown compound is formed with cobalt which causes a significant error[4]. At a pH of 4 copper combines with bathophenanthroline to form the extractable cuprous-monobathophenanthroline ion. This complex is colorless and does not interfere unless insufficient bathophenanthroline is present to complex both the iron and copper present [2,7]. In the presence of large amounts of copper separation prior to determination can be performed by extraction of ferric chloride into ethyl acetate [8], or perhaps more conveniently by complexing the copper with thiourea[9]. Hair and Newman [51] precipitate the copper as cuprous thiocyanate and following filtration determine the iron in an aliquot of the filtrate. Manganese also interferes by competition for bathophenanthroline. Addition of excess bathophenanthroline overcomes this difficulty.

Reduction of Ferric Iron. If all iron present is in an uncomplexed state hydroxylamine hydrochloride is the reducing agent usually used. Ascorbic acid, hydrazine hydrochloride or hydroquinone are also satisfactory

if purified to free them from traces of iron contamination. In the presence of citrate, oxalate, tartrate or fluoride reduction is incomplete with these reducing agents at pH of 4. Sodium hydrosulfite ($Na_2S_2O_4$) is however, satisfactory as a reducing agent even in the presence of the above mentioned ions. The iron contaminate in sodium hydrosulfite may be removed by the addition of bathophenanthroline followed by extraction into chloroform; however, the solution thus purified is not stable and should not be stored more than one-half hour according to Gahler and coworkers [7]. Booth and Evett [10] used stannous chloride as the reducing agent added to a strongly acidic solution, followed by addition of citrate and EDTA to complex other metals present, then addition of buffer to raise the pH to the desired level. Penner and Inman [9] and Cluley and Newman [6] recommend ascorbic acid for the reduction of iron at a low pH. Thioglycolic acid has been used by Tetlow and Wilson [11] to both dissolve particulate iron and reduce any ferric iron present to the ferrous state.

Extraction. Isoamyl, *n*-amyl or *n*-hexyl alcohol may be used for the extraction. The distribution coefficient of the red, bathophenanthroline ion between these alcohols and water could not be readily determined because of the great solubility of the ion in the alcohols. However, the ferrous derivative is more completely removed by one extraction with *n*-hexyl alcohol than by similar treatment with isoamyl or *n*-amyl alcohol. This is probably the consequence of *n*-hexyl alcohol being the least water-soluble alcohol of the group. Under ordinary conditions two extractions with isoamyl alcohol are sufficient to completely recover the iron. *n*-Hexyl alcohol may be preferable where the volume of sample is large and minute amounts of iron are present. The reagent bathophenanthroline as well as the ferrous derivative is extracted quantitatively from essentially neutral aqueous solution by the immiscible alcohols.

Nitrobenzene may also be used to effect the extraction of the ferrous bathophenanthroline ion. The molar extinction coefficient is somewhat greater in nitrobenzene than in the alcohol solvents, 23,300 at the wave length of maximum absorption, 538 mμ. The turbidity which occasionally develops in the isoamyl alcohol extracts, particularly when perchlorate is present, does not appear in nitrobenzene. Repeated extractions can be made with ease if necessary, inasmuch as the nitrobenzene is the lower phase. Although nitrobenzene has a slight yellow color, its absorbancy is zero at 538 mμ and no correction needs to be made for it. After separation of the water and nitrobenzene layers the nitrobenzene solution is diluted with ethanol to the final volume; the ethanol renders miscible any droplets of water carried along with the nitrobenzene and has no effect on the intensity of the color.

Other solvents which have been used to extract the ferrous complex from the aqueous phase are octyl alcohol [12], chloroform [4,5], a chloroform-ethanol mixture [7,13] trichloroethane and isoamyl acetate [14]. In the determination of ferrous iron in the presence of ferric iron [14] Clark found that isoamyl acetate was indeed the only satisfactory solvent with which to carry out the extraction. Chloroform or chloroform-ethanol mixtures are recommended by several workers [4,7,13].

Applications. The general procedure for the determination of iron with bathophenanthroline was worked out by Smith, McCurdy and Diehl[2] who applied it specifically to the determination of iron in raw and treated municipal water supplies. The iron content of boiler water is an important problem industrially and five subsequent papers deal with this problem [11,15,16,17,18]. Iron in gold [20], beryllium [21], bismuth[22], tungsten[4,7,9], molybdenum[5,9], vanadium[7], chromium[7], titanium[7], niobium [7,9], tantalum [7,9] and uranium [7,8] has been determined using bathophenanthroline. The determination of iron in copper was mentioned under interferences. The iron present in aluminum oxide (sapphire and ruby) is of importance in the control of properties of crystals grown for use in masers [23]. Bathophenanthroline has also been applied to the determination of iron in serum [12, 24, 25, 26, 27, 28, 29, 30, 32, 33, 34], in sea water [35], in urine [36, 37, 49], in wine [38], in oil [39], in culture media [40] and in plasma [41]. The determination of ferrous iron in the presence of ferric iron has been the subject of three papers, the determination of ferrous iron in water [42], the determination of ferrous iron in oxides [14], and the determination of ferrous iron in soil and rock [43]. Bathophenanthroline has also been used to determine, indirectly, the amount of a few specific oxidizing agents. In the determination of the tocophenols [44, 45] and corticoids [46] bathophenanthroline is said to be more sensitive and more stable than several other reagents formerly used. Bathophenanthroline has also been applied to the indirect determination of hydrogen peroxide [47]. Bathophenanthroline has been used as a stain for iron bearing tissues [48]. Ferrocyanide has also been determined using this reagent [31].

Zak and coworkers have worked out several methods for simultaneously determining iron and copper in serum using various 1,10-phenanthrolines and tripyridyl-s-triazine. Two reagents are used, one to complex the iron and one to complex the copper. Solubility differences in immiscible solvents are sometimes used to separate the iron and copper and in other cases absorbance measurements are made at two wavelengths and solution of simultaneous equations employed to obtain the amounts of iron and copper present in the sample. Bathophenanthroline was used in conjunction with 2,9-dimethyl-1,10-phenanthroline [28] (results obtained by solution of simultaneous equations), 2,2′-biquinoline [29] (results obtained by solution of simul-

taneous equations), 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline [30] (determination done on separate aliquots) and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonate [30] (iron-bathophenanthroline complex extracted into an immiscible solvent).

IRON IN RAW AND TREATED WATER AND IN SEA WATER. The iron in hard waters will normally run from a few hundredths to two or three parts per million. Iron is present in ground waters as ferrous bicarbonate, $Fe(HCO_3)_2$, having passed into the water along with calcium and magnesium by the attack of the carbonic acid in the water on the minerals, principally limestone, with which it has had contact: $CaCO_3 + H_2CO_3 = Ca(HCO_3)_2$; $FeCO_3 + H_2CO_3 = Fe(HCO_3)_2$. On contact with oxygen the iron is oxidized rapidly to the trivalent state and, the pH being 6 or so, is precipitated as ferric hydroxide. Aeration to remove iron is normally the first step in the water treatment process. Ferric hydroxide is extremely insoluble and the filtration operation in waterworks practice is usually so effective that the iron in treated waters will generally be of the order of a few thousandths of one part per million, that is, in the parts per billion (p.p.b.) range. There are two consequences of this relative to the determination of iron in water: first, attention must be paid that iron is not lost during the sampling process; second, that the method used be suitable to the amount of iron present. If any appreciable time must elapse between the sampling operation and the analysis, the sample should be acidified with hydrochloric acid when taken; this will prevent the precipitation of ferric hydroxide and its loss by adsorption on the walls of the container. Iron in the range 0.5 to 5 p.p.m. can be determined conveniently with 1,10-phenanthroline, although if the iron is less than 1 p.p.m., cells of 2, 4, or 5 cm. path length must be used. For iron in the p.p.b. range, 1,10-phenanthroline is not sufficiently sensitive and bathophenanthroline must be used.

Working in the parts per billion range, the iron introduced by the reagents may be much greater than the iron in the sample. It is the great merit of the bathophenanthroline method that the reagents may first be freed of iron and the blank thus reduced to essentially zero.

The determination of the iron in sea water is basically the same as that for finished or treated water. The iron content of sea water is very low, 2 to 20 p.p.b., and fortunately the large amount of salt present has no effect on the bathophenanthroline method. The determination is somewhat more complicated, however, owing to the presence of organic matter and of "particulate" or filterable iron and "non-particulate" iron, that which is in true solution and passes through a filter of 5 microns pore size. This micro filtration, the bathophenanthroline procedure, the significance of iron in the biochemistry of the sea, and a review of the early literature on the iron in sea water problem will be found in the excellent paper of Lewis and Goldberg[35].

Detailed procedures for the determination of iron in water are given below.

IRON IN WINE. Wines having an iron content greater than 5 to 10 parts per million frequently develop on standing a turbidity called "iron casse". Bottling is delayed until the iron content of the wine is below this value and the rapid and accurate determination of iron in wine is thus a matter of some economic importance. A common method for carrying out this determination has been the colorimetric method using 1,10-phenanthroline following the wet oxidation of the organic matter in the wine with either a mixture of nitric and perchloric acids or with hydrogen peroxide. Bathophenanthroline was adapted to this analysis in 1957 by Banick and Smith[38] who devised two procedures: ashing with nitric and perchloric acids followed by the colorimetric determination with bathophenanthroline, and a direct extraction method based on the solubility of the $Fe(bathophen)_3^{++}$ ion in isoamyl alcohol. Essentially identical results were obtained by the two methods and by the wet ashing-1,10-phenanthroline method on five different wines. The agreement in the series of determinations on the same wine by each method was also good, the spread, for example, from the highest to the lowest result by the direct extraction method being 20 parts in 1000. Working details of the direct extraction method are given below in the section dealing with working procedures.

The paper of Banick and Smith[38] deals also with the determination of copper in wine; this method is also discussed in the booklet of Diehl and Smith, "The Copper Reagents: Cuproine, Neocuproine, Bathocuproine," G. FREDERICK SMITH CHEMICAL COMPANY, Columbus, Ohio, 1958.

In connection with the results obtained for iron in wine by the direct extraction method, see also the discussion of the determination of iron in wine using 2,4,6-tripyridyl-s-triazine, page 46.

IRON IN BLOOD SERUM. Bathophenanthroline can be used to advantage for the determination of the small amounts of iron in serum, normally about one microgram per milliliter. Many procedures for the determination of serum iron with this reagent have been described. In the procedures of Peterson[24] and of Kingsley and Getchell[26] the iron-bathophenanthroline compound is extracted into isoamyl alcohol and the spectrophotometric measurement made on this solution; in the procedure of Peters, Giovanniello, Apt and Ross[27] the measurement is made on the aqueous solution to which propanol has been added to eliminate any turbidity present. The principal problem in the determination of serum iron is to differentiate between hemoglobin and non-hemoglobin or serum iron and to insure for the final

spectrophotometric measurement that no non-hemoglobin iron remains bound with the protein present. Thus, the reagents should not cause the release of iron from any hemoglobin which may remain in the serum and yet should strip away any iron combined with the protein. The Peterson procedure relies on the precipitation of the protein with trichloroacetic acid, thioglycolic acid being present to reduce any ferric iron in the sample and promote its conversion to the final, colored compound for measurement. The Kingsley and Getchell procedure utilizes 1 N hydrochloric acid to release the iron from the protein. The procedure of Peters and Giovanniello, Apt and Ross relies on more dilute, 0.2 N, hydrochloric acid aided by a little thioglycolic acid. Following any of the procedures for the release of iron from protein, bathophenanthroline appears to be a preferred reagent for the final color measurement. Although extraction with isoamyl alcohol would not seem to introduce any great factor of delay or inconvenience, Peters and his coworkers think there is some merit in making the final measurement on the original solution diluted with propanol.

IRON IN URINE. The iron present in urine is normally very low, of the order of a few tenths to one or two parts per million and bathophenanthroline is again the ideal reagent. The procedure of Seven and Peterson[49] calls for wet ashing of the urine with a mixture of sulfuric and nitric acids followed by a clean up with 30 per cent hydrogen peroxide. This procedure is given below; a shorter and cleaner wet ashing procedure using perchloric and nitric acids is also given[36]. The wet ashing by perchloric acid-nitric acid proceeds rapidly and smoothly and only 30 to 45 minutes is required for a 50-ml. sample of urine. Because the perchlorate remaining after neutralization with ammonia tends to cause the development of a turbidity in an isoamyl alcohol extract the procedure utilizes nitrobenzene as the immiscible extraction solvent. Besides calling for a larger sample this procedure directs that the nitrobenzene extracts be diluted to a known volume in a volumetric flask before the final measurement is made and the precision thus obtained is greater than in other procedures.

IRON IN METALLIC BISMUTH. The problems connected with the determination of traces of iron in the presence of a large amount of bismuth were solved by Booth and Evett[22]. The precipitation of bismuth from the neutral solution in which the ferrous-bathophenanthroline compound is formed was prevented by the addition of a mixture of ethylenediaminetetraacetate and citrate. The reduction of ferric iron is somewhat more difficult in the presence of citrate and it is necessary to use stannous chloride rather than the weaker reducing agents hydroxylammonium chloride or hydroquinone.

DETERMINATION OF IRON IN METALLIC COPPER. The determination of iron in copper metal is complicated by the fact that sufficient bathophenanthroline must be added to combine with both the copper and the iron. The method of Diehl and Buchanan[50] circumvents this difficulty by utilizing the violet color of dicyano-bis(4,7-diphenyl-1,10-phenanthroline)iron(II) formed when a solution containing iron and bathophenanthroline is treated with cyanide and extracted with an organic solvent. Copper in either valence state is converted to the colorless, non-dissociated ion, $Cu(CN)_3^{--}$, which is more stable than the cuprous-bathophenanthroline ion. The nature of the ions requires that the ferrous-bathophenanthroline ion be formed first and that the mixture then be treated with cyanide. In the reverse procedure, that is the treatment of a solution of iron with cyanide and then the bathophenanthroline, the desired iron-bathophenanthroline ion is not formed. Some method is further required to prevent the reaction between the bathophenanthroline and the copper at least until the ferrous compound has had an opportunity to form. In the procedure, the copper is removed from the reaction by precipitation as the thiocyanate before the addition of the bathophenanthroline. The cuprous thiocyanate is then redissolved by the addition of cyanide; any iron co-precipitated with it is thus returned to the solution. Of the organic solvents chloroform and nitrobenzene are the most satisfactory for extracting the ferrous-bathophenanthroline cyanide. The method was applied to the determination of iron in copper wire and in National Bureau of Standards Sample No. 45c, Copper Melting Point Standard. The procedure will not work for the determination of iron in brass owing to interference by zinc. Details for carrying out the Diehl and Buchanan procedure are given below.

Procedure for the Determination of Iron. REAGENTS. BATHOPHENANTHROLINE SOLUTION. 0.001 M IN 50 PER CENT ETHANOL. Dissolve 0.0334 g. of 4,7-diphenyl-1,10-phenanthroline (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 108) in 50 ml. of ethyl alcohol and dilute with 50 ml. of iron-free water. Store in a glass bottle with a plastic cap having a polyethylene liner.

HYDROXYLAMMONIUM CHLORIDE. 10 PER CENT AQUEOUS SOLUTION, IRON-FREE. Dissolve 10 g. of hydroxylammonium chloride in 100 ml. of water. Add 3 to 4 ml. of 0.001 M bathophenanthroline. Place the solution in a 125-ml. conical, separatory funnel and add 10 to 20 ml. of isoamyl alcohol. Shake the contents of the funnel and allow 5 minutes for the supernatant alcohol solution of the extracted red compound resulting from iron impurities in the salt to separate. Draw off the colorless, aqueous, lower layer into a second separatory funnel, add 1 ml. of bathophenanthroline solution and repeat the extraction with a further portion of isoamyl alcohol. Store the iron-free hydroxylammonium chloride solution in a flint glass or borosilicate glass bottle with a plastic cap with a polyethylene liner. The pH of this solution will be 1.5 to 1.75 and the solution will be essentially free of bathophenanthroline, that added having passed almost quantitatively into the isoamyl alcohol. The small amount of isoamyl alcohol remaining in the solution is not detrimental.

SODIUM ACETATE. 10 PER CENT AQUEOUS SOLUTION, IRON-FREE. Dissolve 10 g. of sodium acetate in 100 ml. of water in a separatory funnel. Add 3 to 4 ml. of bathophenanthroline solution and 2 ml. of 10 per cent hydroxylammonium chloride solution.

Extract the red iron compound by adding 10 to 20 ml. of isoamyl alcohol and shaking the contents of the separatory funnel thoroughly. Allow the immiscible liquids to separate and draw off the lower aqueous layer into a second separatory funnel. Repeat the extraction to ensure the complete removal of iron. Store the solution in a glass bottle with a plastic cap having a polyethylene liner.

ISOAMYL ALCOHOL. Reagent grade isoamyl alcohol can be used directly. Distil technical grade before use.

IRON-FREE WATER. Pass distilled water through a column of Amberlite MB-3 (mono bed) exchange resin.

STANDARD IRON SOLUTION. 10.0 μg. Fe PER ML.; 1.00 μg. Fe PER ML. Prepare two standard iron solutions, containing respectively 10.0 μg. and 1.00 μg. of iron per ml., starting with ferrous ammonium sulfate hexahydrate, ferrous ethylenediammonium sulfate tetrahydrate, or electrolytically prepared iron metal. If one of the salts is used proceed as follows. Weigh carefully 0.0702 g. of ferrous ammonium sulfate hexahydrate or 0.0684 g. of ferrous ethylenediammonium sulfate tetrahydrate (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 41) and transfer to a 1-liter volumetric flask. Add iron-free water to dissolve the salt, add 2.5 ml. of concentrated sulfuric acid, dilute exactly to the mark with iron-free water and mix well. Pipet 100.0 ml. of this solution into a second 1-liter volumetric flask, add 2.5 ml. of sulfuric acid, dilute to the mark with iron-free water, and mix well. The first of these solutions contains 10 μg. of iron per ml.; the second contains 1 μg. of iron per ml.

Alternately, make up the standard iron solution starting with electrolytic iron (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 226). Weigh accurately about 0.10 g. of iron and transfer to a 500-ml. conical flask. Dissolve the iron in a mixture of 5 ml. of concentrated sulfuric acid and 30 ml. of water. Transfer the solution to a 1-liter volumetric flask and dilute to the mark with iron-free water. Pipet 10.0 ml. of this solution into another 1-liter volumetric flask, add 2.5 ml. of concentrated sulfuric acid and dilute to the mark with iron-free water. Because the electrolytic iron comes in small pieces and it is impractical to weigh exactly 0.1000 g., this final solution will contain only approximately 1 μg. of iron per ml. but the exact value will be known from the weight taken and the two dilutions made.

GENERAL PROCEDURE. PREPARATION OF CALIBRATION CURVE. Carry several solutions through the process together; for example, two samples of the unknown to be analyzed, three or four standards, and a blank. Once a calibration curve has been established, further standards need not be run except as an occasional check. For the standards, use various amounts of the standard iron solution containing 1.00 μg. of iron per ml., for example: 1.00, 2.00, 5.00, 10.0, and 15.0 ml., corresponding to 1.00, 2.00, 5.00, 10.0 and 15.0 μg. of iron. For the sample choose a volume which will contain between 1 and 15 μg. of iron.

Pipet the sample (water being tested or standard iron solution) into a 60-ml. separatory funnel. If the sample taken was less than 10 ml. add sufficient iron-free water to bring the volume to 10 ml. Use 10 ml. of iron-free water for the blank. To each sample add 2 ml. of 10 per cent hydroxylammonium chloride solution. Add 4 ml. of 10 per cent sodium acetate solution. If the original sample of the water had been acidified with hydrochloric acid when taken add an additional 4 ml. of sodium acetate solution. Add 4 ml. of 0.001 M bathophenanthroline and mix. Add 6.0 ml. of isoamyl alcohol, stopper the funnel, and shake the mixture well. Allow the liquids to separate. After not less than 5 minutes after adding the bathophenanthroline and after the liquids have cleanly separated into two layers, draw off and discard the lower aqueous layer. Shake away any of the aqueous layer remaining in the separatory funnel. Drain the isoamyl alcohol layer into a 10-ml. volumetric flask. If more than 10 ml. are required to fill the cell of the colorimeter to be used later, use a 25 ml. volumetric flask rather than a 10-ml. flask at this point. Wash out the separatory funnel with 2 to 3 ml. of ethyl alcohol added from a pipet in such a manner that the upper stopper of the funnel and the walls of the funnel are uniformly washed at least twice by a film of alcohol as it drains from the top to the bottom. Transfer this wash alcohol to the volumetric flask. Dilute the solution in the flask to mark with ethyl alcohol and mix by shaking. At this point the solution in the volumetric flask should

be clear with no turbidity, the ethyl alcohol added being sufficient to render miscible the isoamyl alcohol and the few droplets of water which are carried along in separating the layers.

Measure the absorbancy of the solution. If a spectrophotometer is used make the measurement at 533 mμ. If the measurement is made with a photoelectric colorimeter, use (in addition to the Aklo infrared filter) a blue green filter; the Corning filters 4-64, or 4-68 are especially recommended, but certain others for example, Corning 4-65 and 4-67, are satisfactory and render the method only slightly less sensitive.

Plot the data obtained on the standards after subtracting the absorbancy of the blank, plotting absorbancy or transmittancy against concentration. Use the calibration curve to obtain the amount of iron in the sample analyzed.

Nessler Tube Procedure for Iron in the Range 0.01 to 0.1 p.p.m. Carry several solutions through the process together; for example, two samples of the unknown, a blank, and three or four standards. The colorimetric comparison is later to be made in Nessler tubes, and for routine work a series of standards can be made and preserved for a long period.

Pipet 100 ml. of the water to be tested into a 125-ml. separatory funnel. For the standards pipet the desired volumes, for example, 1.00, 2.00, 3.00, ..., 10.0 ml., of the standard iron solutions containing 1 μg. of iron per ml. into 100 ml. of iron-free water in a 125-ml. separatory funnel. Add 2 ml. of iron-free, 10 per cent hydroxylammonium chloride solution. Add 4 ml. of iron-free, 10 per cent sodium acetate solution; if the sample taken contained any free acid, add additional sodium acetate solution. Add 4 ml. of 0.001 M bathophenanthroline and mix well. Add 10 ml. of isoamyl alcohol. Shake the mixture well and then allow it to stand for 5 minutes. Draw off the aqueous layer into a second 125-ml. separatory funnel. Add to this 10 ml. of isoamyl alcohol, shake well and allow to stand until the layers have separated. Draw off and discard the aqueous layer. Transfer both colored isoamyl alcohol extracts to a 50-ml. volumetric flask, rinsing both separatory funnels with alcohol, and mix thoroughly. Transfer to a 50-ml. Nessler tube. Carry out the comparison by looking down through the full length of the tubes toward a sloping, white, reflecting background such as provided in the conventional Nessler rack.

Inasmuch as unknown and standards were diluted to the same volume, the unknown contains the same weight of iron as that in the standard which it matches. From this weight and the volume of sample taken, calculate the concentration of iron in the sample in p.p.m.

Determination of Iron in Raw and Treated Waters. Untreated, or raw, well water will normally contain from one-tenth of a part to five parts per million of iron. For this range, the general procedure given above, using a 1 to 10-ml. sample of the water, is applicable. Municipal water plants generally supply city water mains with a product that is sufficiently free from iron to fall within the range covered by the Nessler tube procedure given above. A sample of 100 ml. is used.

Sampling of Raw Water. It is important to sample well water at the source and to perform the analysis immediately after taking the sample. If the latter is not possible, fill the sample bottle completely full to help retard oxidation of iron by contact with atmospheric oxygen. If an appreciable length of time is likely to elapse between sampling and analysis, add 2 ml. of iron-free hydrochloric acid to the sampling bottle before sampling to ensure that no precipitation of iron occurs.

Procedure for the Determination of Iron in Wine. BATHOPHENANTHROLINE-DIRECT EXTRACTION METHOD OF BANICK AND SMITH[38]. In this procedure, carry a blank through the same operations omitting the wine. Pipet into a 100-ml. beaker: 1. 5.00 ml. of wine, 2. 5.0 ml. of sodium acetate-acetic acid buffer (approximately 10 per cent sodium acetate adjusted to pH 4 by the addition of acetic acid), 3. 2.0 ml. of 10 per cent hydroxylammonium chloride, 4. 2.0 ml. of 0.001 M bathophenanthroline, and 5. (for red wines only, omit for white wines) 2.0 ml. of 95 per cent ethanol. Heat the mixture to a gentle boil and boil for one minute. Transfer the solution while still hot to a 60 ml., glass-stoppered separatory funnel. Add 6 ml. of isoamyl alcohol and shake for 30 seconds. Allow the mixture to stand for three minutes to permit the liquids to

separate and then draw off the lower, aqueous layer. Rinse the original beaker with 1 ml. of 95 per cent ethanol and two 5-ml. portions of water, transferring the rinsings to the separatory funnel containing the isoamyl alcohol layer. Add 10 ml. of buffer solution (pH 4) and shake the mixture for 30 seconds. Allow five minutes for the liquid phases to separate and draw off the lower layer. Transfer the isoamyl alcohol layer quantitatively to a 10-ml. volumetric flask and dilute to the mark with 95 per cent ethanol. Measure the absorbancy of this solution at a wave length of 533 mμ. Obtain the amount of iron present from a calibration curve prepared in the same manner using appropriate volumes of a standard iron solution. Subtract the amount of iron in the blank and convert the net weight of iron in μg. per 5.00 ml. of sample to parts per million of iron for reporting.

Procedure for the Determination of Iron in Serum. TRICHLOROACETIC ACID-THIOGLYCOLIC ACID-BATHOPHENANTHROLINE METHOD OF PETERSON[24]. Pipet 1 to 2 ml. of serum or plasma into a 15-ml. centrifuge tube, the volume depending upon the anticipated amount of iron. Dilute to a volume of 6 ml. with iron-free water and mix. Add 2 ml. of a solution containing 20 g. of redistilled trichloroacetic acid and 1 g. of thioglycolic acid per 100 ml. Mix and allow to stand 5 to 10 minutes. Place in a water bath at 90 to 95° for 10 to 15 minutes and then centrifuge. Decant the supernatant liquid into a 20-ml. glass-stoppered test tube or a 25-ml. glass-stoppered graduated cylinder. To the precipitate in the centrifuge tube add 2 ml. of water and 0.5 ml. of the trichloroacetic acid-thioglycolic acid reagent and mix. Return this tube to the water bath at 90 to 95° for 5 to 10 minutes. Remove, centrifuge, and decant the supernatant liquid into the first supernatant liquid. Add 2 ml. of the saturated sodium acetate solution to the combined supernatant liquid, bringing the pH of the solution to 4.0 to 5.0. Add 2 ml. of 0.0025 M bathophenanthroline in isoamyl alcohol, 0.5 ml. of the reagent being required for each microgram of iron present. Add isoamyl alcohol to a total volume of 6 ml. Stopper the tube and shake vigorously. Pipet the isoamyl alcohol layer into a spectrophotometer cell and measure the absorbancy at 533 mμ. Carry standards containing 2 to 4 μg. of iron through the same procedure.

Procedure for the Determination of Iron in Serum. METHODS OF PETERS, GIOVANNIELLO, APT AND ROSS[27]. Pipet 2.0 ml. of serum or plasma (fresh or stored, citrated, oxalated, or heparinized) into a test tube. Add 3.0 ml. of 0.2 N hydrochloric acid and 1 drop of an 80 per cent solution of thioglycolic acid. Mix and allow to stand 30 minutes at room temperature. Add 1.0 ml. of a 30 per cent solution of redistilled trichloroacetic acid. Mix with a stirring rod and allow to stand 15 to 30 minutes at room temperature, covered with a paraffin film. Centrifuge for about 15 minutes at high speed. Pipet 4.0 ml. of the supernatant liquid into a colorimeter cell. Add 0.5 ml. of saturated sodium acetate solution and 2.0 ml. of bathophenanthroline solution prepared by dissolving 0.020 g. of 4,7-diphenyl-1,10-phenanthroline in 100 ml. of a mixture consisting of three parts of isopropyl alcohol and one part of isoamyl alcohol. Mix well. Measure the absorbancy at 535 mμ or with one of the green filters recommended above.

Carry known amounts of iron, obtained by measuring out portions of the standard solution described under General Procedure, through the determination and prepare a suitable calibration curve.

Micro Procedure for the Determination of Iron in Serum. METHOD OF FORMAN[13]. Pipet 100 μl. of serum into a 400 μl. micro-test tube and add 50 μl. of 0.4N hydrochloric acid and 1 drop of 80 per cent thioglycolic acid. Mix and allow to stand 5 minutes. Add 50 μl. of 30 per cent trichloroacetic acid. Mix well with a micro-mixer centrifuge for 3 minutes at 10,000 rpm. Transfer 180 μl. of the clear supernatant liquid to a micro-test tube and add 25 μl. of potassium acetate (50 g. per 100 ml.) and 20 μl. of .025 per cent bathophenanthroline in isopropyl alcohol. Mix thoroughly. After 10 minutes, extract with 200 μl. of ethanol-chloroform solvent (1:4). Shake and centrifuge. Transfer the lower layer to a micro cell and measure the absorbance at 535 mμ. Carry blank and standards through the same procedure.

Procedure for the Determination of Iron in Urine. SULFURIC ACID-NITRIC ACID-HYDROGEN PEROXIDE-WET OXIDATION-BATHOPHENANTHROLINE METHOD OF SEVEN AND PETERSON[49]. Pipet 5 to 10 ml. of urine into a 250-ml. conical flask, add 0.75 ml. of concentrated sulfuric acid and 5 ml. of concentrated redistilled nitric acid. Insert a reflux head (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 181) and digest on a hot plate at a temperature of about 250° until the thick brown fumes almost disappear and the remaining solution is nearly colorless. Cool partially and wash down the reflux head and the walls of the flask with iron-free water. Add 5 ml. of 30 per cent hydrogen peroxide through the reflux head and heat until the gas evolution stops. Cool partially and add 2 ml. of 30 per cent hydrogen peroxide. Heat for 1 hour at an intermediate temperature not over 250°, making certain that the liquid condensed is being returned to the boiling solution. Cool, wash down the reflux head and remove it. Wash the walls of the flask until at least 20 ml. of iron-free water has been added. Add 0.2 per cent potassium permanganate dropwise (usually 1 to 3 drops) until a faint pink color persists. Add 3 ml. of 10 per cent hydroxylammonium chloride. Mix the solution and add 15 ml. of saturated sodium acetate. Mix and add 4.00 ml., pipetted carefully, of 0.0025 M bathophenanthroline in isoamyl alcohol and immediately close the flask with a rubber or glass stopper. Shake vigorously until no further red color development is apparent, usually 60 to 90 seconds. When alcohol and aqueous phases have completely separated, aspirate the aqueous layer and discard. Close the flask immediately. Draw off enough isoamyl alcohol-bathophenanthroline solution to fill a cuvette and measure the absorbancy at a wave length of 533 mμ.

Carry a blank through the same procedure.

Procedure for the Determination of Iron in Urine. WET ASHING-BATHOPHENANTHROLINE-NITROBENZENE EXTRACTION METHOD OF COLLINS AND DIEHL[36]. REAGENTS. 0.001 M BATHOPHENANTHROLINE. 10 PER CENT HYDROXYLAMMONIUM CHLORIDE, IRON-FREE. 10 PER CENT SODIUM ACETATE, IRON-FREE. STANDARD IRON SOLUTION. See directions given above at the beginning of the section devoted to working procedures.

NITRIC ACID. Redistil reagent grade nitric acid from an all-glass still and store in a Pyrex bottle.

PERCHLORIC ACID. Use doubly distilled perchloric acid. (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 67).

AMMONIUM HYDROXIDE. Use reagent grade ammonium hydroxide, or to reduce the blank to the very minimum, distil anhydrous ammonia into iron-free water.

PROCEDURE. Rinse all glassware with hydrochloric acid (1:1) before using to remove iron. Run a reagent blank along with the samples in exactly the same manner. Pipet 50.0 ml. of the urine into a 250-ml. conical flask. Add 25 ml. of nitric acid and 10 ml. of perchloric acid. Place a reflux head on the flask, heat to fumes of perchloric acid and continue the digestion for 10 minutes. If a stone or Transite hood is not available for the wet ashing use a glass fume eradicator (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 184). After the flask and contents have cooled to room temperature rinse the reflux head with water and wash down the sides of the flask. Heat the solution to boiling to dissolve the precipitate of ammonium perchlorate and to remove chlorine. While still hot, transfer the solution to a 125-ml. separatory funnel and add 2.0 ml. of 10 per cent hydroxylammonium chloride and 5.0 ml. of 0.001 M bathophenanthroline. Place a small piece of Congo Red paper in the solution and add dropwise ammonium hydroxide until the paper turns red. Complete the adjustment of pH by adding 5.0 ml. of 10 per cent sodium acetate solution. After the solution has cooled to room temperature add 4.0 ml. of nitrobenzene and shake vigorously for 1 minute. Allow the phases to separate and gently swirl to dislodge any droplets of nitrobenzene clinging to the upper walls of the funnel. Collect the nitrobenzene layer in a 10-ml. volumetric flask and repeat the extraction two more times using 2.0-ml. portions of nitrobenzene. Dilute the combined extracts to exactly 10 ml. with ethanol and mix. Determine the absorbancy of the solution at 538 mμ using 1 cm. cells. Use a mixture of nitrobenzene and ethanol (4:1) as the reference solution. Correct the absorbancy of the unknown solution by subtracting from it the absorbancy of the reagent blank.

Prepare a calibration curve by pipetting various volumes from 0 to 25 ml. of the standard iron solution (about 1 μg. Fe per ml.) into 125-ml. separatory funnels. Add 10 ml. of 10 per cent ammonium perchlorate, 2.0 ml. of 10 per cent hydroxylamonium

chloride, 5.0 ml. of 0.001 M bathophenanthroline and 8.0 ml. of 10 per cent sodium acetate solution and proceed with the extraction as directed in the preceding paragraph. Use the extract from the solution to which no iron was added as the reagent blank and subtract its absorbancy from the absorbancy of each of the other solutions. Prepare a plot of absorbancy vs. concentration.

Procedure for the Determination of Iron in Copper Metal. METHOD OF DIEHL AND BUCHANAN[50]. SEE ALSO HAIR AND NEWMAN[51]. Two of the solutions required, BATHOPHENANTHROLINE, 0.001 M IN 50 PER CENT ETHANOL, and STANDARD IRON SOLUTION, 1 μg FE PER ML., are the same as those described earlier, page 21, The sodium acetate and hydroxylammonium chloride solutions used in this determination are, however, more concentrated than those used in procedures given in preceding pages.

SODIUM ACETATE. 50 PER CENT AQUEOUS SOLUTION, IRON-FREE. Dissolve 50 g. of sodium acetate trihydrate in 100 ml. of water. Add 10 ml. of hydroxylammonium chloride, 5 ml. of bathophenanthroline solution and extract with isoamyl alcohol until the iron has been completely removed. Extract the iron-free solution three times with chloroform to remove the isoamyl alcohol.

HYDROXYLAMMONIUM CHLORIDE. 50 PER CENT AQUEOUS SOLUTION, IRON-FREE. Dissolve 50 g. of hydroxylammonium chloride in 100 ml. of water. Add 10 ml. of sodium acetate, 5 ml. of bathophenanthroline solution and extract with isoamyl alcohol until the iron has been completely removed. Extract the iron-free solution three times with chloroform to remove isoamyl alcohol.

AMMONIUM THIOCYANATE. 50 PER CENT AQUEOUS SOLUTION, IRON-FREE. Dissolve 50 g. of ammonium thiocyanate in 100 ml. of water. Add 10 ml. of hydroxylammonium chloride solution, 10 ml. of sodium acetate solution, and 5 ml. of bathophenanthroline solution. Extract with isoamyl alcohol until all of the iron has been removed and then three times with chloroform to remove the isoamyl alcohol.

PROCEDURE. Weigh 1.00 g. of the sample into a 125-ml. conical flask, add 10 ml. of distilled 6 N hydrochloric acid and 5 ml. of 30 per cent hydrogen peroxide. After the vigorous reaction subsides heat the solution to boiling and hold at this temperature until dissolution is complete. (In the case of samples of very large particle size additional hydrogen peroxide may be added to aid dissolution.) After the sample has completely dissolved, evaporate the solution to near dryness, take up again in 25 ml. of distilled water, and transfer to a 125-ml. separatory funnel. Add 10 ml. of 50 per cent, iron-free hydroxylammonium chloride solution and sufficient distilled ammonium hydroxide to render the solution neutral or slightly alkaline. Add 10 ml. of 50 per cent, iron-free sodium acetate solution, followed by 10 ml. of 50 per cent, iron-free ammonium thiocyanate solution. If the solution is sufficiently alkaline it will have the color and consistency of thick cream at this point. Otherwise add additional ammonium hydroxide. Add 3 ml. of 0.001 M bathophenanthroline solution and mix well. Add 2 g. of potassium cyanide and extract at once with 15 ml. of chloroform. Allow the mixture to stand for 5 minutes and then draw off the lower layer into a 25-ml. volumetric flask. Dilute the solution to the mark with chloroform, add a few crystals of potassium cyanide and mix well. Read the absorbancy at 600 mμ within 2 hours and determine the amount of iron present by comparison with the calibration curve.

PREPARATION OF CALIBRATION CURVE. Using the standard iron solution, 1.0 μg Fe per ml., introduce into a series of 125-ml. separatory funnels quantities of iron varying between 1 and 10 μg. To each funnel add 10 ml. of iron-free copper and treat the solution according to the procedure given above for the determination of iron in copper metal. Roughly the same weight of copper must be added to each standard as used in the sample for analysis, that is about 1 g. Iron free copper metal may be obtained by electrodeposition on a platinum electrode through a slightly acidic solution of EDTA containing 1 g of EDTA for each 5 g of copper. The electrodeposition is repeated several times to insure freedom from iron contamination.

SECTION II. BIBLIOGRAPHY
BATHOPHENANTHROLINE
(4,7-Diphenyl-1,10-Phenanthroline)

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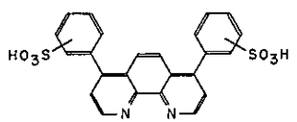
SECTION III

BATHOPHENANTHROLINEDISULFONIC ACID

4,7-Diphenyl-1,10-phenanthrolinedisulfonic Acid



$C_{24}H_{14}N_2(SO_3H)_2$
Mol. Wt.: 492.5

$C_{24}H_{14}N_2(SO_3Na)_2$
Mol. Wt.: 536.5

Molar Extinction Coefficient of ferrous derivative 22,140 at 535 mμ.

G. Frederick Smith Chemical Company, Catalog Item No. 286

One difficulty encountered in the use of bathophenanthroline in water solution is its low solubility in water. The reagent is soluble in alcohol and also in water as the hydrochloride, but in the neutral solutions needed for maximum color development with iron, the excess reagent tends to precipitate, rendering the solutions turbid. This turbidity causes no trouble if the iron derivative, together with the excess reagent, is extracted into isoamyl alcohol for the measurement of absorbance as prescribed in the operating procedure of the original publication of Smith, McCurdy and Diehl[1]. The extraction is an extra step, however, and burdensome when the work load is heavy. Trinder at the Royal Infirmary, Sunderland, County Durham, solved the problem by treating bathophenanthroline with chlorosulfonic acid; the resulting sulfonated derivative was not isolated but the water soluble product retained the sensitivity of bathophenanthroline toward iron and was free from the turbidity difficulty.

The initial application of sulfonated bathophenanthroline by Trinder[2] was to the determination of iron in serum and this particular determination remains the principal use. The series of papers by Zak and coworkers [3,4,5] also deals with this determination and with the simultaneous determination of copper. A later paper, Callahan,[6] also deals with the determination of iron in serum, and another, Goodwin[7], deals with the determination of iron in urine. The two other papers, Blair and Diehl[8] and Cryberg and Diehl[9], which complete the bibliography on bathophenanthrolinedisulfonic acid, have to do with the preparation of the sulfonated derivative in a solid form suitable as a reagent, the nature of the sulfonated compound, the use as a spectrophotometric reagent for iron, and the use in the form of its ferrous derivative as an oxidation-reduction indicator.

Properties of Bathophenanthrolinedisulfonic Acid. The nature and characteristics of the sulfonation product of bathophenanthroline were worked out in detail by Blair and Diehl[8]. The material was isolated as the disodium salt. An ammonium salt of stoichiometric composition could not be obtained. The disodium salt is light tan in color when prepared free from contamination by iron. It is extremely soluble in water and somewhat hygroscopic. It can be dried at 110° and in fact shows no loss in weight up to 275° (thermobalance study). It shows a light blue fluorescence under ultraviolet light.

The structure of bathophenanthrolinedisulfonic acid has not been determined precisely. 1,10-Phenanthroline cannot be sulfonated under the same conditions, so that it is reasonable to conclude that the sulfonic groups have entered into the phenyl groups of the bathophenanthroline, and because a sulfonic group on a phenyl ring tends to hinder further addition to the ring, that one sulfonic group has entered each ring.

The neutralization titration of bathophenanthrolinedisulfonic acid takes place in two steps, Figure 1, the respective acid dissociation

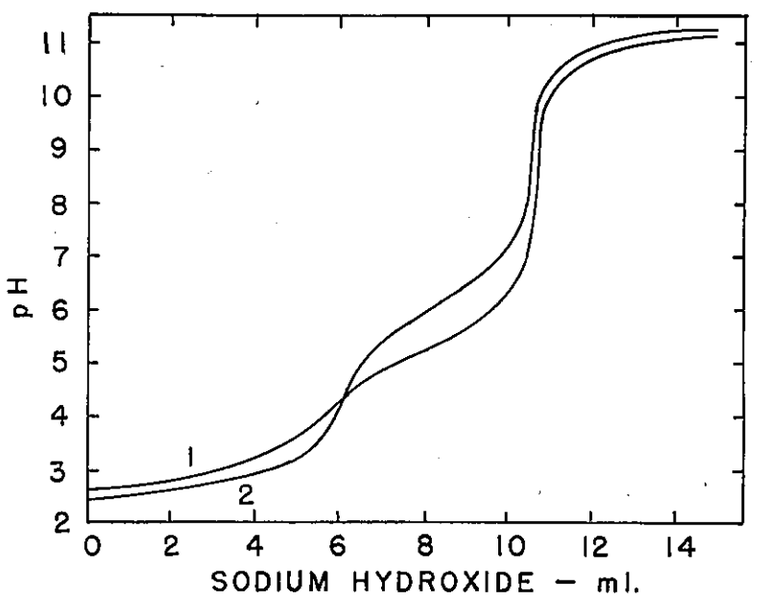


Figure 1. Titration of bathophenanthrolinedisulfonic acid (curve 1) and bathocuproinedisulfonic acid (curve 2). Glass electrode. *Data of Blair and Diehl*[8].

constants being $pK_1 = 2.83$ and $pK_2 = 5.20$. The molecule behaves as a zwitter ion. 1,10-Phenanthroline and its derivatives act as monoacidic bases presumably because the spatial arrangement permits only one proton to enter the area of the ring nitrogen atoms easily. In this disulfonic acid it might be expected that one proton would be transferred to the ring nitrogen giving the molecule a zwitter ion structure. The first replaceable hydrogen, then, is that of the free sulfonic group, the second the proton on the nitrogen.

Properties of the Ferrous Derivative. Bathophenanthrolinedisulfonic acid reacts with ferrous iron to produce an intense red color. The absorption spectrum of this colored ion was measured on a solution of ferrous sulfate containing also hydroxylammonium chloride, sodium acetate, and a ten-fold excess of bathophenanthrolinedisulfonic acid, Figure 2; at the wavelength of maximum absorption, 535 mμ, the molar extinction coefficient is 22,140.

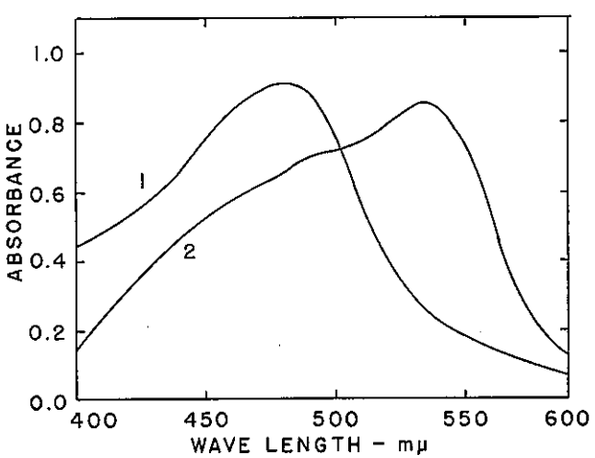


Figure 2. Absorption spectra of ferrous tris(bathophenanthrolinedisulfonic acid), curve 1, and of cuprous di(bathocuproinedisulfonic acid), curve 2. *Data of Blair and Diehl*[8].

The combining ratio of the ferrous ion and bathophenanthrolinedisulfonic acid was determined by a spectrophotometric titration, the iron concentration being held constant in a series of solutions and the concentration of reagent varied. Straight lines were obtained for the rising and horizontal portions of the curve and the sharp break fell at the ratio Fe:bathophenanthrolinedisulfonic acid = 1:3.17.

The pH range over which ferrous tris(bathophenanthrolinedisulfonic acid) is stable was determined by preparing a series of solutions, each

solution having the same amount of iron, sodium sulfite, and an excess of bathophenanthrolinedisulfonate; the pH was adjusted with hydrochloric acid or sodium hydroxide and after 1 hour the absorbance of each solution was measured. The results are presented graphically in Figure 3.

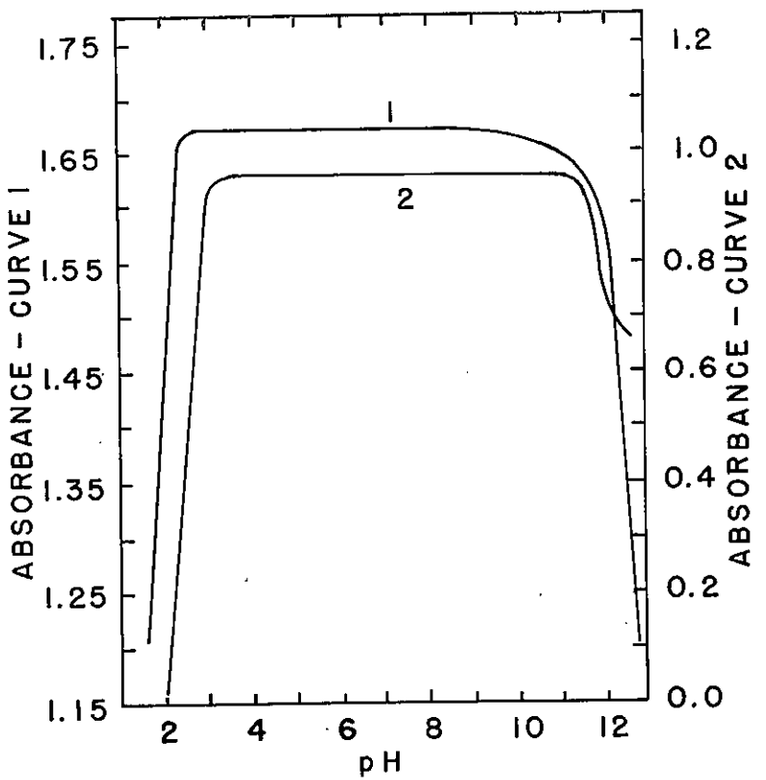


Figure 3. Effect of pH on the stability of ferrous tris(bathophenanthrolinedisulfonic acid), curve 1, and of cuprous bis(bathocuproinedisulfonic acid), curve 2. *Data of Blair and Diehl*[8].

DETERMINATION OF IRON WITH BATHOPHENANTHROLINEDISULFONIC ACID. Bathophenanthrolinedisulfonic acid has about the same sensitivity toward iron as the parent bathophenanthroline and is eminently suited for the colorimetric determination of iron. The determination must be carried out in water, of course, but can be made in solutions containing perchlorates, such as those resulting from the wet ashing of plant and animal material.

The disturbing effect of various ions on the spectrophotometric determination of iron was studied by Blair and Diehl[8]. The tests were made on

TABLE 1. EFFECT OF VARIOUS IONS ON THE DETERMINATION OF IRON WITH BATHOPHENANTHROLINEDISULFONIC ACID

Data of Blair and Diehl[8]

Ion	*Concentration of Interfering Ion p.p.m.*	*Form in Which Added*	*Relative error[a] Per Cent of 0.726 p.p.m. Fe taken*
Cu^{2+}	0.9	$CuSO_4$	+2.2
Cu^{2+}	9.4	$CuSO_4$	+3.2[b]
Co^{2+}	1.2	$CoSO_4$	+1.1
Co^{2+}	5.8	$CoSO_4$	0.0[b]
Ni^{2+}	1.4	$NiSO_4$	+1.1
Ni^{2+}	5.5	$NiSO_4$	0.0[b]
Zn^{2+}	13.8	$ZnSO_4$	+0.3
Mn^{2+}	25.3	$MnSO_4$	—1.1
Cr^{3+}	5.8	$K_2Cr_2O_7$	+1.6
Cr^{3+}	29.2	$K_2Cr_2O_7$	+4.7
Be^{2+}	14.6	$Be(ClO_4)_2$	+2.2
Al^{3+}	121.3	$Al_2(SO_4)_3$	Prec.
Mg^{2+}	58.7	$MgSO_4$	+0.3
Ca^{2+}	189	$Ca(C_2H_3O_2)_2$	—1.1
Sr^{2+}	571	$Sr(NO_3)_2$	0.0
Cd^{2+}	35.5	$Cd(NO_3)_2$	0.0
Sn^{4+}	307	$SnCl_4$	Prec.
Th^{4+}	546	$Th(NO_3)_4$	+0.3
UO_2^{2+}	653	$UO_2(NO_3)_2$	+1.6
Li^+	627	LiCl	0.0
ClO_4^-	17780	$NaClO_4$	+2.7
CN^-	536	KCN	No color
PO_4^{3-}	1122	KH_2PO_4	0.0
F^-	543	NaF	+1.1
$C_2H_3O_2^-$	15320	$NH_4C_2H_3O_2$	0.0
Br^-	895	KBr	—2.3
I^-	1040	NaI	—0.8
Cl^-	5180	KCl	—0.8
NO_2^-	873	$NaNO_2$	—2.0
SO_4^{2-}	3470	$(NH_4)_2SO_4$	0.0
ClO_3^-	818	$KClO_3$	—0.8
SCN^-	1200	NaSCN	—1.7
$S_2O_3^{2-}$	1074	$Na_2S_2O_3$	Prec.
BO_3^{3-}	1800	$Na_2B_4O_7$	+0.3
BrO_3^-	1380	$KBrO_3$	+0.3
MoO_4^{2-}	93.2	Na_2MoO_4	0.0
$C_6H_5O_7^{3-}$	1627	$H_3C_6H_5O_7$	—1.7
$S_2O_8^{2-}$	1626	$(NH_4)_2S_2O_8$	—9.7

[a]Calculated using

$$\text{Per Cent Relative Error} = \frac{C_1 \frac{A_2}{A_1} - C_1}{C_1} 100$$

where C and A refer to concentration and absorbance, respectively, and subscripts 1 and 2 to solutions without and with the interfering ion, respectively.

[b]Solutions containing a large excess (2.5×10^{-5} moles) of bathophenanthrolinedisulfonate.

solutions made up to contain 6.5×10^{-7} moles of iron and 9.5×10^{-6} moles of bathophenanthrolinedisulfonate in a total volume of 50.0 ml. In all cases the order of addition of the reagents was the same, first the interfering ion, second the iron, next the hydroxylammonium chloride, then the sulfonated reagent, and finally the sodium acetate buffer. The results of this study are given in Table I.

The spectrophotometric determination of iron is thus straightforward, there is no problem at all with large amounts of perchlorate, and the method has a fair tolerance for copper, cobalt, nickel and chromium. One disadvantage is that the reagent solutions necessary for the determination cannot be purged of iron by treatment with the reagent and extracting. This can, however, be done with bathophenanthroline and fortunately the excess bathophenanthroline is extracted along with the tramp iron into isoamyl alcohol. Thus the solutions of hydroxylammonium chloride and sodium acetate needed, and also the distilled or deionized water used for dilution, can be freed of iron contamination and the sulfonated bathophenanthroline then used for the determination of submicrogram amounts of iron.

This method for the spectrophotometric determination of iron was checked by Blair and Diehl[8] by the analysis of yeast, with and without added iron. An unexpected difficulty arose in that not all of the yeast was destroyed by digestion with nitric acid and perchloric acid. The results for iron were erratic and only somewhat better using the ternary mixture of nitric, perchloric and sulfuric acids. Complete destruction of the organic

TABLE 2. DETERMINATION OF IRON IN DRY YEAST WITH BATHOPHENANTHROLINEDISULFONIC ACID

Data of Blair and Diehl[8]

Sample Number	*Iron Added mg.*	*Iron Found mg.*	*Iron in Yeast p.p.m.*	
1	none	0.100	50.0	
2	none	0.102	51.0	
3	none	0.101	50.5	
4	none	0.101	50.5	
5	none	0.101	50.5	
6	none	0.099	49.5	
		Ave. 0.101	Av. 50.3	
			Iron recovered mg.	*Error mg.*
7	0.036	0.139	0.038	+0.002
8	0.036	0.143	0.042	+0.006
9	0.036	0.138	0.037	+0.001
10	0.072	0.172	0.071	—0.001
11	0.072	0.169	0.068	—0.004
12	0.072	0.173	0.072	0.000

matter and satisfactory results for iron were only obtained by first charring the yeast with hot sulfuric acid and then subsequently digesting the black carbonaceous residue with nitric and perchloric acids. The procedure is given below; the results obtained by Blair and Diehl[8] are given in Table 2.

Procedure for the Determination of Iron in Yeast. METHOD OF BLAIR AND DIEHL[8]. Transfer a weighed sample of about 2 g. of the yeast to a 250-ml. conical flask. Add 5 ml. of concentrated sulfuric acid and insert a reflux head in the neck of the flask. Carry along simultaneously a blank, starting with the sulfuric acid. Char the yeast by heating the mixture for 15 minutes on a hot plate. Cool, and then add 20 ml. of a mixture of equal volumes of 72 per cent perchloric acid and 70 per cent nitric acid. Replace the reflux head and boil the mixture in such a fashion that the water and nitric acid are expelled in about 15 minutes and perchloric acid begins to condense on the walls of the flask. Continue to reflux smoothly, without undue escape of perchloric acid, for 10 minutes. Cool the mixture, remove the reflux head, and wash it and the flask with approximately 30 ml. of deionized water.

To this solution add 5 ml. of a 10 per cent solution of hydroxylammonium chloride and 10 ml. of a 0.1 per cent solution of disodium bathophenanthrolinedisulfonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 286). Add ammonium hydroxide until the pH of the solution reaches 7 to 8 as shown by pH paper. Bring the pH to between 4 and 5 by the dropwise addition of perchloric acid. Transfer the solution to a 100-ml. volumetric flask, dilute to the mark with deionized water, and mix. Measure the absorbance in a 1-cm. cell at 535 mμ.

Prepare a calibration curve following the procedure just given, starting with various volumes of a standard iron solution (0.036 mg. of iron per ml.), prepared by dissolving electrolytic iron ignited in moist hydrogen (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 226) in sulfuric acid, diluting, and aliquoting, and again diluting as appropriate. The calibration curve should be linear over the range 0 to 3.6 p.p.m. of iron.

DETERMINATION OF IRON IN SERUM. The first application of bathophenanthrolinedisulfonic acid was to the determination of iron in serum, by Trinder[2]. Protein in the serum was first precipitated with trichloroacetic acid and centrifuged away. The pH of the filtrate was adjusted by the addition of sodium acetate and the color developed and measured. The method was checked by the addition of known amounts of iron to a serum; the recovery was satisfactory, Table 3. Proof that all of the iron originally

TABLE 3. RECOVERY OF IRON ADDED TO SERUM. Trichloroacetic Acid Precipitation of Protein and Bathophenanthrolinedisulfonic Acid as Color Reagent Data of Trinder[2]

Ferric Iron Added μg./100 ml.	*Iron Found μg./100 ml.*	*Iron Recovered μg./100 ml.*	*Recovery Per Cent*
0	98	—	—
140	237	139	99.5
210	312	214	102
280	383	285	102

present was being extracted was also offered. Ten samples of serum analyzed by both the trichloroacetic acid-bathophenanthrolinedisulfonic acid method and an earlier method of Ramsay *(Biochem. J.*, 53, 227 (1954); 57, xvii) gave essentially identical results. The more intense red color with bathophenanthrolinedisulfonic acid lends a great deal more certainty to the determination for the amount of iron normally present in serum is low and provides only a pale color with bipyridine. The addition of 120 μg. of haem iron per 100 ml. of serum was found to have no effect on the results of the bathophenanthrolinedisulfonic acid method.

The simultaneous determination of iron and copper in serum using bathophenanthrolinedisulfonic acid in conjunction with 2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid[4,5] and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline[3] has been worked out by Zak and coworkers.

Procedure for the Determination of Iron in Serum. METHOD OF TRINDER[2]. PREPARATION OF IRON REAGENT. Dissolve 160 mg. of disodium bathophenanthrolinedisulfonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 286) in 20 ml. distilled water. Add 1 ml. of thioglycolic acid and add sufficient water to bring the volume to 100 ml.

DETERMINATION OF IRON. Transfer 2 ml. of serum to a 15-ml. cylindrical centrifuge tube and add 2.5 ml. of water and 1.5 ml. of 20 per cent trichloroacetic acid. Mix by lateral shaking. Cover the tube with an aluminum cap and heat for 10 minutes in a water-bath maintained at 90° to 95°. After 5 minutes mix the contents of the tube by lateral shaking and mix again just before removal from the water-bath. Cool and centrifuge for a few seconds to dislodge droplets of condensed water and shake the tube to mix the contents. Centrifuge (still covered with the aluminum cap) at 4,000 r.p.m., radius 6 in., for 15 minutes. Transfer 4 ml. of the clear supernatant liquid to a test tube. Prepare a blank by heating a mixture of 3 ml. of water and 1 ml. of 20 per cent trichloroacetic acid at 90° to 95° in a test tube covered with an aluminum cap. Heat the mixture for 10 minutes and cool. To each tube add 0.2 ml. of iron reagent, 0.6 ml. of 40 per cent sodium acetate, and 0.4 ml. of 1 to 1 sulfuric acid in that order, mixing the contents of the tubes after each addition. Measure the absorbance in a 20-mm. cell, setting the instrument at zero with the blank, and making the measurement at 535 mμ. Use a green filter if a photoelectric colorimeter is used for the measurement.

In a similar manner prepare a calibration curve by treating 3 ml. of 20 per cent trichloroacetic acid, and so on. The quantities of iron used in the standards correspond to serum iron values of 52.5 to 420 μg. per 100 ml., and if 20-mm. cells are used to obtain the readings the corresponding absorbances are 0.1 to 0.8.

Ferrous tris(bathophenanthrolinedisulfonic acid) as an oxidation-reduction indicator. Because the ferrous derivative of bathophenanthrolinedisulfonic acid does not form an insoluble perchlorate it has a distinct advantage over the corresponding 1,10-phenanthroline and 5-nitro-1,10-phenanthroline iron complexes as an oxidation-reduction indicator, with the exception that the redox potential is 0.15 v. lower than 5-nitro-1,10-phenanthroline. The formal reduction potential was measured by Blair and Diehl[8] in both 1M sulfuric acid and 1M perchloric acid. The formal reduction potential is about the same as that of 1,10-phenanthroline, around 1.1 volt on the hydrogen scale.

USE OF FERROUS TRIS(BATHOPHENANTHROLINEDISULFONIC ACID) IN THE DETERMINATION OF IRON. As an oxidation-reduction indicator ferrous tris(bathophenanthrolinedisulfonic acid) is very advantageous in cerate oxidimetry inasmuch as it is soluble in solutions containing perchlorate, whereas the other ferroin indicators are only sparingly soluble. The color change is vivid and the end-point sharp. The indicator was tested by Blair and Diehl[8] in the analysis of three standard iron ores. The indicator was used later by Miller and Diehl in the analysis of iron ores prepared at the G. Frederick Smith Chemical Company[a] for analysis by students. Briefly the method involves placing the ore in solution with a mixture of perchloric and phosphoric acids, reducing the iron by passage through an amalgamated zinc reductor, and titrating the ferrous iron with sulfatoceric acid.

Procedure for the Determination of Iron in Iron Ore by Titration with Four-Valent Cerium Using Ferrous Tris(bathophenanthrolinedisulfonic Acid) as Indicator. MODIFIED METHOD OF BLAIR AND DIEHL[8]. Weigh accurately into a 500-ml. conical flask 0.3 to 0.35 g. of the iron ore. For the standardization of the oxidizing agent, weigh accurately about 0.22 to 0.24 g. of electrolytic iron (G. FREDERICK SMITH

[a] *G. Frederick Smith Chemical Company, Items No. 450 through 490, Iron Ore Samples; a brochure is available giving the preparation, methods of analysis, and the composition of these samples.*

CHEMICAL COMPANY, Item No. 226) and transfer it to a 500-ml. conical flask. Carry ore and electrolytic iron through the following procedure in identical fashion. Add 20 ml. of a mixture of equal volumes of 70 per cent perchloric acid and 85 per cent phosphoric acid. Swirl the mixture until all particles are wetted and free from the bottom of the flask. Place a reflux head in the neck of the flask and heat, preferably on a gas-fired or electrically-heated hot plate. Boil the solution gently until the ore has dissolved and only a fine, white residue remains. The solution may be pink in color owing to the presence of trivalent manganese. The dissolution will be complete in five to ten minutes but a few additional minutes of boiling will cause the liquid refluxing on the walls of the flask to wash down any solid particles and insure the complete dissolution of the sample. Cool the mixture and remove the reflux head rinsing it with water. Dilute with water to a volume of 100 to 125 ml. Pass the solution through an amalgamated zinc reductor (amalgamated zinc column about 2.5 cm. in diameter by 22 cm. long, 500-ml. suction flask as receiver) previously well washed with 2 per cent sulfuric acid. Adjust the flow to a rate just somewhat faster than the drops can be counted. Use suction if necessary. In manipulating the reductor during the washing, during the passage of the iron-bearing solution and during the subsequent washing do not draw air into the zinc column, in order to avoid the formation of hydrogen peroxide. Transfer the iron-bearing solution quantitatively to the reductor, washing with 2 per cent sulfuric acid and allowing the level of the liquid in the reductor to drop to the top of the zinc column after each washing. Finally wash the column with five 15 ml. portions of 2 per cent sulfuric acid. Add 6 drops of 0.002 M ferrous tris(bathophenanthrolinedisulfonic acid) and titrate with 0.1 N sulfatoceric acid to the color change from pink to light green.

Prepare the 0.002 M ferrous tris(bathophenanthrolinedisulfonic acid) indicator by dissolving 0.078 g. of ferrous ammonium sulfate and 0.32 g. of disodium bathophenanthrolinedisulfonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 286) in 50 ml. of water and mixing well.

Indirect Determination of Ferrocyanide Using Iron and Bathophenanthrolinedisulfonic Acid. The iron in ferrocyanide and in ferricyanide is so tightly bound that it does not react with 1,10-phenanthroline or other phenanthroline reagents. Ferrocyanide is oxidized by ferric iron and the ferrous ion so produced will react with the phenanthrolines. The oxidation is complete and indirectly ferrocyanide can be determined by measuring the intensity of the ferrous-phenanthroline color. The method using bathophenanthrolinedisulfonic acid, devised by Avron and Shavit[10], is based on an earlier method, Krogman and Jagendorf, *Plant Physiol.*, 32, 373 (1957), using the same chemistry, but employing 1,10-phenanthroline. As expected, Avron and Shavit found the bathophenanthrolinedisulfonic acid method about twice as sensitive as the 1,10-phenanthroline method. Ferricyanide is used as a so-called "electron acceptor" in enzymology and a measurement of the extent of the reduction is of importance, for example, in the photolysis of water by cell-free preparation of photosynthetic tissue (Hill reaction).

Ferricyanide does not produce a color so that ferrocyanide can be determined accurately even in the presence of a large excess of ferricyanide. The decrease in intensity of the color of ferricyanide itself (at 420 mμ) has been used in biological studies but is far less sensitive than even the 1,10-phenanthroline method.

Citric acid is added to hold the ferric iron in solution at the pH at which the determination is made. Variation of pH in the range 2.5 to 6.5 does not affect the intensity or stability of the color developed; at high pH the intensity of the color slowly increases with time. Exposure to light causes an increase in the intensity of the color and the cuvette in which the reaction (and the absorbance measurement) is carried out should be kept in the dark.

In the procedure given below a high concentration of sodium acetate is specified to permit freedom in the acid content of the sample.

As much trichloroacetic acid as 6 per cent may be present in the sample without affecting the results.

Procedure for the Indirect Determination of Ferrocyanide. METHOD OF AVRON AND SHAVIT[10]. REAGENTS. SODIUM ACETATE, 3 M. Dissolve 25 g. of sodium acetate in 100 ml. of water and adjust the pH to between 6.0 and 6.5.

CITRIC ACID. 0.2 M. Dissolve 3.8 g. of citric acid in 100 ml. of water.

FERRIC CHLORIDE. 0.0033 M. Dissolve 0.89 g. of ferric chloride hexahydrate in 1 liter of water.

BATHOPHENANTHROLINEDISULFONATE. 0.33 PER CENT IN AQUEOUS SOLUTION. Dissolve 0.33 g. of disodium bathophenanthrolinedisulfonate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 286) in 100 ml. of water.

1,10-PHENANTHROLINE. 0.33 PER CENT AQUEOUS SOLUTION (IF USED). Dissolve 0.33 g. of 1,10-phenanthroline monohydrate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 162) in 100 ml. of water.

DETERMINATION OF FERROCYANIDE. Place the sample, of such size as to contain 0.002 to 0.1 μ mole of ferrocyanide, in a 1-cm. cuvette of 3 ml. capacity. Fill the cuvette to 2.10 ml. with water. Prepare, just before use, a mixture of 0.3 ml. of 25 per cent sodium acetate, 0.3 ml. of 0.2 M Citric acid, 0.15 ml. of 0.0033 M ferric chloride, and 0.15 ml. of 0.33 per cent bathophenanthrolinedisulfonate. Add 0.90 ml. of this mixture to the cuvette and mix. Allow the mixture to stand 5 minutes. Measure the absorbance at 535 mμ using as reference a blank (stopped at time zero when working with biological material). Calculate the result using the relation[a]

$$(\text{Absorbance})\ (0.145) = \mu \text{ moles of ferrocyanide}$$

All volumes may be reduced to one third those given, the final volume in the 1-cm. cuvette then being 1.0 ml., and as little as 0.001 μ mole of ferrocyanide can be determined.

In the above procedure the disodium bathophenanthrolinedisulfonate may be replaced by 1,10-phenanthroline with a loss of about one-half of the sensitivity. The measurement of absorbance is then made at 510 mμ.

[a] *Assumes a value of 20,500 for the molar extinction coefficient.*

SECTION III. BIBLIOGRAPHY. BATHOPHENANTHROLINEDISULFONIC ACID. (4,7-Diphenyl-1,10-Phenanthrolinedisulfonic Acid)

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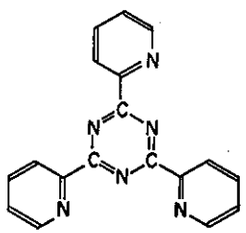
SECTION IV

2,4,6-TRIPYRIDYL-S-TRIAZINE
TPTZ



$C_{18}H_{12}N_6$

Mol. Wt.: 242.25

Molar Extinction Coefficient of $Fe(TPTZ)_2^{++}$ in water: 22,600 at 593 mμ; of $Fe(TPTZ)_2(ClO_4)_2$ in nitrobenzene: 24,100 at 595 mμ

G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 291

2,4,6-Tripyridyl-s-triazine (2,4,6-tripyridyl-1,3,5-triazine) reacts with the ferrous ion to yield an intense violet color which is eminently suited to the spectrophotometric determination of iron. This reagent has the same high sensitivity toward iron of bathophenanthroline and bathophenanthroline-disulfonic acid (molar extinction coefficients of the order of 22,000); like bathophenanthroline it forms a ferrous derivative which can be extracted into immiscible solvents (in this case as the perchlorate into nitrobenzene); it is highly specific for iron; and it is relatively easy to prepare. 2,4,6-Tripyridyl-s-triazine is the best of a number of pyridyl substituted triazines first synthesized by Case and Koft[1]. The fundamental chemistry of its reaction with iron and the applications of it to the determination of iron in water, wine, urine, silicates, refractories and limestone was worked out by Collins, Diehl and Smith[2, 3, 4]. It has since been applied to the determination of iron in serum [5, 6, 7, 8], the determination of tocophenols[9], and the determination of EDTA[10]. Zak, Cavanaugh and Williams[5] applied TPTZ to a spectrophotometric microdetermination of iron and copper on a single aliquot in conjunction with bathocuproinedisulfonic acid disodium salt. The designation TPTZ has been adopted as a shorter name for the reagent.

The absorption spectrum of $Fe(TPTZ)_2^{++}$ in nitrobenzene is shown in Fig. 1. The molar extinction coefficient is 22,600 in water solution and somewhat greater, 24,100, in nitrobenzene. These values were determined[2] by measurements of the absorbancy of a series of solutions of varying iron content and the slope of the Beer's law plot (absorbancy against concentra-

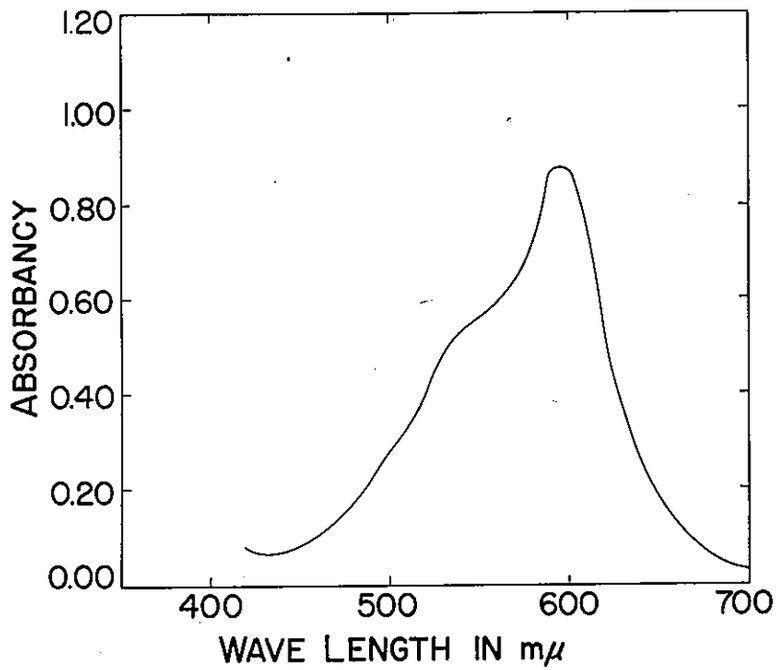


Fig. 1. Absorption spectrum of Fe(2,4,6-tripyridyl-s-triazine)$_2$(ClO$_4$)$_2$ in nitrobenzene. 2.08 P.p.m. of iron. 1-cm. cell.

tion expressed in moles per liter). The absorption peaks lie at essentially the same wave length in the two solvents, 593 mμ in water and 595 mμ in nitrobenzene. The spectra in both solvents show a shoulder on the short wave length side of the absorption maximum as do most of the ferrous derivatives of the ferroin reagents, that is, of the 1,10-phenanthrolines, the 2,2′-bipyridines, and the 2,2′,2″-terpyridines. In both water and nitrobenzene the color conforms to Beer's law over the useful range of spectrophotometers, up to about 6×10^{-5} M iron in this case. The formation constant of Fe(TPTZ)$_2^{++}$ was found by Crichton[11], using the spectrophotometric method, to be log K = 10.83.

The color is stable, no change having been observed[2] in a water solution for 32 hours or in a nitrobenzene solution in 12 hours.

The pH range over which the violet compound is completely formed in water solution is 3.4 to 5.8. With extraction of the perchlorate into nitrobenzene the pH range is greater, 2.7 to 7.0. Although this pH range is not as wide as for many of the 1,10-phenanthroline and polypyridine reagents, a suitable pH can be obtained easily by the use of an acetate buffer.

The violet Fe(TPTZ)$_2^{++}$ ion forms water soluble salts with the common cations which are not extracted from the water layer by isoamyl alcohol, *n*-hexyl alcohol, benzene, chloroform, or ethyl acetate; this is true whether the anion present be sulfate, chloride, nitrate, iodide, acetate, or perchlorate. In the presence of perchlorate or iodide, the color is extracted rapidly and completely by nitrobenzene.

Specificity. Interferences. An extensive study was made[2] of the effects of various cations and anions on the colorimetric determination of iron with TPTZ. The tests were carried out in the following manner. A 10.00-ml. portion of a solution 1.000×10^{-4} M in iron was pipetted into a 50-ml. volumetric flask containing a solution of the ion to be tested. TPTZ was added in the various amounts shown in Table 1 and was followed by 1.0 ml. of 10 per cent hydroxylammonium chloride and 5.0 ml. of 20 per cent sodium acetate. The solution was then diluted to volume and its absorbancy determined at 593 mμ. The solutions containing the various ions to be tested were prepared from the reagent grade compounds listed in Table 1. In testing the possible interference of silver and chloride ion, hydroxylammonium sulfate was substituted for the chloride salt. To test the possible interference of sodium or acetate ions a solution containing sodium acetate was compared with a solution containing no sodium acetate, the pH being adjusted with dilute ammonium hydroxide.

The results obtained in this study of interferences are shown in Table 1. The method of calculating the relative error is based on the relations

$$C_2 = C_1 \frac{A_2}{A_1}$$

$$\text{Per Cent Relative Error} = \frac{C_2 - C_1}{C_1} \times 100$$

in which C_1 and A_1 refer to the concentration of iron and absorbancy of the solution containing no interfering ion and A_2 is the absorbancy of the solution containing the possible interference.

Of the ions tested only Cu^{++}, Co^{++}, Ni^{++}, Cr^{+++}, Ag^{+}, Hg^{++}, Bi^{+++}, MoO_4^{--}, CN^{-}, $C_2O_4^{--}$, and NO_2^{-} interfere significantly. The interference of Co^{++}, Cu^{++}, and Ni^{++} is due to the formation of colored compounds with TPTZ; however, 2.5 p.p.m. of Cu^{++}, 2.4 p.p.m. of Co^{++} or 5.3 p.p.m. of Ni^{++} results in a relative error of less than 2 per cent in the determination of iron. A precipitate is formed in the presence of Ag^{+}, Hg^{++} and Bi^{+++} and the other ions retard color development or interfere due to the color of the ion. In the presence of most of the transition metals, if an excess of TPTZ is not present the color development is retarded.

TABLE 1. EFFECT OF VARIOUS IONS ON THE FORMATION OF THE VIOLET $Fe(TPTZ)_2^{++}$ ION

Ion	*Concentration p.p.m.*	*Source*	*Relative Error Per Cent*	*TPTZ Added Moles x 10^5*
Cu^{++}	1.3	$Cu(NO_3)_2$	+0.7	0.5
Cu^{++}	2.5	$Cu(NO_3)_2$	+1.4	0.7
Cu^{++}	6.3	$Cu(NO_3)_2$	+4.8	0.9
Co^{++}	1.2	$CoSO_4$	+0.9	0.5
Co^{++}	2.4	$CoSO_4$	+1.8	0.7
Co^{++}	4.8	$CoSO_4$	+3.6	1.1
Ni^{++}	2.7	$Ni(ClO_4)_2$	+0.4	0.8
Ni^{++}	5.3	$Ni(ClO_4)_2$	+1.5	1.3
Ni^{++}	10.6	$Ni(ClO_4)_2$	+2.7	2.3
Zn^{++}	99.4	$ZnCl_2$	+0.2	10.0
Mn^{++}	110	$MnSO_4$	+0.2	2.0
Cr^{+++}	10.4	$K_2Cr_2O_7$-SO_2	+0.6	0.5
Cr^{+++}	20.8	$K_2Cr_2O_7$-SO_2	+2.4	0.5
Be^{++}	73.0	$Be(ClO_4)_2$	0.0	0.5
Al^{+++}	100	$AlCl_3$	0.0	0.5
Mg^{++}	100	$MgSO_4$	0.0	0.5
Ca^{++}	100	$CaCO_3$-HCl	0.0	0.5
Sr^{++}	99	$Sr(ClO_4)_2$	+0.2	0.5
Ba^{++}	101	$BaCl_2$	+0.2	0.5
Cd^{++}	100	$Cd(NO_3)_2$	-0.7	5.0
Hg^{++}	100	$HgCl_2$	precipitate	0.5
Bi^{+++}	100	$Bi(NO_3)_3$	precipitate	0.5
Sn^{++}	100	$SnCl_2$	-0.2	0.5
Pb^{++}	101	$Pb(NO_3)_2$	+0.2	2.0
Th^{++++}	120	$Th(NO_3)_4$	+0.2	0.5
UO_2^{++}	115	$UO_2(C_2H_3O_2)_2$	+0.4	0.5
Li^+	1020	LiCl	0.0	0.5
K^+	1067	KCl	+0.4	0.5
NH_4^+	1033	NH_4Cl	0.0	0.5
Na^+	5600	$NaC_2H_3O_2$	-0.2	0.5
Ag^+	102	$AgNO_3$	precipitate	0.5
CN^-	500	NaCN	very large	0.5
PO_4^{---}	528	KH_2PO_4	+0.2	0.5
F^-	502	NaF	+0.2	0.5
$C_2H_3O_2^-$	14,400	$NaC_2H_3O_2$	-0.2	0.5
Br^-	556	NaBr	+0.2	0.5
I^-	497	KI	0.0	0.5
NO_3^-	504	KNO_3	+0.2	0.5
NO_2^-	500	KNO_2	large	0.5
SO_4^{--}	512	K_2SO_4	0.0	0.5
ClO_4^-	524	$NaClO_4$	0.0	0.5
ClO_3^-	548	$NaClO_3$	+0.2	0.5
$S_2O_5^{--}$	538	$Na_2S_2O_5$	0.0	0.5
SCN^-	507	KSCN	+0.2	0.5
$S_2O_3^{--}$	528	$Na_2S_2O_3$	+0.4	0.5
BO_3^{---}	545	H_3BO_3	0.0	0.5
BrO_3^-	499	$KBrO_3$	0.0	0.5
MoO_4^{--}	34	$(NH_4)_2MoO_4$	very large	2.0

Applications. IRON IN LIMESTONE, SILICATES AND REFRACTORIES. One of the main difficulties in the determination of the iron content of silicate-bearing materials is the problem of decomposing the sample completely without loss of iron. Treatment with hydrofluoric acid often leaves a residue that is insoluble in hydrochloric acid so that a fusion of the sample may be required. During fusions in platinum crucibles, iron is lost to the platinum crucible, evidently by reduction and alloying. This alloyed iron is difficult to recover completely and affects subsequent analyses inasmuch as it is sometimes partially released at a later time. This subject was studied by Shell[12] and his findings were confirmed by Collins, Diehl and Smith[2] and by others. To overcome this absorption of iron by platinum, Shell suggested the use of a silver crucible and a flux consisting of equal amounts of sodium carbonate and sodium borate. Collins, Diehl and Smith also made use of a heavy-walled silver crucible and the same sodium carbonate-sodium borate flux, completing the determination by the TPTZ spectrophotometric method. Some attack of the silver occurred and any silver which passed into solution on taking up the melt was removed immediately as silver chloride.

The TPTZ procedures, working details of which are given below, involve fewer operations and call for the addition of fewer reagents and ones which can be freed of iron prior to use. The procedures were checked on a number of samples of the National Bureau of Standards and on the G-1 Granite and W-1 Diabase of the United States Geological Survey; the results reported by Collins, Diehl and Smith[2] are reproduced in Table 2. For many of the samples analyzed, the iron content as determined by the various TPTZ procedures is lower than the average reported by the Bureau. In general the precision obtained by the TPTZ methods is better, but a comparison of values reported in the second and fourth columns of Table 2 is not completely fair as the latter gives the range of values reported by several analysts. The possibility that iron was present in the precipitate of silica and silver chloride filtered off after taking up the melt was checked by Collins, Diehl and Smith[2] during the analysis of NBS 76 Burnt Refractory; no significant amount of iron was found. The conclusion seems warranted that there is some inherent difficulty in the older analyses. These usually employed procedures involving lengthy separations and the use of relatively large amounts of reagent grade chemicals which frequently contain appreciable amounts of iron. In the TPTZ methods, most of the reagents can be easily freed of iron by virtue of the extractability of the iron derivative of TPTZ into nitrobenzene. This reduces the blank to almost zero. No separations are necessary in the analysis of the silicates usually encountered.

Preliminary analyses of the G-1 Granite were carried out using 150 mg. samples. Fe found: 1.70, 1.93, 1.73, 1.82 per cent. The poor precision obtained was attributed to inhomogeneity of the sample inasmuch as more precise results had been obtained by a similar procedure on NBS samples. On examination of the material, it was noted that black particles are scattered throughout the sample which are attracted to a magnet. Presumably these particles are magnetite and distribution through the mass is not absolutely uniform. In subsequent analyses of the granite larger samples, 3 g., were taken and the results were quite satisfactory (Table 2).

IRON IN WINE. 2,4,6-Tripyridyl-s-triazine can be used to advantage in the determination of iron in wine (Collins and Diehl[3]). Just as in the use of bathophenanthroline for the determination of iron in wine (page 17) the colorimetric measurement can be made following wet ashing of the organic matter with nitric and perchloric acids, or by direct extraction of the iron with an immiscible solvent. Results obtained by Collins and Diehl[3] on various American and European wines are shown in Table 3.

It is apparent that the determination of iron in wine by the direct TPTZ method usually, but not always, gives values which are lower than those obtained by wet ashing procedures. This is presumably due to the presence of a very stable iron compound which is not broken by TPTZ. If this is the case, it may well be that the "complexed iron" is inactive in forming a turbidity in wine and that the results obtained for "uncomplexed iron" by this direct method may be more useful to the wine producer than a knowledge of the total iron content. Various conditions, such as length of heating and ethanol concentration, were changed; but in no case was it possible to recover completely the iron from certain wines by the direct procedure. The iron which is not recovered remains in the aqueous phase as shown in one case by a wet ashing of the remaining water solution.

The iron content of the Medoc Bordeaux Red Wine was also determined by the bathophenanthroline-extraction method of Banick and Smith and found to be 6.46 mg. of iron per liter, almost identical with the TPTZ extraction method.

The procedure employing preliminary wet ashing followed by determination of the iron with TPTZ does have distinct advantages over the usual 1,10-phenanthroline method. A smaller sample may be used because of the greater sensitivity of the reagent and the extractability of the iron derivative into nitrobenzene. Iron may be easily removed from the reagents and the blank reduced to essentially zero. Also, it is unnecessary to remove the excess perchloric acid as is the case in the determination employing 1,10-phenanthroline.

TABLE 2. DETERMINATION OF IRON IN VARIOUS SILICEOUS MATERIALS

Sample No. and Description	*Iron Found Per Cent Fe_2O_3*	*Average Value Reported by NBS Per Cent Fe_2O_3*	*Range of Values Reported by NBS Per Cent Fe_2O_3*
NBS 76 Burnt Refractory	2.11, 2.11, 2.11, 2.12, 2.08 Average: 2.11	2.38	2.22 to 2.50
NBS 77 Burnt Refractory	0.82, 0.81, 0.82 Average: 0.82	0.90	0.79 to 1.39
NBS 78 Burnt Refractory	0.71, 0.71, 0.70 Average: 0.71	0.79	0.70 to 1.17
NBS 1a Argillaceous Limestone	1.59, 1.57 Average: 1.58	1.63	1.57 to 1.69
NBS 88 Dolomite	0.083, 0.083, 0.084 Average: 0.083	0.084	0.082 to 0.086
NBS 97 Flint Clay	0.92, 0.92, 0.93, 0.92 Average: 0.92	0.98	0.92 to 1.01
NBS 98 Plastic Clay	1.97, 1.97, 1.99, 1.97, 2.00 Average: 1.98	2.05	2.00 to 2.11
NBS 81 Glass Sand	0.074, 0.076, 0.075, 0.074 Average: 0.075	0.073	0.067 to 0.077
NBS 91 Opal Glass	0.073, 0.074, 0.073, 0.074, 0.073, 0.076 Average: 0.074	0.081	0.070 to 0.095
NBS 93 Borosilicate Glass	0.078, 0.078, 0.079 Average: 0.078	0.076	0.07 to 0.078
G-1 Granite	1.85, 1.85, 1.84, 1.85 Average: 1.85	1.86	1.29 to 2.99
W-1 Diabase	10.91, 10.94, 10.87 Average: 10.91	11.09	10.70 to 12.19

TABLE 3. DETERMINATION OF IRON IN WINE USING TPTZ

(Each result given is the average of at least three determinations)

Wine	*Wet Ashing—1,10-Phenanthroline*		*Wet Ashing—TPTZ*		*Direct TPTZ—Extraction*	
	Fe Found mg./liter	*Average Deviation mg./liter*	*Fe Found mg./liter*	*Average Deviation mg./liter*	*Fe Found mg./liter*	*Average Deviation mg./liter*
Italian Swiss Colony California Sherry	2.54	.07			2.51	.01
Meier's Ohio State Tawny Port	3.79	.15			3.80	.04
Medoc Bordeaux Red Wine	7.53	.10	7.56	.04	6.44	.03
Virginia Dare White Wine	4.42	.01	4.48	.01	4.16	.08
Virginia Dare Red Wine	5.81	.02	5.82	.03	5.37	.11
Ambassador California Burgundy	4.73	.07	4.69	.01	4.24	.02
Richelieu California Port	5.15	.01	5.12	.02	4.68	.01
Homestead Piestengel Rhubarb Wine	2.27	.00	2.27	.01	2.28	.02

IRON IN TREATED WATER AND IN SEA WATER. For the amounts of iron found in ground waters, 0.1 to 3 p.p.m., the commonly used 1,10-phenanthroline method is satisfactory. For the much smaller amounts of iron in the finished water from water treatment plants and in sea water, 1,10-phenanthroline is not sensitive enough and either bathophenanthroline or 2,4,6-tripyridyl-s-triazine must be used. The bathophenanthroline method is discussed earlier in Section II of this booklet; 2,4,6-tripyridyl-s-triazine is equally good.

TPTZ procedure, working details of which are given below, was tested on synthetic sea water containing 10 p.p.m. of fluoride, 3 per cent sodium chloride, and various amounts of iron. The results are shown in Table 4. Essentially this is the data for a calibration curve. There is a

TABLE 4. DETERMINATION OF IRON IN SYNTHETIC SEA WATER

Iron Added μg./liter	*Iron Found μg./liter*	*Absolute Error μg./liter*
0.70	0.70	0.00
3.49	3.40	0.09
6.98	7.35	0.37
10.47	10.4	0.1
13.96	14.2	0.2

little scatter as will be observed in plotting the data, but yet very small amounts of iron can be measured with fair accuracy. This success arises from the sensitivity of the reagent, the concentration effected by the extraction, and the essentially zero blank obtained by cleaning up the reagents. In the recommended procedure a concentration factor of four is obtained by the extraction; this can be increased by using a larger separatory funnel and a larger sample.

IRON IN URINE. The small amounts of iron in urine can be determined with 2,4,6-tripyridyl-s-triazine very nicely. The procedure works well on the sample after ashing with a mixture of nitric and perchloric acids. Inasmuch as the presence of perchlorate is necessary in the water layer for the extraction into nitrobenzene to occur, removal of the remaining perchloric acid by evaporation is not needed, the excess acid being simply neutralized with ammonium hydroxide and sufficient water added to dissolve all of the ammonium perchlorate. Results obtained by Collins and Diehl for a urine spiked with various amounts of iron are reported in Table 5.

TABLE 5. RECOVERY OF IRON ADDED TO URINE

Iron Added μg.	*Absorbancy*	*Iron Found* μg.	*Iron Originally Present* μg./50 ml.
0	0.027	0.61	0.61
0	0.028	0.67	0.67
3.47	0.171	4.02	0.55
6.94	0.329	7.60	0.66
10.41	0.473	11.05	0.64

IRON IN SERUM. The determination of iron in serum may be made using TPTZ following the release of iron by heating the sample with hydrochloric acid and the precipitation of the protein with trichloroacetic acid. The absorbance of the aqueous solution is measured. Caraway[7] reports that the recovery of added iron varies only slightly, if at all, from 100 per cent. Several reducing agents have been used: ascorbic acid[6,7] and hydroxylammonium chloride[8]. The procedure of Caraway[7] is given below.

TOTAL IRON-BINDING CAPACITY OF SERUM. The total iron-binding capacity of serum is related to the concentration of beta$_1$-globulin capable of binding iron. The determination of this capacity involves the addition of sufficient ferric iron to produce saturation and the removal of the excess iron by passage through an ion exchange column or by absorption on magnesium carbonate. The latter is generally preferred as it is most easily carried out. Following this preparation, the determination is made using one of the usual methods for iron in serum. The procedure is described below.

DETERMINATION OF ETHYLENEDIAMMONIUMTETRAACETIC ACID WITH BIS (2,4,6-TRIPYRIDYL-S-TRIAZINE) IRON (II). The determination of microgram amounts of EDTA utilizes the decrease in color which accompanies the abstraction of iron from the $Fe(TPTZ)_2^{++}$ ion by EDTA. The method was devised by Kratochvil and White[10], who applied it to the determination of EDTA in urine. As EDTA is now being used to treat individuals with abnormally high heavy metal concentrations, a measure of the uncomplexed EDTA which is eliminated is very desirable. The alkali metal and alkaline-earth metal compounds are determined as free EDTA. This procedure is given below.

Procedure for the Determination of Iron. REAGENTS. 2,4,6-Tripyridyl-s-triazine (TPTZ). 0.001 M. Dissolve 0.312 g. of 2,4,6-tripyridyl-s-triazine (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 291) in a few drops of hydrochloric acid and dilute to 1 liter with de-ionized water.

HYDROXYLAMMONIUM CHLORIDE. 10 PER CENT SOLUTION, IRON-FREE. Dissolve 10 g. of hydroxylammonium chloride in 100 ml. of water. Add 1 ml. of 0.001 M TPTZ and 1 g. of sodium perchlorate. Add 10 ml. of nitrobenzene and shake the mixture for one minute. Allow the mixture to stand in a separatory funnel for a few minutes and then draw off the lower, nitrobenzene layer and discard. Store the water solution of the iron-free hydroxylammonium chloride in a glass bottle with a plastic cap with a polyethylene liner.

SODIUM ACETATE-ACETIC ACID BUFFER. 2 M SODIUM ACETATE—2 M ACETIC ACID, IRON-FREE. Dissolve 16.4 g. of sodium acetate and 11.5 ml. of acetic acid in 100 ml. of water. Add 1 ml. of 0.001 M TPTZ, 1 ml. of 10 per cent hydroxylammonium chloride solution, and 1 g. of sodium perchlorate. Add 10 ml. of nitrobenzene and shake the mixture for one minute. Allow the mixture to stand in a separatory funnel for a few minutes and then draw off the lower, nitrobenzene layer and discard. Store the solution in a glass bottle with a plastic cap with a polyethylene liner.

SODIUM PERCHLORATE. 10 PER CENT SOLUTION, IRON-FREE. Dissolve 10 g. of sodium perchlorate in 100 ml. of water. Add 1 ml. of 0.001 M TPTZ and 1 ml. of 10 per cent hydroxylammonium chloride solution. Add 10 ml. of nitrobenzene and shake the mixture for 1 minute. Allow the mixture to stand in a separatory funnel for a few minutes and then draw off the lower, nitrobenzene layer and discard. Store the solution in a glass bottle with a plastic cap with a polyethylene liner.

STANDARD IRON SOLUTION. 10.0 μg. Fe PER ML.; 1.00 μg. Fe PER ML. Follow the directions given on page 22.

DEIONIZED WATER. Pass distilled water through a column of Amberlite MB-3 (monobed) exchange resin.

Procedure for the Determination of Iron in Burnt Refractories, Silicates and Argillaceous Limestone. METHOD OF COLLINS, DIEHL AND SMITH[2]. Weigh a sample of such size as to contain 3 to 5 mg. of ferric oxide into a silver crucible (available from the G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 236) and add 1.0 g. of sodium carbonate and 1.0 g. of sodium borate decahydrate. Mix thoroughly and then gently heat the crucible and contents over a Meker burner until the water of the sodium borate has been vaporized. Gradually increase the heat to melt the flux and continue heating until the sample has been completely decomposed. Rotate the crucible while cooling to cause the melt to solidify on the sides of the crucible. After the crucible has cooled to room temperature, add 10 ml. of water and 5 ml. of hydrochloric acid, cover with a watch glass and gently heat on a hot plate until the melt has dissolved. It may be necessary to add more hydrochloric acid to obtain complete dissolution of the residue (a precipitate of silver chloride and silica will remain which is later removed). Transfer the contents of the crucible to a 250-ml. volumetric flask and dilute to the mark. Mix well and filter or centrifuge a portion of the solution to remove any suspended silver chloride and silica.

Pipet a 5.00 ml. aliquot of this solution into a 50-ml. volumetric flask; add 2.0 ml. of 10 per cent hydroxylammonium chloride solution, 5.0 ml. of 0.001 M TPTZ and 10 ml. of 2 M sodium acetate-2 M acetic acid buffer. Dilute the solution to exactly 50 ml. and measure the absorbancy at 593 mμ using a 1-cm. cell. Run a blank on the reagents and silver crucible in exactly the same manner.

Procedure for the Determination of Iron in a Silicate of Low Iron Content (For Example, Granite G-1)[2]. Fuse a 3.0 g. sample with 5.0 g. of sodium carbonate and 5.0 g. of sodium borate decahydrate in a 50-ml. silver crucible (available from the G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 236) and continue heating until a clear melt is obtained. Rotate the crucible while cooling and place it in a 600-ml. beaker. Add 100 ml. of hydrochloric acid and 200 ml. of water and heat until the melt has been completely dissolved. Cool, remove the crucible with washing and dilute the solution to exactly 1 liter in a volumetric flask. Pipet a 25.0 ml. aliquot of this solution into a 250-ml. beaker, add 5 ml. of hydrochloric acid and heat for several hours to precipitate silica. Cool the solution, transfer to a 250-ml. volumetric flask and dilute to the mark. Filter a portion of this solution (no washing) to remove silica and silver chloride. Pipet a 15.00 ml. aliquot of the filtered solution into a 50-ml. volumetric flask; add 2.0 ml. of 10 per cent hydroxylammonium chloride solution, 5.0 ml. of 0.001 M TPTZ, and 10 ml. of 2 M sodium acetate-2 M acetic acid buffer. Dilute the solution to exactly 50 ml. and measure the absorbancy at 593 mμ using a 1-cm. cell. Run a blank on the reagents and silver crucible in exactly the same manner.

***Procedure for the Determination of Iron in a Silicate of High Iron Content (For Example, Diabase W-1)*[2].** Mix 0.22 g. of the sample with 1.0 g. of sodium carbonate and 1.0 g. of sodium borate decahydrate in a silver crucible (available from the G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 236) and fuse. Continue heating until the decomposition of the sample is complete (about 15 minutes), cool and add 20 ml. of water and 10 ml. of hydrochloric acid. Heat the crucible on a hot plate until the melt has completely dissolved (a residue of silica and silver chloride will remain), cool and dilute to exactly 500 ml. in a volumetric flask. Pipet a 50.0 ml. aliquot of this solution into a 500-ml. volumetric flask, dilute to volume and centrifuge a portion of the final solution to remove silica and silver chloride. Place 15.00 ml. of this solution in a 50-ml. volumetric flask, add 2.0 ml. of 10 per cent hydroxylammonium chloride, 5.0 ml. of 0.001 M TPTZ, and 10.0 ml. of 2 M sodium acetate-2 M acetic acid and dilute to volume. Mix well and determine the absorbancy of the solution at 593 mμ. Run a blank through the entire procedure.

***Procedure for the Determination of Iron in Glass and Glass Sand*[2].** Free a platinum crucible of iron by repeated heating to 1000° to 1200° in a muffle furnace and leaching with hot hydrochloric acid. Weigh a sample of such size as to contain 0.5 to 1.0 mg. of ferric oxide into the iron-free platinum crucible. Add 2 ml. of water and 4 ml. of hydrofluoric acid if the sample is a glass or 4 ml. of hydrofluoric acid if the sample is a glass sand. After the reaction has subsided, add 1 ml. of perchloric acid and evaporate to dryness on a hot plate without boiling. Cool, add 2 ml. of hydrofluoric acid and again evaporate to dryness. Place the crucible and contents in a 250-ml. beaker, add 20 ml. of hydrochloric acid and 50 ml. of water and heat. If complete solution is obtained, cool, transfer the solution to a 250-ml. volumetric flask, dilute to volume and continue the determination as directed in the next paragraph. If an insoluble residue remains, filter the solution into a 250-ml. volumetric flask using a medium porosity filter paper. Wash first with dilute hydrochloric acid (1:100) and then with water. Ash the filter in a silver crucible (available from G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 236). Add 1 g. of sodium carbonate and 1 g. of sodium borate decahydrate and heat until a clear melt is obtained. Cool to room temperature, add 5 ml. of hydrochloric acid and 10 ml. of water, cover with a watch glass and heat on a hot plate until the residue dissolves. A precipitate of silver chloride and silica will remain. Transfer the contents of the crucible to the 250-ml. volumetric flask containing the filtrate and dilute to volume. After mixing, centrifuge or filter a portion of this solution to remove any silica and silver chloride.

Pipet a 25.0 ml. aliquot of the solution into a 50-ml. volumetric flask and add 2.0 ml. of 10 per cent hydroxylammonium chloride and 5.0 ml. of 0.001 M TPTZ. Dropwise add ammonium hydroxide until the violet color of the iron derivative remains on mixing, add 10 ml. of the 2 M sodium acetate-2 M acetic acid buffer and dilute to the mark. Measure the absorbancy at 593 mμ using a 1-cm. cell. Run a blank on reagents and crucible in exactly the same manner.

***Procedure for the Determination of Iron in Limestone*[2].** Weigh a sample of such size as to contain 0.5 to 1.0 mg. of ferric oxide into a 250-ml. beaker. Cover with a watch glass, add 20 ml. of water and 10 ml. of hydrochloric acid and heat gently. After the reaction is completed, filter the solution into a 250-ml. volumetric flask using a medium porosity paper. Wash the filter with dilute hydrochloric acid (1:100) and water. Place the filter in a silver crucible (available from the G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 236), ash, cool and add 1.0 g. of sodium carbonate and 1.0 g. of sodium borate. Heat gently at first and then more strongly to melt the flux and decompose the residue. Rotate the crucible while cooling to cause the melt to solidify on the sides of the crucible. After cooling, add 10 ml. of water and 5 ml. of hydrochloric acid and warm to dissolve the residue. After complete dissolution (a precipitate of silica and silver chloride will remain), transfer the contents of the crucible to the 250-ml. volumetric flask containing the original filtrate. Dilute the solution to volume, filter or centrifuge a portion of the solution. Pipet a 25.0 ml. aliquot of the solution into a 50-ml. volumetric flask and add 2.0 ml. of 10 per cent hydroxylammonium chloride solution and 5.0 ml. of 0.001 M TPTZ. Add dropwise ammonium hydroxide until the violet color of the iron derivative remains on mixing, add 10 ml. of the 2 M sodium acetate-2 M acetic acid buffer and dilute to the mark. Measure the absorbancy at 593 mμ using a 1-cm. cell. Run a blank on reagents and crucible in exactly the same manner.

Procedure for the Determination of Iron in Wine. WET ASHING-TPTZ METHOD AND TPTZ-DIRECT EXTRACTION METHODS OF COLLINS AND DIEHL[3]. See page 50 for preparation of reagent solutions.

PREPARATION OF CALIBRATION CURVE. Pipet various volumes of the standard iron solution into 125-ml. separatory funnels to cover the range from 0 to 40 μg. of iron. To each solution add 2.0 ml. of 10 per cent, iron-free hydroxylammonium chloride, 5.0 ml. of 0.001 M TPTZ, 5.0 ml. of 2 M sodium acetate-2 M acetic acid buffer, and 1.0 ml. of 10 per cent sodium perchlorate. Extract each solution three times using 4.0, 2.0 and 2.0 ml. portions of nitrobenzene. Collect the extracts of each solution in a 10-ml. volumetric flask, dilute to the mark with ethanol and determine the absorbancy at 595 mμ using 1-cm. cells.

PROCEDURE FOR IRON BY THE WET ASHING-TPTZ METHOD. Pipet 3.00 ml. of the wine into a 250-ml. conical flask (preferably one of Vycor) and insert a reflux head (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 181). Add 10 ml. of nitric acid and 5 ml. of perchloric acid and heat to fumes of perchloric acid. Cool the solution, add 20 ml. of water and heat to boiling to remove any chlorine. After cooling the solution, add 2.0 ml. of 10 per cent hydroxylammonium chloride, 2.0 ml. of sodium acetate-acetic acid buffer, and 5.0 ml. of 0.001 M TPTZ. Neutralize the solution with ammonium hydroxide to pH 4 to 5 using a pH meter or pH indicating paper. Transfer the solution to a 125-ml. separatory funnel, add 4.0 ml. of nitrobenzene and shake vigorously for one minute. Allow the phases to separate and gently swirl to remove drops of nitrobenzene clinging to the upper walls of the funnel. Repeat the extraction using 2.0 ml. portions of nitrobenzene. Collect the extracts in a 10-ml. volumetric flask, dilute to the mark with ethanol and measure the absorbancy at 595 mμ using a 1-cm. cell. Run a blank through the entire procedure and subtract the iron so found from that obtained in the analysis.

PROCEDURE FOR IRON BY THE TPTZ-DIRECT EXTRACTION METHOD. Pipet 3.00 ml. of the wine into a 100-ml. beaker. Add 2.0 ml. of 10 per cent, iron-free hydroxylammonium chloride solution, 5.0 ml. of ethanol, 5.0 ml. of acetate buffer and 5.0 ml. of 0.001 M TPTZ. Heat the solution to boiling for five minutes, cool and transfer to a 125-ml. separatory funnel. Wash the beaker with 20 ml. of ethanol and 1.0 ml. of 10 per cent, iron-free sodium perchlorate and add the washings to the separatory funnel. Extract the solution three times using one 4.0 ml. and two 2.0 ml. portions of nitrobenzene and collect the extracts in a 10-ml. volumetric flask. Dilute to volume with ethanol and determine the absorbancy of the solution at 595 mμ using a 1-cm. cell. Run a blank through the entire procedure and subtract the iron so found from that obtained in the analysis.

Procedure for the Determination of Iron in Treated Water and in Sea Water (Parts Per Billion Range). TPTZ-EXTRACTION METHOD OF COLLINS AND DIEHL[4]. REAGENTS. The reagent solutions required are the same as those described on page 50 and in addition another standard iron solution of lower iron content.

STANDARD IRON SOLUTION. 0.050 μg. Fe PER ML. Pipet 50.0 ml. of the standard iron solution containing 1.00 μg. Fe PER ML., into a 1-liter volumetric flask. Add 2.5 ml. of sulfuric acid, dilute to the mark with deionized water, and mix well. This solution will contain 0.05 μg. Fe per ml.

CLEANING OF GLASSWARE. Clean the glassware to be used with concentrated nitric or concentrated hydrochloric acid or a mixture of the two, preferably by soaking the glassware overnight. Rinse the glassware using only deionized water. Between determinations do not subject the apparatus to any cleaning operations, but merely rinse it with distilled water and stopper and allow to stand without drying. It will be observed frequently in dealing with microgram quantities of iron that consistent results will only be obtained after the first one or two analyses following a cleaning of the glassware; that is, the first few analyses condition the glassware by removing adsorbed iron taken up from previous use.

PROCEDURE. Pipet 100.0 ml. of the water into a 125-ml. separatory funnel. Add 2.0 ml. of 10 per cent, iron-free hydroxylammonium chloride solution, 2.0 ml. of 10 per cent, iron-free sodium perchlorate solution, 5.0 ml. of 0.001 M TPTZ, and 5.0 ml. of 2 M sodium acetate-2 M acetic acid buffer. If the previous treatment of the sample, such as wet ashing, has introduced much acid, neutralize with ammonium hydroxide to pH 4 to 5 as determined with a bit of indicator paper. Add 10 ml. of nitrobenzene, shake for one minute, allow the phases to separate and gently swirl the funnel to dislodge any drops of nitrobenzene clinging to the upper walls. Drain the nitrobenzene layer into a 25-ml. volumetric flask and repeat the extraction with another 10-ml. portion of nitrobenzene. Dilute the combined extracts to 25.0 ml. with ethanol. Measure the absorbancy of the solution at 595 mμ using a 5-cm. cell and a mixture of nitrobenzene and ethanol (4:1) in the solvent cell. Run a reagent blank through the entire operation and subtract the absorbancy found for it from that of the unknown solution.

Prepare a calibration curve following the above procedure, but using various volumes from 0 to 50 ml. of the standard iron solution containing 50.0 μg. Fe per liter.

Procedure for the Determination of Iron in Urine. WET ASHING-TPTZ METHOD OF COLLINS AND DIEHL. REAGENTS. 0.001 M 2,4,6-TRIPYRIDYL-S-TRIAZINE. HYDROXYLAMMONIUM CHLORIDE SOLUTION, 10 PER CENT, IRON FREE. 2 M SODIUM ACETATE-2 M ACETIC ACID BUFFER, IRON-FREE. SODIUM PERCHLORATE, 10 PER CENT, IRON-FREE. STANDARD IRON SOLUTION, 1 μg. FE PER ML. See directions given above at the beginning of the section devoted to working procedures.

NITRIC ACID. Redistil reagent grade nitric acid from an all-glass still and store in a bottle with a ground-glass stopper. (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 63).

PERCHLORIC ACID. Use doubly distilled perchloric acid. (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 67).

AMMONIUM HYDROXIDE. Use reagent grade ammonium hydroxide, or to reduce the blank to the very minimum, distil anhydrous ammonia into iron-free (deionized) water.

PROCEDURE. Rinse all glassware with hydrochloric acid (1:1) and wash with deionized water. Run a reagent blank along with the sample in exactly the same manner. Pipet 50.0 ml. of the urine into a 250-ml. conical flask. Add 25 ml. of concentrated nitric acid and 10 ml. of 70 per cent perchloric acid. Place a reflux head on the flask, heat to fumes of perchloric acid and continue the digestion for 10 minutes. If a stone or Transite hood is not available for the wet ashing use a glass fume eradicator (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 184). Wash down the sides of the flask and reflux head with water and heat to boiling to remove chlorine while still hot, transfer the solution to a 125-ml. separatory funnel, cool the solution and add 2.0 ml. of 10 per cent, iron-free hydroxylammonium chloride and 5.0 ml. of 0.001 M 2,4,6-tripyridyl-s-triazine. Place a small piece of Congo Red paper in the solution and add ammonium hydroxide dropwise until the paper turns red. Complete the adjustment of pH by adding 5.0 ml. of 2 M sodium acetate-2 M acetic acid buffer. Dilute the solution to about 100 ml. with water to dissolve the precipitate of ammonium perchlorate. Add 4.0 ml. of nitrobenzene and shake vigorously for 1 minute. Allow the phases to separate and gently swirl to dislodge any droplets of nitrobenzene clinging to the upper walls of the funnel. Collect the nitrobenzene layer in a 10-ml. volumetric flask and repeat the extraction two more times using 2.0-ml. portions of nitrobenzene. Dilute the combined extracts to exactly 10.0 ml. with ethanol, mix and determine the absorbancy of the solution at 595 mμ using a 1-cm. cell. Use a mixture of nitrobenzene and ethanol (4:1) as the reference solution.

Prepare a calibration curve by pipetting various volumes from 0 to 20 ml. of the standard iron solution, 1.00 μg. Fe per ml., into 125-ml. separatory funnels. Add 10 ml. of 10 per cent, iron free ammonium perchlorate, 2.0 ml. of 10 per cent, iron free hydroxylammonium chloride, 5.0 ml. of 0.001 M 2,4,6-tripyridyl-s-triazine and 10 ml. of 2 M sodium acetate-2 M acetic acid buffer. Proceed with the extraction as directed in the preceding paragraph. Use the extract from the solution to which no iron was

added as the reagent blank and subtract the absorbancy found for it from that of the other solutions. Plot absorbancy against concentration.

Procedure for the Determination of Iron in Serum. METHOD OF CARAWAY[7]. Pipet 2.0 ml. of serum into a small test tube, add 1 ml. of 0.2N hydrochloric acid-1 per cent ascorbic acid solution, mix, and allow to stand 5 minutes. Add 1 ml. of 20% trichloroacetic acid made from redistilled, iron-free acid (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 390), and 1 ml. of chloroform. Stopper and shake for 10 to 15 seconds. Centrifuge for 10 minutes. Carefully decant the supernatant liquid into another small test tube. Pipet 2.0 ml. of the clear liquid into a 12 mm. cuvette or test tube. In identical tubes place a blank and a standard.

The blank is prepared by mixing 1.0 ml. of water, 0.5 ml. of 0.2N hydrochloric acid-1 per cent ascorbic acid and 0.5 ml. of 20 per cent trichloroacetic acid. The standard is prepared by mixing 1.0 ml. of the iron standard (2.00 μg. Fe per ml.), 0.5 ml. of 0.2N hydrochloric acid-1 per cent ascorbic acid, and 0.5 ml. of 20 per cent trichloroacetic acid.

To each of the three solutions thus prepared add 0.2 ml. of 0.1 per cent TPTZ. Mix and add 0.2 ml. of 40 per cent ammonium acetate solution and mix again. Using the blank as a reference measure the absorbance at 590 mμ within five minutes if possible.

The final volume obtained is 2.4 ml. For instruments which require 3 ml. of solution, 0.8 ml. of 10 per cent ammonium acetate may substituted for the 0.2 ml. of 40 per cent.

The results may be calculated using the relation:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200 = \mu\text{g Fe per 100 ml. of serum}$$

For the microdetermination of serum iron, all volumes are reduced by a factor of 20 and the techniques of ultra micro-chemistry are applied.

Procedure for the Determination of Total Iron Binding Capacity of Serum (TIBC) METHOD OF CARAWAY[7]. Pipet 2 ml. of serum into a small test tube, add 4.0 ml. of ferric iron solution (5.0 μg Fe per ml.), mix, and allow to stand for 5 minutes. Add 0.5 g. of anhydrous magnesium carbonate powder, stopper, and shake for 10 to 15 seconds. Allow the solution to stand for 30 minutes but remix 4 or 5 times during this interval. Centrifuge for 10 minutes. Pipet 2.0 ml. of the clear supernatant liquid into a test tube and analyze for serum iron as described above.

The TIBC may be calculated using the relation:

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 600 = \text{IBC in } \mu\text{g Fe per 100 ml. of serum}$$

For the microdetermination of TIBC, all volumes are reduced by a factor of 20.

Procedure for the Determination of Microgram Quantities of EDTA in Urine. METHOD OF KRATOCHVIL AND WHITE[10]. REAGENTS. $Fe(TPTZ)_2^{++}$. $5x10^{-4}$M IN 0.1M SODIUM ACETATE-0.1M ACETIC ACID. Dissolve 0.228 g. of TPTZ in a few milliliters of deionized water containing several drops of concentrated hydrochloric acid. Add 50 ml. of 1M sodium acetate-1M acetic acid buffer. Add 0.0995 g. of ferrous ethylenediammonium sulfate tetrahydrate (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 41) and dilute to 500 ml.

STANDARD EDTA SOLUTION $1.00x10^{-4}$. M DISODIUM DIHYDROGEN ETHYLENEDIAMINE-TETRAACETATE. Dissolve 0.3362 g. of primary standard EDTA (G. FREDERICK SMITH CHEMICAL COMPANY, Item No. 365) in deionized water, transfer quantitatively to a 1-liter volumetric flask, dilute to the mark and mix well. Transfer 10.0 ml. of this solution to a 100 ml. volumetric flask, dilute to the mark and mix.

Place the sample containing 0.1 to 1 μ mole of EDTA in a 25 ml. volumetric flask, add 2.00 ml. of the $5x10^{-4}$M solution of $Fe(TPTZ)_2^{++}$, dilute to volume with deionized water and mix well. Measure the absorbance against a deionized water blank at 593 mμ. A calibration curve is prepared using 0-10ml. portions of the $1.00x10^{-4}$M EDTA solution in place of the sample. If any metals other than alkali metals are present, measure the absorbance as soon as possible after the addition of the $Fe(TPTZ)_2^{++}$ solution and at 1 minute intervals for 2 or 3 minutes, and extrapolate back to zero time to obtain the amount of uncomplexed EDTA.

SECTION IV. BIBLIOGRAPHY
2,4,6-TRIPYRIDYL-S-TRIAZINE (TPTZ)

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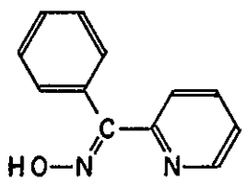
SECTION V

PHENYL-2-PYRIDYL KETOXIME
syn-Phenyl-2-pyridyl Ketoxime

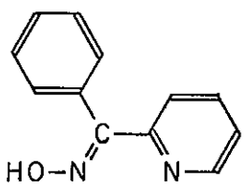


$C_{12}H_{10}N_2O$
Mol. Wt.: 198.22

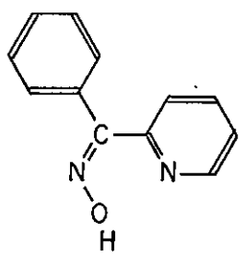
Molar extinction coefficient of $[Fe(\text{Phenyl-2-pyridyl Ketoxime})_3]^-$ in isoamyl alcohol: 15,600 at 550 mμ

G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 295

Phenyl-2-pyridyl ketoxime exists in two stereoisomeric forms



syn-
m.p. 150.5-152°



anti-
m.p. 165-167°

designated *syn-* and *anti-* with reference to the position of the hydroxyl with respect to the phenyl group. That the lower melting isomer formed metal derivatives was first observed by Tschugaeff[1].

The correct assignment of configuration, the lower melting isomer being the *syn-* form, was made by Huntress and Walker[3] in 1948. The oxime group exists in a tautomeric form[2], the so-called *nitrone* form $=N\overset{H}{\underset{O}{}}$, which is the form in which it reacts with the metals by replacement of the hydrogen ion; the iron atom is thus attached directly to the nitrogen atom of the oxime group as well as to the nitrogen atom of the pyridyl group.

Properties. *syn*-Phenyl-2-pyridyl ketoxime is a white, crystalline solid. It is soluble in benzene, chloroform, dioxane, isoamyl alcohol, and hot ethyl alcohol. It is slightly soluble in cold ethyl alcohol, and is insoluble in either hot or cold water.

Solutions of phenyl-2-pyridyl ketoxime are stable when protected from direct sunlight. When exposed to direct sunlight for any length of time the solution takes on a yellow color. The solid oxime is stable when protected from direct sunlight; when exposed to it, the exposed surfaces turn grey.

Reaction with Various Metals. Phenyl-2-pyridyl ketoxime forms colored, water soluble compounds with a number of the transition elements in neutral and alkaline solution. The metals and the colors produced and the lowest concentration of the metal which will produce a color are:

Fe(II)	Red 0.1 p.p.m.	Mn(II)	Yellow
Fe(III)	Red 5	Cr(III)	No colored product
Cu(I)	Orange 4	Pd(II)	Yellow
Cu(II)	Green 4	Pt(II)	Yellow
Co(II)	Yellow 1	UO_2^{++}	Yellow

All of the colored, metal derivatives can be extracted into chloroform or carbon tetrachloride, with the exception of the platinum and uranyl compounds. Phenyl-2-pyridyl ketoxime has been applied to the determination of iron[4,5,6], palladium[7], gold[8], and rhenium[9].

Reaction with Ferrous Iron. Phenyl-2-pyridyl ketoxime reacts with the ferrous ion to form a red, water soluble compound which, in aqueous solution exhibits a single absorption peak in the visible region, at 545 mμ. This red compound is formed in neutral solutions and in basic solutions of concentrations as high as saturated sodium hydroxide. The red compound can be extracted from solutions more than 1 M in sodium hydroxide into isoamyl alcohol or chloroform. If the solution is more than 3 M in sodium hydroxide the compound can be extracted into dioxane. Two extractions are necessary to quantitatively remove the iron compound using chloroform or dioxane. A single extraction suffices in the case of isoamyl alcohol if a few milliliters of ethyl alcohol are also added. In isoamyl alcohol the iron compound exhibits a single absorption peak in the visible region, this being at 550 mμ. The absorption spectrum of the iron compound in isoamyl alcohol is shown in Figure 1.

Aqueous solutions of the iron compound are relatively unstable, a red precipitate appearing on standing several hours. Stability is decreased by exposure to strong light. Chloroform solutions of the iron compound are quite light sensitive, the color intensity diminishing noticeably on even short

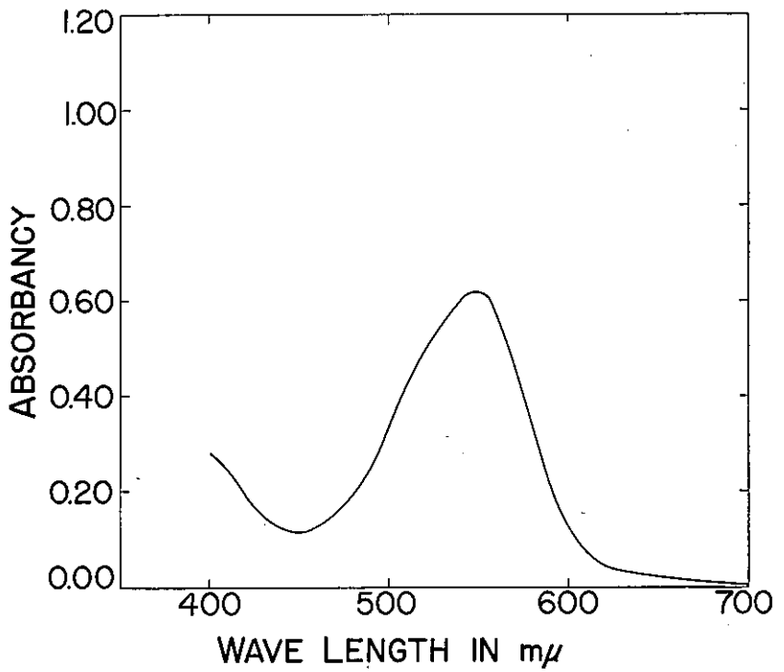


Fig. 1. Absorption spectrum of Fe(phenyl-2-pyridyl ketoxime)$_3^{++}$ in isoamyl alcohol. 2.23 P.p.m. of iron. 1-cm. cell.

exposure to strong light. Solutions of the iron compound in isoamyl alcohol are stable, an insignificant change in absorbancy occurring on 24 hours standing under normal laboratory conditions, or on one hour standing in direct sunlight.

The method of continuous variations was used to determine the ratio of phenyl-2-pyridyl ketoxime to iron. The ratio was found to be three molecules of oxime for each atom of iron when an excess of oxime was present. When the concentration of oxime is less than three times that of the iron, apparently another species is also present.

Specificity. Interferences. As phenyl-2-pyridyl ketoxime forms extractable compounds with many transition elements, high specificity toward iron is not expected, but the tolerance to the transition metals closely associated with iron is sufficiently great for its application to be useful. Cluley and Newman[5], found that recoveries of iron were slightly low in the presence of equal weights of cobalt, copper, manganese and nickel. No interference was found in the presence of cerium (III), mercury (I), aluminum, calcium and zinc when the extraction procedure is followed.

Low results were obtained in the presence of large amounts of cadmium, lead and magnesium. The following anions do not interfere: chloride, sulfate, nitrate, perchlorate, acetate, citrate, orthophosphate, and borate. Arsenate, fluoride, molybdate, oxalate and tartrate interfere if present in relatively high concentrations.

THE DETERMINATION OF IRON IN STRONG ALKALIES. Highly satisfactory reagents are known for iron in neutral solutions; bathophenanthroline, bathophenanthrolinedisulfonic acid and tripyridyl-s-triazine, for example, are more sensitive than phenyl-2-pyridyl ketoxime. The principal work with phenyl-2-pyridyl ketoxime was designed to take advantage of its reaction with iron in and extraction from alkaline solution.

In aqueous solution the time required for the attainment of maximum color intensity was found to vary with the alkalinity. At pH 10 maximum color intensity was reached immediately, while in 5 M sodium hydroxide 10 minutes was required.

In aqueous solution the range of conformity to Beer's law and the molar extinction coefficient are both functions of alkalinity as shown in Table 1.

TABLE 1. MOLAR EXTINCTION COEFFICIENT OF TRIS(PHENYL-2-PYRIDYL KETOXIME)IRON(II) AS A FUNCTION OF THE CONCENTRATION OF ALKALI

Alkalinity	*Molar Extinction Coefficient*	*Limiting Concentration for Beer's Law*
pH 10	10,700	10×10^{-5} M
1 M NaOH	14,100	6×10^{-5} M
3 M NaOH	15,200	5×10^{-5} M
5 M NaOH	13,800	5×10^{-5} M

After extraction into isoamyl alcohol the molar extinction coefficient is no longer a function of the alkalinity of the aqueous phase. In isoamyl alcohol the compound conforms to Beer's Law up to concentrations as high as 1×10^{-4} M. The molar extinction coefficient is 15,600. Satisfactory extractions are difficult from solutions less than 2 M in alkali, and impossible from solutions less than 1 M, since below this concentration the iron compound is precipitated in the aqueous phase rather than being extracted. The working procedure for the determination is given below.

THE DETERMINATION OF "OXIDIZED" IRON IN STRONG ALKALIES. *syn*-Phenyl-2-pyridyl ketoxime was used by Trusell and Diehl[4] for the determination of iron in sodium hydroxide, potassium hydroxide, lithium hydroxide, and sodium carbonate. The results are shown in column 2 of Table 2. The results showed good reproducibility, but the value obtained

on the sample of lithium hydroxide was appreciably lower than that found by Dr. R. B. Ellestad of the Lithium Corporation of America. For this reason the sodium and lithium hydroxides were analyzed for iron by the phenyl-2-pyridyl ketoxime method and also by the 1,10-phenanthroline method following neutralization with twice-distilled nitric acid. In addition, the lithium hydroxide was analyzed using the phenyl-2-pyridyl ketoxime reagent following neutralization with twice distilled nitric acid and the addition of iron-free sodium hydroxide. The results are reported in Table 2.

TABLE 2. IRON CONTENT OF ALKALI HYDROXIDES BY DIFFERENT PROCEDURES

Material	*Phenyl-2-pyridyl Ketoxime Direct Extraction p.p.m. Fe*	*1,10-Phenanthroline Preliminary Neutralization p.p.m. Fe*	*Phenyl-2-pyridyl Ketoxime Preliminary Neutralization p.p.m. Fe*
NaOH	3.5	4.6	
	3.5	4.6	
	3.5	4.4	
LiOH	2.4	9.5+	9.5
	2.2	9.5	9.5
	2.5	9.5	9.5
KOH	3.1		
	3.1		
	3.1		
Na_2CO_3	0.3		
	0.3		
	0.3		

+ Lithium Corporation of America value, 11.0 p.p.m.

The reproducible discrepancy between the direct extraction and preliminary neutralization procedures indicated that two forms of iron were present in the hydroxides analyzed. Preliminary experiments indicated that metallic iron reacted slightly if at all with phenyl-2-pyridyl ketoxime. That even the slight reaction observed was due to surface oxide was proved by the following experiment.

A porcelain boat containing 0.5 g. of iron filings, 30 mesh and finer, was placed in a borosilicate tube and ignited in a stream of hydrogen. Boat and metal were allowed to cool in the atmosphere of hydrogen, and with the stream of hydrogen flowing rapidly through the tube the boat and its contents were quickly transferred to a solution of phenyl-2-pyridyl ketoxime and iron-free sodium hydroxide which had been deaerated by the passage of nitrogen for 30 minutes. The container was closed with a glass stopper and allowed to stand for several hours. No red color formed

to indicate a reaction between the iron metal and the oxime. Even the briefest exposure of the reduced iron to the air before treatment with alkaline phenyl-2-pyridyl ketoxime reagent resulted in some surface oxidation as indicated by the formation of the red color.

A 0.1 g. sample of finely ground magnetite was added to 25 ml. of 10 M iron-free sodium hydroxide. On treatment with 2 ml. of 10 per cent sodium hydrosulfite and 5 ml. of 0.2 per cent phenyl-2-pyridyl ketoxime, the characteristic red color developed. A 0.1 g. sample of hematite was treated in a similar manner, with the production of the red color.

It is thus apparent that the phenyl-2-pyridyl ketoxime reagent permits the determination of what we term "oxidized" iron, either the hematite or magnetite forms of iron oxide, in the presence of metallic iron. Working directions for carrying out this determination are given below.

THE DETERMINATION OF IRON IN GLASS SAND AND SILICEOUS MATERIALS. That "oxidized" iron reacts directly with the reagent is fortunate, for this permits the determination of iron in silicate materials directly after a sodium carbonate-sodium borate fusion. Results on National Bureau of Standards Sample No. 81, Glass Sand, are shown in Table 3. Details of the procedure are given below.

TABLE 3. THE DETERMINATION OF IRON IN GLASS SAND, NATIONAL BUREAU OF STANDARDS SAMPLE No. 81

Weight of Sample (mg.)	*Absorbancy at 550 mμ, 1-cm. cell*	*Iron Found*	
		μg.	*Per Cent*
102.8	0.607	52.5	0.0510
102.9	0.605	52.4	0.0509
114.7	0.660	57.1	0.0512
		Average	0.0511
		NBS Value	0.051

DETERMINATION OF IRON IN ETHYLENE AMINES. Chernin and Simonsen[6] found that the reaction of ferrous iron and phenyl-2-pyridyl ketoxime in aqueous ethylene amine solutions yields bluish-purple compounds. The absorption spectrum shows three maxima, at 588, 509, and 405 mμ. The band at 588 mμ is used for quantitative measurements. The limit of detection is 0.5 μg. of iron per gram of amine using 1 cm. cells. The molar extinction coefficients vary slightly with the various amines. With solutions containing 120 μg. of iron, 1000 μg. amounts of aluminum, copper, nickel and zinc did not interfere. The procedure is given below.

Procedure for the Determination of Iron in Strong Alkalies. METHOD OF TRUSELL AND DIEHL[5]. REAGENTS. PHENYL-2-PYRIDYL KETOXIME. 0.2 PER CENT. Dissolve 2 g. of *syn*-Phenyl-2-pyridyl ketoxime (G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 295) in 1 liter of 0.1 M hydrochloric acid.

SODIUM HYDROSULFITE. 10 PER CENT SOLUTION, IRON-FREE. Prepare fresh each day. Dissolve 2 g. of sodium hydrosulfite in 10 ml. of water, add 5 ml. of 0.2 per cent phenyl-2-pyridyl ketoxime and 5 ml. of 10 M sodium hydroxide, iron free, and allow to stand 10 minutes. Extract the iron compound thus formed into 10 ml. of isoamyl alcohol and 5 ml. of ethyl alcohol.

SODIUM HYDROXIDE. 10 M, IRON-FREE. Dissolve 400 g. of sodium hydroxide in 800 ml. of water. Add 10 ml. of sodium hydrosulfite and 50 ml. of 0.2 per cent phenyl-2-pyridyl ketoxime. Allow the mixture to stand for 10 minutes and then extract the red compound thus formed into 100 ml. of isoamyl alcohol and 25 ml. of ethyl alcohol. Dilute the colorless, aqueous phase to 1 liter.

STANDARD IRON SOLUTION. 5×10^{-4} M. Dissolve 0.5585 g. of pure iron (for example, the electrolytic iron ignited in hydrogen, G. FREDERICK SMITH CHEMICAL COMPANY, Catalog Item No. 226) in a few ml. of hydrochloric acid and dilute to 1 liter. Pipet 50.0 ml. of this solution to a 1-liter volumetric flask and dilute to the mark with distilled water.

Procedure for Oxidized Iron in Strong Alkalies. Weigh a sample of such size as to give a solution 4 to 5 molar in alkali when dissolved in 250 ml. of water. Dissolve the sample in deionized water and dilute to 250 ml. Pipet 25.0 ml. into a 100 ml. beaker and add 2.0 ml. of 10 per cent sodium hydrosulfite and 5.0 ml. of 0.2 per cent phenyl-2-pyridyl ketoxime. Heat the mixture 5 minutes on a steam plate and transfer to a separatory funnel. Add 5 ml. of ethyl alcohol and 10 ml. of isoamyl alcohol, shake, and allow the layers to separate. Draw off the aqueous layer. Transfer the alcohol layer to a 25-ml. volumetic flask, dilute to the mark with isoamyl alcohol, and measure the absorbancy at 550 mμ.

Prepare a calibration curve by adding known amounts of the standard iron solution, up to 10 ml., to 25 ml. of 10 M iron-free sodium hydroxide. Add 2.0 ml. of 10 per cent sodium hydrosulfite, 5.0 ml. of 0.2 per cent phenyl-2-pyridyl ketoxime, dilute to approximately 50 ml., and heat on a steam plate for five minutes. Transfer the solution to a separatory funnel and extract the red iron compound into 5 ml. of ethyl alcohol and 10 ml. of isoamyl alcohol. Draw off the aqueous layer. Transfer the alcohol layer to a 25-ml. volumetric flask, dilute to the mark, and determine the absorbancy at 550 mμ.

Procedure for Iron in Glass Sand. METHOD OF TRUSELL AND DIEHL[4] as modified by Cluley and Newman[6]. Dry the sample for two hours at 110°. Weigh 100 mg. into a platinum crucible which has been previously freed of iron by alternate ignition to red heat and treatment with hot hydrochloric acid. Add one g. of an equimolar mixture of sodium carbonate and sodium tetraborate, and blend the contents thoroughly. Fuse in the usual manner. After cooling, dissolve the melt in 10 ml. of water. Transfer the solution to a small beaker with 25 ml. of 10 M sodium hydroxide, treat with 2 ml. of 10% sodium hydrosulfite and 5 ml. of 0.2 per cent phenyl-2-pyridyl ketoxime and heat on a steam plate five minutes. Transfer the solution to a separatory funnel and extract with a mixture of 5 ml. of ethyl alcohol and 10 ml. of isoamyl alcohol. Transfer the alcohol layer to a 25-ml. volumetric flask, dilute to the mark, and measure the absorbany at 550 mμ.

Procedure for Iron in Ethylene Amines. METHOD OF CHERNIN AND SIMONSEN[6]. Introduce a 10 ml. portion of the sample (less if the iron content is greater than 20 p.p.m.) into a 50 ml. graduated mixing cylinder. Dilute to 25 ml. with distilled water, mix and transfer the solution to a 100 ml. beaker. Add 2 ml. of 10 per cent, iron-free sodium hydrosulfite and allow the mixture to stand a few minutes. Add 5 ml. of 0.2 per cent phenyl-2-pyridyl ketoxime and heat for 10 minutes in a hot water bath (80-100°C). Cool and transfer the solution to a separatory funnel. Add 15 ml. of isoamyl alcohol-ethanol (15:2), shake and separate. Filter the alcohol layer into a 25 ml. volumetric flask and rinse the paper with isoamyl alcohol. Dilute to the mark, mix, and measure the absorbance at 588 mμ against a reagent blank. A calibration curve is prepared by adding known amounts of iron to 10 ml. samples of iron-free ethylene amine and treating in the same manner.

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