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The Evaluation of Certain Serum Iron Analyses by Atomic Absorption Spectroscopy

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THE EVALUATION OF CERTAIN SERUM IRON ANALYSES BY ATOMIC ABSORPTION SPECTROSCOPY

by

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INTRODUCTION

Twyman (1) gives a historical account of the elucidation of the basic principles of atomic spectra from the original studies by Kirchhoff (2) in 1860, up to 1950. In 1955, Walsh (3), Alkemade and Milatz (4,5) in independent studies established the general applicability of atomic absorption in flames to the analysis of trace metals. The techniques which they set forth are based upon the principle that when a solution of an element is aspirated into a flame, a large proportion of the metal atoms will populate the flame as neutral ground state atoms. The passage of a beam of characteristic monochromatic radiation through the flame will result in the attenuation of its intensity arising from its absorption by specific atomic vapor species. Since the absorption is proportional to the concentration of ground state atoms, this measurement can be used as a quantitative determination of the specific element in the original solution. Fuwa and Vallee (6) discuss the pertinence of the Beer-Lambert-Bouguer Law to atomic absorption. Various reviews (7,8,9,10,11,12) have been written describing the instrumentation and listing numerous applications of atomic absorption spectroscopy to the analysis of agricultural, industrial, metallurgical, geochemical, and biological materials. This technique is being adopted with increasing confidence in clinical analysis. Emphasis has been placed on the determination of calcium and magnesium (13,14,15) as well as the trace metals

zinc (16,17), copper (16,17,18), lead (17,19,20), and iron (16,17,21,22). Zettner (23) has recently reviewed the applications of this technique in the clinical laboratory.

In spite of the many successes achieved by atomic absorption analysis, problems often arise when applying these techniques to different sample materials. For example, the determination of iron in urine has proven to be reasonably simple (24); however, difficulties are encountered when the same procedure is applied to serum iron. Significantly different sensitivities and detection limits are reported for serum iron analysis which seem to be related to techniques of sample treatment. Numerous explanations can be and often are given in order to explain these discrepancies. Unfortunately, a systematic study of this problem relative to atomic absorption techniques appears to be notably absent in the current literature. It has been the purpose of this study to attempt to isolate and clarify the specific causes of these apparent discrepancies.

INSTRUMENTATION

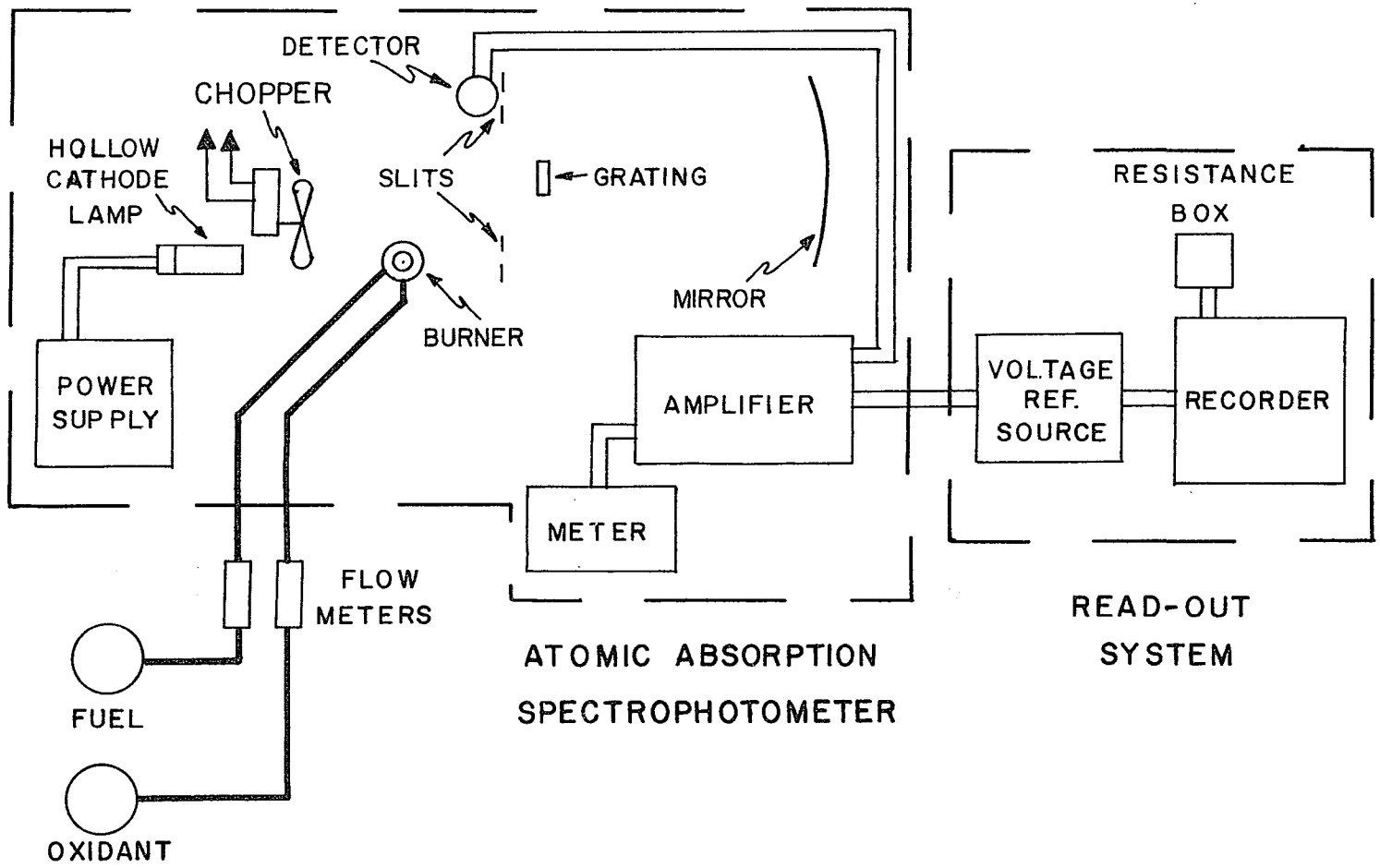
A Jarrell-Ash Atomic Absorption Unit, model number 82-536 with interchangeable fixed slits, was used to obtain all the atomic spectral data in this study. This unit employs a 0.5-meter Ebert Scanning Spectrometer with a 150-mm. diameter concave mirror and a replica grating of 1180 grooves per mm. blazed at 3000 Å. The effective aperature ratio of the spectrometer is f/8.6 and its reciprocal linear dispersion at the exit slit is 16 Å per mm. in the first order. A graded (Jarrell-Ash number R212) photomultiplier tube with an ultraviolet transmitting window was used as the detector.

The read-out system, Figure 1, consisted of a Model EUW 16A voltage reference source (Heath Company), a decade resistance box (Leeds and Northrup serial number 1183257), and a 120 millivolt strip chart recorder (E. H. Sargent Company, Model SRL).

The compressed oxygen and air were maintained at 50 p.s.i. by means of two stage regulators (Linde Company R201 and National Cylinder Gas 10-61) at the cylinders while the flow rates were monitored by a flow meter (Matheson Company 606). Acetylene pressure was kept at 15 p.s.i. by a single stage regulator (Matheson Company 11 p) while the hydrogen used a two stage regulator (Linde Company R219) to maintain a pressure of 50 p.s.i. Both the acetylene and hydrogen flow rates were monitored with a flow meter (Matheson Company 605 - steel float).

FIGURE 1

INSTRUMENTAL BLOCK DIAGRAM



The hollow cathode lamp power supply is a moderately regulated current source with the provision for operating certain lamps, e.g. Se or As, at high intensity. In the normal mode of operation a diode bridge circuit is used for full wave rectification along with a modified L section filter. The high intensity mode uses half wave rectification to obtain a pulsed direct current. Here the root mean square current will be less than the maximum current rating of the lamp but the maximum current will exceed the maximum current rating of the lamp for a short period of time during each cycle. In both modes of operation, the current is regulated by means of a resistor in series with the gas filled hollow cathode lamp.

A multiple pass optical system, Figure 2, is utilized to increase the effective cell length to a maximum of 12.5 cm. (25). One, three, or five passes may be made through the flame. Five passes is the maximum practical limit because more radiation will be lost through reflection off additional mirrors than the absorbance gained by increasing the number of passes.

An A.C. amplifier was used to amplify the signal from the photomultiplier tube, thus necessitating modulation of the light beam by means of a mechanical chopper for the normal mode of lamp operation. The high intensity mode of lamp operation requires no chopper. The amplifier incorporates a wide band pass filter at its input, peaked at 90 cycles per second corresponding to the chopper frequency. Two advantages are gained by use of the A.C. amplifier

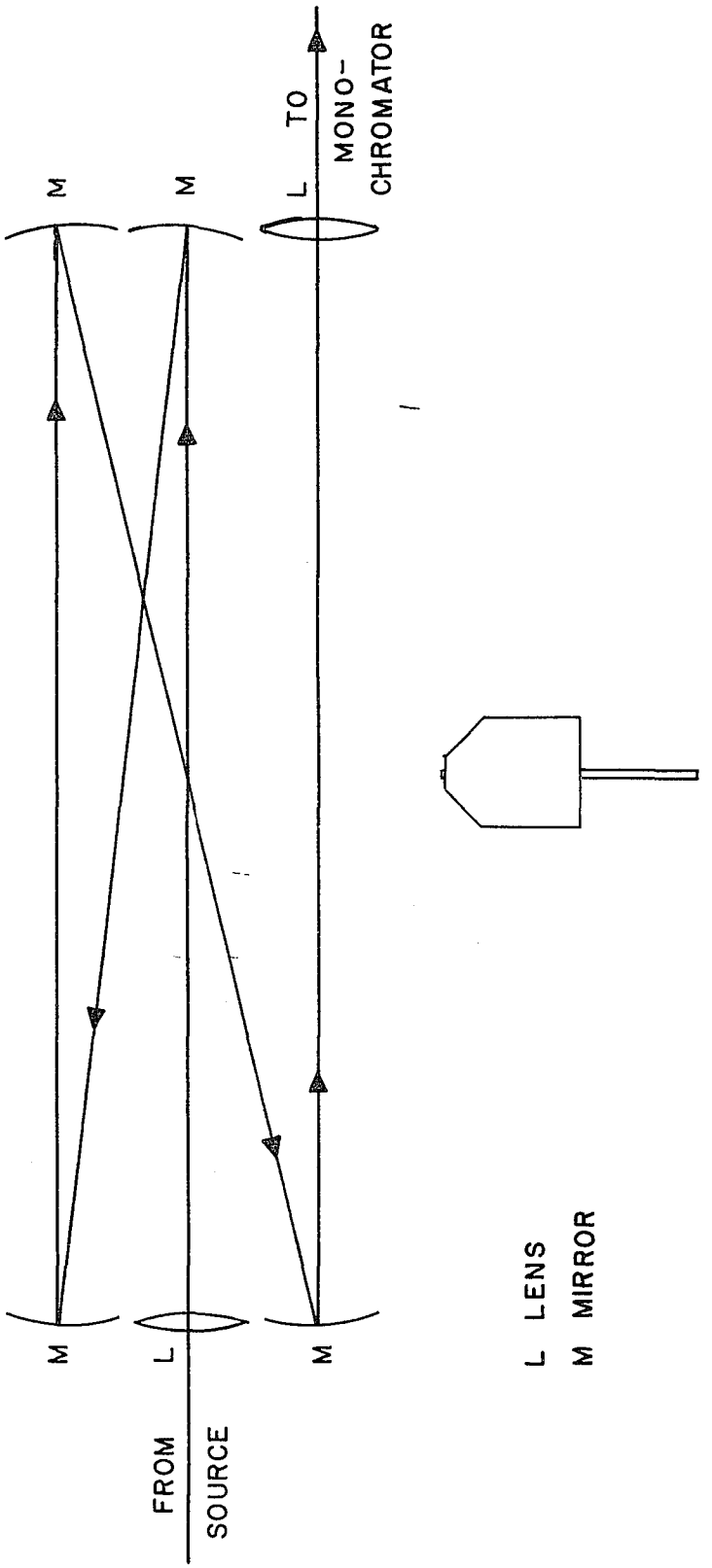


FIGURE 2
MULTI-PASS OPTICS

system over the conventional D.C. amplifier, these being greater stability and removal of continuous D.C. flame emission signals.

The Heath voltage reference source was connected between the amplifier output of the atomic absorption instrument and the input of the recorder in order to buck out a known portion of the signal and to give a four-fold scale expansion. The decade resistance box was used to shunt the resistance of the slide wire potentiometer of the recorder.

A Hetco (Ditric Corporation) triflame burner was used in this study. It may be operated as a total consumption burner or as a laminar flow burner with a 5 or 10 cm. slot. The burner mount was moved 1/4 inch forward from the manufacturer's preset position to allow the burner to be moved completely through the optical path and positioned at the point of maximum absorption. A 31.7 cm. capillary tubing was attached to the burner to facilitate changing of samples.

SOLUTIONS AND REAGENTS

All reagents used in this study were of analytical reagent quality except where indicated. A colorimetric procedure was used to determine if there were any iron impurities in the reagents. All of the distilled water used in this study was redistilled from alkaline permanganate to remove iron contaminants.

A 500 p.p.m. standard iron solution was prepared by dissolving 500.2 mg. of 99.95% iron wire in 14 ml. of dilute nitric acid and diluting to 1 liter with distilled water. A solution of approximately 8.8 p.p.m. iron was prepared by dissolving 72.4 mg. of ferric perchlorate hexahydrate in a total volume of 1 liter of distilled water.

Other solutions used in the study were as follows:

Trichloroacetic acid (U.S.P.) - 30% (weight/volume) aqueous solution.

Sodium citrate - 25% (weight/volume) aqueous solution.

1,10-phenanthroline - 0.5% (weight/volume) aqueous solution.

Mercaptoacetic acid (Thioglycolic acid) - 80% aqueous solution.

Bovine blood - local packing company.

PROCEDURE

Instrumental

There are many variable parameters in atomic absorption spectroscopy, some pertaining to sample preparation and others related to optimum instrument performance. Instrumental variables include hollow cathode current, photomultiplier voltage, dampening and gain of both amplifier and recorder, slit width, type of burner, position of burner, fuel-oxidant systems and their flow rates. A series of experiments was carried out to determine the effect of these variables upon the absorption signal of iron at 2482 Å. The Hetco total consumption burner was positioned to give the optimum absorption while burning an acetylene-oxygen mixture and aspirating an aqueous solution of ferric-perchlorate at approximately 9 p.p.m. of iron. The optimum values found were :

Hollow cathode lamp.....	14 - 22 milliamps
Photomultiplier voltage.....	580 - 640 volts
Flow rate of O_2.....	7.0 S.L.P.M.
Flow rate of C_2H_2.....	9.3 S.L.P.M.

The net effect of decreasing the slit width is to increase the signal-to-noise ratio because the flame noise varies as the square of the slit width while the spectral line intensity varies linearly with the slit width. Due to the attenuation of the signal,

the photomultiplier dynode voltage must be increased, thus increasing the shot effect noise. Consequently, a 100μ entrance and 150μ exit slits were found to give the best signal-to-noise ratio.

Serum Analysis

Due to the relatively low level of iron, 0.5 to 1.5 p.p.m. (26), in normal human serum, the voltage reference source was used to expand the effective output signal four-fold thus allowing greater instrumental sensitivity. Investigation proved the air-hydrogen flame to be more sensitive for iron analyses than the oxygen-acetylene flame. Due to the increased sensitivity gained from the read-out system and use of the air-hydrogen flame, the hollow cathode lamp and the photomultiplier voltage required re-adjustment to obtain a better signal-to-noise ratio.

Bovine serum was used in place of human serum because it is more readily available and its iron content is similar to that found in human beings (27). Since this problem does not concern itself with the absolute concentration of iron, but the relative amounts found by different methods of sample treatment, it was felt that the use of bovine serum should not affect the validity of the study.

Two procedures were used to separate the serum from the whole blood. Procedure 1 was to allow the blood to clot at room temperature, then break up the clot and remove the coagulated

protein by means of vacuum filtration. The filtrate was placed in a Sorvall RC-2 refrigerated centrifuge (10°C) for 25 minutes at 16,000 x g. The supernatant liquid was refrigerated overnight, centrifuged (5°C) for 30 minutes at 27,000 x g, decanted and then frozen. In procedure 2 (28) the whole blood is allowed to clot at room temperature in glass bottles supported at an angle of approximately 30° to 45°. The bottles were then placed in a refrigerator overnight, decanted the following morning and centrifuged (5°C) for 25 minutes at 1500 x g. The supernatant liquid was refrigerated for immediate use rather than frozen as in procedure 1. The serum obtained from both procedures exhibited a reddish-yellow color. Microscopic examination of the sera obtained from both procedures indicated the absence of any red cells or cell fragments.

A slight modification of the colorimetric method described by Peters and Giovanniello (29) was used to determine the concentration of iron. Two ml. of serum, 1 ml. of 0.6N HCl and 1 drop of mercaptoacetic acid were placed in a small test tube, mixed well and allowed to stand for 30 minutes. Next, 1 ml. of fresh 30% trichloroacetic acid was added and mixed with the solution and allowed to stand for 15 minutes followed by centrifugation for 15 minutes using a semimicro centrifuge. To 4 ml. of the supernatant liquid 18 drops of sodium citrate (final pH$\sim$3.5) and 1 ml. of 0.5% 1,10-phenanthroline were added. The color was allowed to develop overnight and the absorbance was read on a Cary Model 14

Spectrophotometer at 511 mμ . All standard iron solutions and blanks were treated in an identical manner. From the data of the standards a calibration curve of absorbance versus concentration of iron was constructed. The concentration of serum iron was then determined as 1.66 p.p.m. In order to cover a range of concentrations, the serum was diluted with distilled water to obtain stock solutions of serum containing 0.55, 0.83, 1.11, 1.39 and 1.66 p.p.m. of iron.

Three methods for the determination of iron in blood serum by atomic absorption techniques were studied. The first of these is a modification of the Peters and Giovanniello precipitation procedure (29) where 1 ml. of 0.6N HCl and 1 drop of mercaptoacetic acid were added to 2 ml. of serum and diluted to 5 ml. The solution was mixed and then transferred to a test tube. After 30 minutes 1 ml. of fresh 30% trichloroacetic acid was added and mixed in the solution and allowed to stand for 15 minutes. Following centrifugation for 15 minutes, the supernatnat liquid was analyzed for iron with the atomic absorption instrument. A blank was also carried through the same procedure.

A second procedure for the analysis of iron in blood serum was recently reported by Sprague and Slavin (16). This procedure involves the dilution of the serum with an equal volume of distilled water and its direct analysis by atomic absorption spectroscopy. In this case distilled water is used as a blank.

The third procedure is a wet-oxidation method whereby 2 ml. of a nitric-perchloric acid mixture (one to two parts by volume) are added to 1 ml. of serum contained in a 25-ml. erlenmyer flask. The flask and contents are placed on a hot plate (approximately 260°C) for 15 minutes. After cooling, the solution was transferred to a 5-ml. volumetric flask, diluted to volume with distilled water and analyzed for iron. A water blank was carried through the same procedure.

The 2483 Å iron line was used for the analysis. The air flow rate was 8.4 S.L.P.M. while the hydrogen flow rate was 53 S.L.P.M. and the top of the burner was positioned 4.1 cm. below the optical path. The hollow cathode lamp current was adjusted to 6.0 ma. while the photomultiplier voltage was 595 volts with full gain and the instrument fully dampened. The recorder, resistance box and voltage reference source were adjusted so that the recorder would give a full scale reading for 25% absorption.

DISCUSSION

The iron absorption line at 2483 Å gives the best analytical sensitivity (30). Zettner, Sylvia and Capacho-Delgado (22) indicate that narrow slits must be used to separate this line from the 2488 Å line since the latter line is not as sensitive. The absorption at 2483 Å is the result of a transition from a^5D_4 (0.000 electron volts) ground state level to the $x^5F_5^\circ$ (4.991 electron volts) state, a resonance absorption. The transition at 2488 Å is from the a^5D_3 (0.052 electron volts) state to the $x^5F_4^\circ$ (5.033 electron volts) level, a non-resonance absorption. Assuming a Boltzmann distribution (31,32), there should be more atoms available for absorption in the lower energy state thus accounting for its greater sensitivity.

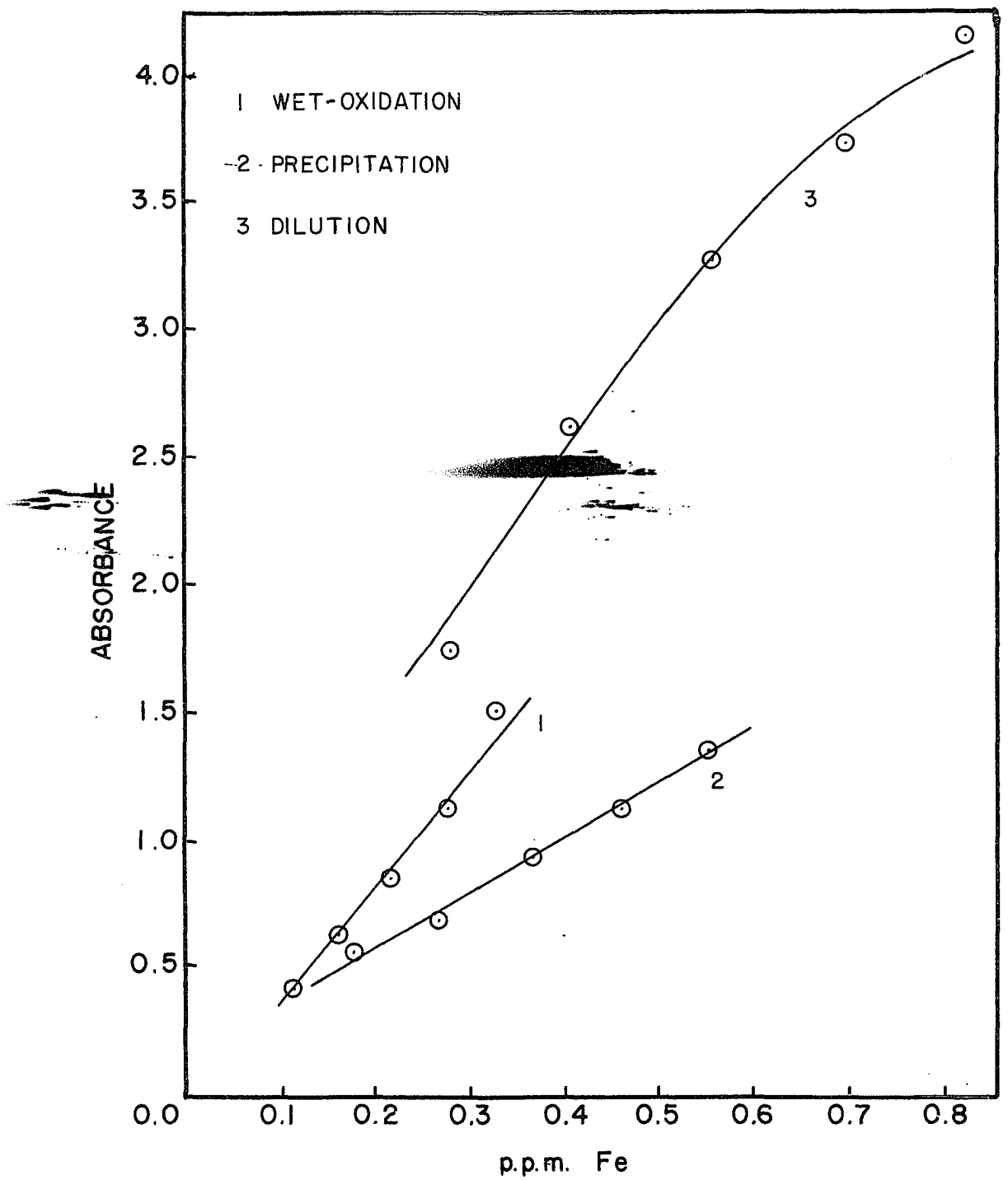
The data for the three methods of sample treatment are summarized in Table I. The final concentration, A, was calculated by taking 1/3, 1/2, and 1/5 of the original concentration for the precipitation, dilution and wet-oxidation methods respectively. These fractions represent the dilution factors of each method. Figure 3 shows the relationship of one method to another. It was felt that the low absorbance values for the precipitation method were due to a loss of iron either through adsorption or occlusion. Therefore, the precipitates from this method were carried through the wet-oxidation procedure and analyzed for iron. The precipitates were found to contain iron equivalent to 0.44 to 0.91

TABLE I

Method	Conc. Fe (p.p.m.)			Number of Detn's	(Abs. $\pm\sigma$) x 10^3
	Initial	Final			
		A	B		
	1.66	0.55		5	13.1 ± 1.2
	1.39	0.46		5	11.0 ± 1.5
Precipitation	1.11	0.37		4	9.1 ± 1.0
	0.82	0.27		5	6.7 ± 0.6
	0.55	0.18		3	5.4 ± 0.4
	1.66	0.83	1.90	4	41.2 ± 0.4
	1.39	0.70	1.58	5	37.1 ± 0.5
Dilution	1.11	0.56	1.27	5	32.6 ± 1.4
	0.82	0.41	0.95	5	24.8 ± 0.7
	0.55	0.28	0.63	5	17.3 ± 0.4
	1.66	0.33	0.76	5	14.8 ± 3.1
	1.39	0.28	0.63	5	11.0 ± 0.8
Wet-oxidation	1.11	0.22	0.51	4	8.2 ± 1.1
	0.82	0.16	0.32	5	6.1 ± 1.3
	0.55	0.11	0.25	5	3.9 ± 0.8

FIGURE 3

GRAPH OF RELATIVE ABSORBANCE
VERSUS FINAL CONCENTRATION OF IRON

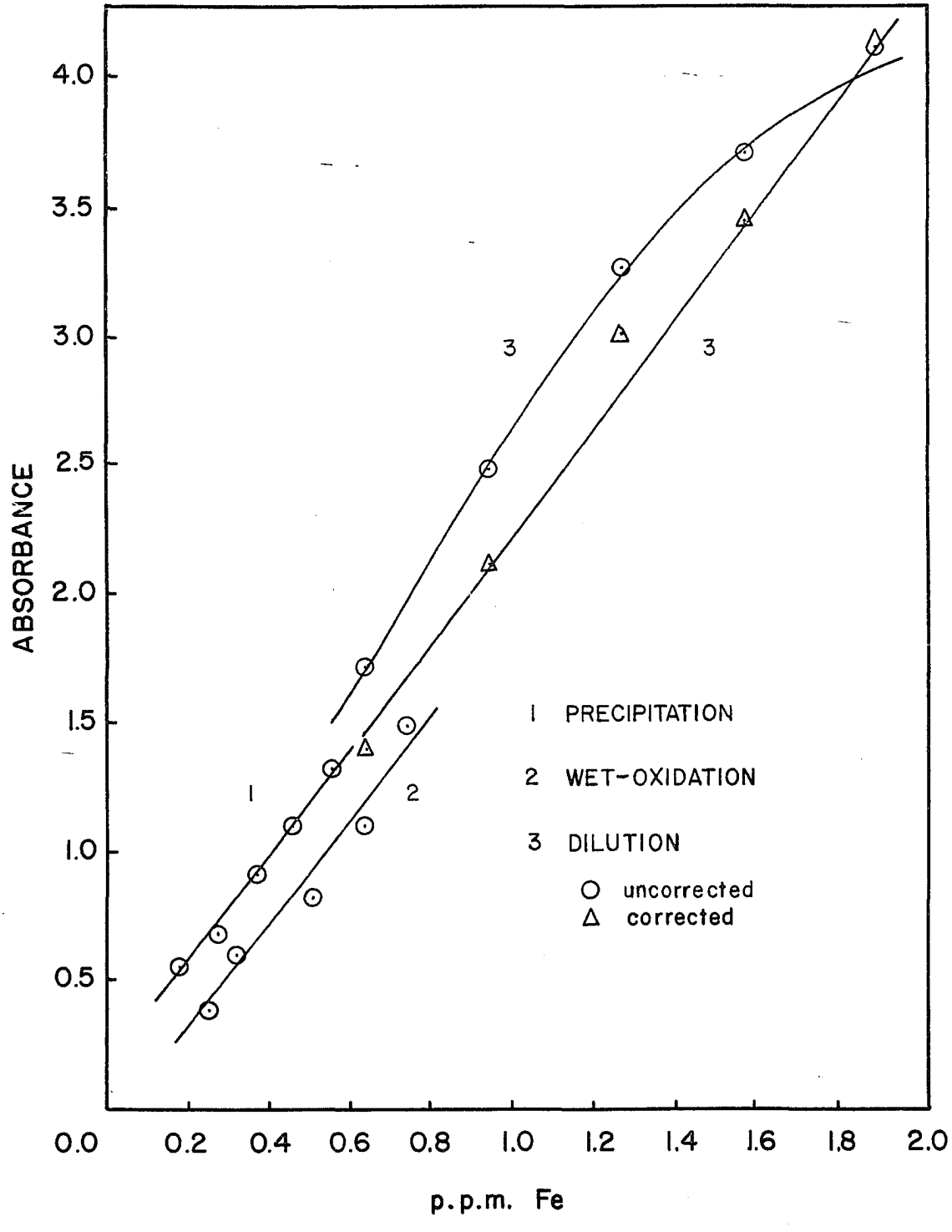


p.p.m. in the final solution. This could possibly be due to the presence of hemoglobin iron from erythrocytes that may have been ruptured during the separation of the serum. One should also consider the possibility of the adsorption or occlusion of iron with the precipitated protein. Therefore, a second sample of blood was obtained and the serum was separated by procedure 2 in the hope of eliminating hemolysis. This sample was analyzed by the dilution and precipitation methods. There was no significant difference in the results obtained for the two methods used to separate the serum. This does not rule out the possibility of hemolysis taking place but does indicate that the results are independent of which procedure is used for separating the serum from the whole blood.

The colorimetric determination of iron in the serum employed a precipitation method and therefore does not indicate the total amount of iron present. The total iron was obtained by carrying standard iron solutions through the wet-oxidation method. From this calibration curve the total iron concentration for the undiluted stock serum was found to be 3.80 p.p.m. The concentrations of the stock solutions were recalculated and multiplied by the dilution factors for the dilution and wet-oxidation methods to obtain the final concentration, B, in Table I. In Figure 4 this recalculated data is plotted along with the original precipitation method. Over the concentration range studied all of the lines have equal slopes within experimental error which would indicate that the

FIGURE 4

GRAPH OF RELATIVE ABSORBANCE
VERSUS FINAL CONCENTRATION OF IRON



three methods have equal sensitivities. The dilution method gives a greater absorbance value than either the precipitation or the wet-oxidation method for the same iron content. A possible explanation of this may be related to the differences in the matrix of the samples. Iron readily forms oxides in the flame which could result in a diminution of atomic vapor thereby giving rise to lower absorbances. This should especially be true in the oxidizing perchloric acid media. In the dilution method the iron is bonded to the protein which may tend to inhibit oxide formation and more easily facilitate the conversion of iron to the atomic state thus leading to an enhancement.

The dilution method shows considerable curvature at higher concentrations. This could be interpreted as being related to changes in viscosity since the stock serum was diluted with distilled water to obtain the lower concentrations. If this is true it should be possible to modify the Beer-Lambert-Bouguer Law to incorporate a viscosity dependence.

The Beer-Lambert-Bouguer Law states that the absorbance, A, is a function only of the cell length, b, and the concentration, c, or

$$dA = \left(\frac{\partial A}{\partial c}\right)_b dc + \left(\frac{\partial A}{\partial b}\right)_c db \qquad (1)$$

Malmstadt and Chambers (33) have indicated that the absorbance is directly proportional to the flow rate or the volume per unit time, $\frac{v}{t}$.

$$A = k' \left(\frac{v}{t}\right) \qquad (2)$$

Poiseuille's Law states that the viscosity, η, of a liquid flowing through a capillary is inversely proportional to the flow rate.

$$\eta = \frac{\pi p r^4 t}{8 l v} \tag{3}$$

where

p = difference in pressure at the ends of the tube

r = radius of the tube

l = length of the tube

t = time

v = volume

Using the relationship that the density, d, is equal to mass, m, per unit volume and substituting this into Equation 3 yields

$$\eta = \frac{\pi p r^4 t d}{8 l m} \tag{4}$$

For a given tube with constant pressure differential and constant density, Equation 4 reduces to

$$\eta = k_1 \frac{t}{m} \tag{5}$$

Defining the term D equal to $\frac{m}{t}$ yields

$$\eta = \frac{k_1}{D} \tag{6}$$

By introducing the viscosity term, Equation 1 becomes

$$dA = \left(\frac{\partial A}{\partial c}\right)_{b,\eta} dc + \left(\frac{\partial A}{\partial b}\right)_{c,\eta} db + \left(\frac{\partial A}{\partial \eta}\right)_{c,b} d\eta \tag{7}$$

$$A = f(c) = k_2 c; \quad b \text{ and } \eta \text{ are constant} \tag{8a}$$

$$A = f(b) = k_3 b; \quad c \text{ and } \eta \text{ are constant} \tag{8b}$$

$$A = f(\eta) = k_4 \eta^{-1}; \quad c \text{ and } b \text{ are constant} \tag{8c}$$

$$\left(\frac{\partial A}{\partial c}\right)_{b,\eta} = k_2 = \frac{A}{c} \tag{9a}$$

$$\left(\frac{\partial A}{\partial b}\right)_{c,\eta} = k_3 = \frac{A}{b} \tag{9b}$$

$$\left(\frac{\partial A}{\partial \eta}\right)_{c,b} = k_4 \eta^{-2} = -\frac{A}{\eta} \tag{9c}$$

Substituting Equations 9a, b, c, into Equation 7, we obtain

$$dA = \frac{A}{c} dc + \frac{A}{b} db - \frac{A}{\eta} d\eta \tag{10}$$

After dividing by A and integrating, Equation 10 becomes

$$\ln A = \ln \frac{k_5 bc}{\eta} \tag{11}$$

or

$$A = \frac{k_5 bc}{\eta} \tag{12}$$

Substituting Equation 6 into Equation 12 yields

$$A = \frac{k_5 bcD}{k_1} = k_6 bcD \tag{13}$$

or

$$A = kcD; \quad \text{for constant } b \tag{14}$$

In applying this correction to the dilution method, the densities and D were measured. To ascertain that the density was essentially constant, the weight of a 10 ml. sample of the most and least concentrated solutions was determined. The D values were determined by aspiration of a known weight of sample for a given time rather than measuring viscosity directly. In this technique the aspirator serves as a simple viscometer. These results are summarized in Table II.

TABLE II

Concentration Fe p.p.m.	Number of Determinations	Average D (gm./sec.)	Average d (gm./ml.)
1.90	3	0.0665	1.0054
1.58	3	0.0719	------
1.27	3	0.0726	------
0.95	3	0.0781	------
0.63	3	0.0814	0.9983

A relative difference in the density of 0.7% was considered insignificant since it lies within the limits of experimental error. The data for the determination of k and the absorbances normalized to an uptake rate of 0.0665 gm./sec. are tabulated in Table III.

TABLE III

Final Concentration Fe p.p.m.	Experimental Absorbance	$k = \frac{A}{cD}$	Corrected Absorbance
1.90	0.0412	0.326	0.0412
1.58	0.0371	0.327	0.0343
1.27	0.0326	0.354	0.0299
0.95	0.0248	0.334	0.0211
0.63	0.0173	0.337	0.0141
		$\bar{k} = 0.331$	
		$\pm \sigma = 0.005$	

A plot of the corrected absorbance values versus concentration of iron appears in Figure 4. This figure shows that, over the concentration range covered and within the experimental error, viscosity is the greatest contributing factor causing the curvature in the plot of absorbance versus concentration for the dilution method.

Erroneous results may be obtained in atomic absorption spectroscopy if the particles in the flame scatter the incident radiation. The detector will sense the decreased intensity of the

incident beam and record this as absorbance. In this study the most concentrated solutions of each method were analyzed at the non-resonance 2788 Å iron line to check for scattering. This transition from the a^5F_5 (0.859 electron volts) state to the $y^5G_6^\circ$ (5.304 electron volts) level was chosen because a standard 5 p.p.m. iron solution gave no absorption and also due to its relative close proximity to the 2483 Å line. The only change in instrumental parameters was a change in the photomultiplier dynode voltage from 595 volts to 645 volts. The results of this study indicated that there was no detectable scattering.

CONCLUSIONS

Summary

It has been shown that there is a significant difference between three of the methods used to detect serum iron by atomic absorption spectroscopy. All three methods use 2 ml. or less of serum and, within experimental error, all have the same sensitivities but different detection limits.

Winefordner and Vickers (34) have derived an expression relating the minimum detectable concentration to the experimental conditions. Using this relationship the detection limits for the dilution, precipitation and wet-oxidation methods are 0.01, 0.05 and 0.21 p.p.m. respectively.

When a large number of samples are to be analyzed, the time needed per analysis is important. An analysis can be effected in less than 5 minutes by the dilution method. The wet-oxidation method requires 25 minutes while approximately 75 minutes are needed for the precipitation method.

Dilution and wet-oxidation procedures determine the total iron content in the sample. Therefore, it is important to obtain an effective separation of the serum without hemolysis occurring. As indicated by the iron found in the precipitates, the precipitation method did not determine the total iron present. Goodwin and Murphy (35) have used a precipitation technique to determine non-

hemoglobin-bound urinary iron. It is conceivable that hemoglobin iron was present in our serum samples and consequently was responsible for the iron found in the precipitates. Further work should be done to determine the exact nature of the precipitated iron, e.g. hemolyzed, adsorbed, occluded or iron arising from some other source.

The dependence of absorbance on viscosity has only been applied to one system and over a limited range of viscosities. These studies should be extended to other systems and also the limitations of this dependence should be determined.

Recommendations

As a result of the expanded scale readout system, it is felt that the hollow cathode lamp power supply should be modified for better current regulation. Also a phase-sensitive amplifier would better serve to eliminate transient D.C. signals which are amplified through the broad band pass filter system.

As indicated in Figure 2, the optical path traverses the flame in different regions. The multiple pass optical system would be more effective if it could be confined to a smaller region of the flame or, ideally, have the optical path traverse the flame in the same region.

Other techniques incorporated into the iron analysis procedure should also be investigated. In this respect a number of advantages might be expected by extraction of the iron into an

organic solvent. This should have the effect of concentrating the iron and also enhancing the signal. Signal enhancement by organic solvents has become a widely recognized technique for enhancing flame emission and absorption signals.

Another technique which would seem to offer certain advantages is the electrodeposition of iron at a mercury drop electrode. Subsequent recovery of the iron could be effected by anodic dissolution or acid dissolution of the entire drop depending on the effects of a high concentration of mercury. This method in addition to concentrating the iron could also serve to remove the iron from certain interfering species contained in the original sample.

As stated in the procedure, bovine serum was used in this study. Before any definite conclusions are drawn between this study and iron analysis in human serum, samples of human serum need to be analyzed.

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