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Notes on the Thiocyanate Iron Reaction.

A Modified Procedure for the Quantitative Determination of Iron in Biological Materials.

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SINCE the time of Ossian (1837) cited by Woods and Mellon (1941) the thiocyanate iron method has been tackled by many investigators. However, a review of the literature reveals a most variable, and in some cases contradictory mass of information on the use of the thiocyanate reagent in the determination of iron.

The thiocyanate method is based on the fact that ferric iron and an alkali thiocyanate give a red colour in an acid solution. At present there are still conflicting hypotheses as to the nature of the coloured complex. Schlesinger and Van Valkenburgh (1931) found that an anion $Fe(CNS)_6^{\equiv}$ is responsible for the colour. This concept could in no way be substantiated by Bent and French (1941) who did find definite proof for the colour to be due to a cation $FeGNS^{+\,+}$.

The purpose of the work described in this paper is to throw more light on: (i) The influence of various ions, especially calcium, phosphorus, and copper on the red complex. (ii) The acid best suited, its optimum concentration, and whether absolute control of concentration is necessary. (iii) The influence of temperature on colour intensity. (iv) The effect of the thiocyanate present, and (v) an expedient in the form of an oxidant to obviate colour fading.

HYDROGEN PEROXIDE AS A MEANS OF ARRESTING THE REDUCTION OF FERRIC TO FERROUS IRON.

One of the main objections to the thiocyanate-iron colour system, is the fact that the colour fades in aqueous solution. This fading of the colour is due to the reduction of ferric to ferrous iron by the thiocyanate; a fact which can be easily established by heating the aqueous solution of the coloured salt, thereby accelerating the reduction process, until the colour disappears. If an oxidizing agent is added the colour reappears.

Hydrogen peroxide may be used to excellent advantage as an oxidizing agent to ensure that the iron remains as the ferric ion. Peters *et al* (1939) report that hydrogen peroxide develops a yellow colour with thiocyanate.

The lowest concentration of hydrogen peroxide at which a yellow colour has been noted by the author is 0·12 per cent. Satisfactory results were obtained with a concentration of 0·004 per cent. H_2O_2 (see table 3).

The Acid best suited and its Optimum Concentration.

The test is carried out by some workers in hydrochloric acid solution [Elvehjem (1930), Farrar (1935)]; by others in sulphuric acid solution [Leeper (1930), Scott cited by Daniel and Harper (1934)], and in nitric acid solution [Winter (1931), Woods and Mellon (1941)].

During the present investigation it was found immaterial whether hydrochloric acid or sulphuric acid is used. The effect of nitric acid is variable. By making use of this acid errors of up to a few hundred per cent., depending upon the amount of acid used and other circumstances such as time of contact and temperature, may be made.

Walker (1925) found that ordinary nitric acid gave a red colour with thiocyanate. He further pointed out that the red colour was produced not by the nitric but by the nitrous acid it contained. This statement needs some elucidation. It is known that nitrous acid is the great offender, but although nitric acid does not produce an instantaneous colour with thiocyanate, it has been found that the oxidation-reduction reaction between nitric acid and thiocyanate (potassium thiocyanate was employed) might be " catalyzed " under certain conditions with the liberation of nitrogen dioxide which is also extracted by the amyl alcohol. If the concentration of the nitric acid is kept low, 0·25 N, no colour is obtained, but in such a case the concentration of the acid is too low to act as an efficient oxidizing agent for maintaining the iron in the ferric state.

As is evident from Tables 1 and 2, a relatively wide range of acid concentration is permissible. However, as will be pointed out later on, high concentrations of acid and thiocyanate are essential for efficient colour development in phosphate rich solutions. A concentration of 0·45 N hydrochloric or sulphuric acid, and of 0·4 N potassium thiocyanate in the presence of 0·0025 N hydrogen peroxide is optimal for ferric thiocyanate colour development.

For many wet-ashed samples it has been found immaterial whether the aliquot was first neutralized with strong ammonium hydroxide, and then reacidified, or, whether the colour was developed directly in the sulphuric acid medium. Table 2 shows that a concentration of ammonium sulphate equivalent to 0·6 ml. of concentrated sulphuric acid can be tolerated in a solution free from other interfering substances. However, the total mass action of a high concentration of other substances especially phosphates already present in the testing solution together with the additional ammonium sulphate will cause a greater reduction of the ferric-thiocyanate colour potential. It is thus advisable to develop the colour directly in the solution as prepared from wet ashing.

The Effect of the Thiocyanate Present.

According to the law of mass action it is logical to expect a higher concentration of thiocyanate to be accompanied by a corresponding intensification of the colour (see discussion).

In studying the relationship between colour intensity and thiocyanate concentration Peters and French (1941) came to the conclusion that the colour intensity increases linearly as the CNS/Fe ratio increases. The effect of increasing thiocyanate concentration on colour intensity is clearly presented in Table 1. The linear relationship between colour and CNS/Fe ratio referred to above, could however, not be confirmed. Due to this effect of the thiocyanate it is of the utmost importance to have its concentration the same for both standard and unknown.

As far as orthophosphates are concerned the effect of the thiocyanate present can clearly be seen from the following reaction.

$$FePO_4 + 3\ CNS^- \rightleftarrows Fe(CNS)_3 + PO_4^{\equiv}$$

This is the reason for advocating a relatively high concentration of thiocyanate for phosphate rich solutions in order to shift the equilibrium for the above reaction as much as possible to the right.

Apparatus.

A photo-electric colorimeter (the Leitz type was used in this work) pyrex ignition tubes, 8 × 1 in., and medium sized Kjeldahl flasks for greater samples.

Reagents.

(1) Standard iron solutions. Stock solution.

Any of the following procedures may be followed:—

(*a*) Dissolve 0·5000 gram of " analytical " iron wire in 20 per cent. sulphuric acid to which 3 ml. concentrated nitric acid have been added. Carefully evaporate until copious white fumes appear, cool, and transfer quantitatively to a 1 litre volumetric flask and dilute to volume 1 ml. = 0.1 mg. of iron.

(*b*) Dissolve 0.7022 gram ferrous ammonium sulphate [$FeSO_4\ (NH_4)_2\ SO_4\ 6H_2O$] in 100 ml. doubly distilled water. Add 5 ml. concentrated sulphuric acid. Warm slightly and titrate with 0·1 N $KMnO_4$ solution until just pink. Dilute to 1 litre.

1 ml. = 0·1 mg. of iron.

This latter stock solution was made use of.

Working Standard.—Dilute 100 ml. of the stock solution to 1,000 ml; adding 10 ml. of a 0·1 per cent. hydrogen peroxide solution or its equivalent, prior to adjusting to volume.

1 ml. = 0·01 mg. of ferric iron.

(2) A 50 per cent. potassium thiocyanate solution (50 gram per 100 ml. of solution).

(3) Reagent grade hydrochloric and sulphuric acids, concentrated.
Nitric acid, concentrated, redistilled.
Perchloric acid, 60 per cent, redistilled.

(4) Hydrogen peroxide; 0·1 per cent. solution in doubly distilled water, kept in a brown bottle, in a dark place, preferably in a refrigerator.

(5) Iso-amyl alcohol. Recovered amyl alcohol from residues is not satisfactory.

Preparation of Sample.

It is a familiar fact that dry ignition of biological materials for iron analysis yields erroneous results if special care is not taken. The wet combustion method has proved not only to be safe in the hands of an experienced worker, but advantageous in many respects. All pyro- or metaphosphates are, for instance, hydrolysed to the ortho form, thus doing away with a special hydrolysis during the sample preparation.

The Combustion Procedure.—1 ml. of blood (or ·5 to 2 grams of tissue depending on the suspected iron content) is measured into a 8 × 1 in. pyrex tube. A glass bead, 2 ml. concentrated sulphuric acid, 3 ml. 60 per cent. perchloric acid, and a few drops of concentrated nitric acid (0·3 to 0·5 ml.) are added, and the material digested over a micro flame in a fume cupboard. When the initial, sometimes vigorous, oxidation reaction has subsided, nitric acid is added drop-wise until the oxidation is completed and the solution clarified. The final solution should be colourless or, at most, a greenish-yellow colour. Products of high fat content do not lend themselves so readily to complete oxidation. If the HNO_3 is unable to clarify the solution, the contents are allowed to cool slightly, a further 0·5 to 1 ml. $HClO_4$ added, and the heating continued. A few more drops of HNO_3 may be necessary to clarify the solution. The heating is continued until the appearance of characteristic dense white fumes of sulphur trioxide.

At this stage the solution may be expected to be free from perchloric and nitric acids. However, decomposition products of these acids are sometimes tenaciously retained by the concentrated sulphuric acid (e.g. nitrosylsulphuric acid), even after prolonged boiling. Hydrolysis with about 2 ml. of water, and subsequent boiling to white fumes effect the removal of these products. During the present investigation it was found sufficient to boil the diluted contents just for about one minute. When no evolution of gas takes place upon dilution boiling is unnecessary. Roberts *et al* (1940) are of the opinion that in the case of milk nitrosylsulphuric acid cannot be decomposed by the usual hydrolysis procedure. Refuge has to be taken to 30 per cent. hydrogen peroxide, a dropwise addition of 1 ml. to the warm sulphuric acid solution, completely decomposing the nitrosylsulphuric acid. The digest is allowed to cool, a further 10 ml. redistilled water are added, and quantitatively transferred to a 100 ml. volumetric flask.

Cool to room temperature and make up to volume.

Procedure of Determination.

After a test experiment has been conducted to determine the approximate iron content of the sample, transfer the chosen aliquots to pyrex tubes similar to those used for combustion, marked at 25 ml. Adjust the acid concentration if necessary, add 1 ml. of the 0·1 per cent. hydrogen peroxide solution and fill up to the 25 ml. mark with redistilled water. Add exactly 10 ml. isoamyl alcohol to be followed by 2 ml. of the 50 per cent. potassium thiocyanate solution. Close the tube with a stopper, previously extracted with 10 per cent. hydrochloric acid and shake for 30 seconds. Transfer the amyl alcohol phase to centrifuge tubes, and centrifuge for 5 minutes at 3,500 r.p.m. to get rid of water particles suspended in the alcohol extract. Obtain readings with filter 430, setting a reagent blank at 100 per cent transmission. The iron values are obtained from a standard curve. No unnecessary time should be allowed to elapse between the addition of the thiocyanate and the extraction of the red complex from the aqueous solution.

TABLE 1.

The effect of different concentrations of hydrochloric acid and potassium thiocyanate on the Ferric Thiocyanate complex.

Concentrated HCl.	50 % KCNS.	Transmission.	Concentrated HCl.	50 % KCNS.	Transmission.
Ml.	Ml.	Per Cent.	Ml.	Ml.	Per Cent.
0·2	0·5	54·5	0·5	6·0	44·8
0·2	1·0	49·0	1·0	0·5	59·0
0·2	2·0	48·0	1·0	1·0	49·5
0·2	3·0	47·5	1·0	2·0	48·0
0·2	4·0	47·0	1·0	3·0	45·0
0·2	6·0	47·5	1·0	4·0	44·8
0·5	0·5	57·5	2·0	0·5	60·5
0·5	1·0	49·0	2·0	1·0	50·0
0·5	2·0	48·0	2·0	2·0	48·6
0·5	3·0	45·0	3·0	2·0	51·5
0·5	4·0	45·0	4·0	2·0	54·0

TABLE 2.

The effect of sulphuric acid or a sulphate on the red complex.

Tube No.	$(NH_4)_2SO_4$* Grams.	HCl Concentrated Ml.	Transmission, Per Cent.	Tube No.	H_2SO_4 Concentrated Ml.	Transmission, Per Cent.
1	0·486	1·0	48·2	8	0·2	48·0
2	0·729	1·0	48·0	9	0·3	48·3
3	1·215	1·0	48·3	10	0·5	48·0
4	1·458	1·0	48·0	11	0·6	49·0
5	1·944	1·0	49·0	12	0·8	49·5
6	2·430	1·0	49·0	13	1·0	50·0
7	3·645	1·0	50·0	14	1·5	50·0

* The amount of ammonium sulphate introduced is the equivalent of the sulphuric acid in the second last column.

TABLE 3.

The stabilizing influence of hydrogen peroxide on the ferric thiocyanate complex.

Time Elapsed before Shaken Out of the Aqueous Phase. (Min.)	Transmission.	
	No Hydrogen Peroxide Added.	Hydrogen Peroxide Added. (1 ml. of 0·1 % solution.)
	Per Cent.	Per Cent.
0	48·0	48·0
5	48·2	48·2
10	48·0	48·0
15	48·3	48·2
25	48·5	48·3
35	49·0	48·2
45	50·5	48·0
65	53·5	48·0

The apparent stability of the colour for the first 15 tot 20 minutes in the tubes without added peroxide, is accounted for by a relatively strong oxidizing agent present in Mercks extra pure iso-amyl alcohol. This has been verified, and constitutes an added convenience of considerable value. However, not the least coloration is obtained between this alcohol and thiocyanate even after standing for a few days. When amyl alcohol free from any oxidizing agent, for instance, those of the British Drug Houses (B.D.H.) was used, a marked reduction in colour intensity took place within the first 5 minutes, and proceeded more or less linearly with the time at a constant temperature. The reduction potential is a function of both the time and the temperature.

The Effect of Temperature on Colour Intensity.

A point of interest to note here is that the colour of the thiocyanate complex is a little more intense at a temperature of 15° C. and lower, than at higher temperatures; so much so that a difference of 1 per cent. to 3 per cent. transmission might be obtained. This phenomenon has probably nothing to do with a possible contraction of the amyl alcohol, and hence a corresponding intensification of the colour. Although the temperature of the alcohol was allowed to rise after the extraction of the colour, the transmission still remained the same. A further investigation into the secrets and properties of the coloured complex may perhaps yield an answer for this behaviour.

Table 4.

Tube No.	Temperature of Test Solution.	Transmission.	Tube No.	Temperature of Test Solution.	Transmission.
	°C.	Per Cent.		°C.	Per Cent.
1.....	30	48·0	5.....	20	48·0
2.....	28	48·1	6.....	18	48·0
3.....	26	48·0	7.....	15	47·0
4.....	24	48·2	8.....	5	45·2

The red complex seemed to be fairly stable in the amyl alcohol. In no cases were increases in transmissions noted of samples read within 30 minutes after extraction of the colour provided the temperature was not higher than 27° C. On a few occasions not the slightest difference in transmission was obtained even at the end of 24 hours. However, it is absolutely essential not to make use of recovered alcohol that has been used before. Iso-amyl alcohol recovered from residues is liable to errors varying from 10 to 20 per cent. within the first 5 minutes after extraction.

Phosphate Interference.

Various statements occur in the literature as to the interference caused by phosphates in the formation of the colour of ferric thiocyanate. It is evident from Table 5 that the effect of orthophosphates has been exaggerated. Results concordant with the control were obtained with aliquots containing up to the equivalent of 87 mg. of phosphorus. Leeper (*loc. cit.*) on the other hand gave 100 mg. P_2O_5 (i.e. 43·7 mg.P) as the safe limit. Walker (*loc. cit.*) quoted 50 mg. P_2O_5 (i.e. 21·8 mg. P) as the limit. A lower tolerance for

phosphate can without exception be accounted for by a lower concentration of acid and thiocyanate. It is alleged by Winter (*loc. cit.*) that with a proper acidity and a sufficient excess of thiocyanate no difficulty is encountered from phosphates.

TABLE 5.

Effect of orthophosphate on the recovery of iron.

Sample No.	Iron Present.	Phosphorus Added.*	Per Cent. Iron Recovered.
	Mg.	Mg.	
1	0·01	17·4	100·0
2	·01	52·5	100·0
3	·01	87·0	100·0
4	·025	17·4	100·0
5	·025	52·2	99·2
6	·025	87·0	92·3
7	0·03	17·4	99·5
8	·03	52·2	96·0
9	·03	87·0	90·5

* The phosphorus was added as $Na_2HPO_42H_2O$ with an iron content of 0·1 mg. per cent.

It is quite clear that the lower the concentration of iron the greater the tolerance for phosphate. Farrar (*loc. cit.*) presented results to indicate that " concentrations of orthophosphate of 70 mg. per cent. or greater cause fading of the ferric thiocyanate color with amyl alcohol, however, permits easy colorimetric estimation of 0·01 mg. of iron ". Thus it is always advisable especially where a high concentration of phosphate is suspected to carry out the determination with an aliquot rather low in iron content.

Both pyro- and metaphosphates prevent the reaction between thiocyanate and iron. [Woods and Mellon (loc. cit.)]. They are, however, quantitatively hydrolysed and changed to orthophosphates by the strong acids during wet ashing. This can be seen from the data of Table 6 which are very similar to those of Table 5.

TABLE 6.

Recovery of iron from pyrophosphate solutions treated with the wet ash procedure.

Sample No.	Iron Present.	Phosphorus Added.*	Per Cent. Iron Recovered.
	Mg.	Mg.	
1	·01	13·9	100·0
2	·01	41·6	99·8
3	·01	69·4	100·0
4	·025	41·6	99·5
5	·025	69·4	94·5
6	·03	41·6	96·6

* Phosphorus was added as $Na_4P_2O_7.10H_2O$.

Calcium has been reported by Daniel and Harper (1934) to interfere with the quantitative recovery of iron if present in excess of 10 mg. per 100 ml. of an hydrochloric acid solution. Recovery data presented in Table 7 indicate that calcium interference is negligible when the proposed method is applied.

TABLE 7.

*The joint effect of calcium and phosphorus or calcium alone on the recovery of Iron.**

Sample.	Kind of Acid Used.	Calcium† Added.	Phosphorus‡ Added.	Per Cent Iron Recovered.
		Mg.	Mg.	
1	HCl	20	None	100·0
2	HCl	40	None	99·8
3	HCl	200	None	100·3
4	HCl	400	None	100·5
5	HCl	20	17·4	99·8
6	HCl	20	52·2	98·8
7	H_2SO_4	20	17·4	99·8
8	H_2SO_4	20	52·2	98·5
9	H_2SO_4	80	None	99·9
10	H_2SO_4	200	None	100·2

* 0·025 mg. of iron added to all solutions.
† Calcium added as $CaCO_3$.
‡ Phosphorus added as $Na_2HPO_4.2H_2O$.

With samples of high calcium content the possibility does exist that iron may be occluded by the fine precipitate of $CaSO_4$. No marked occlusion occurred for the few cases studied in Table 7. A well-defined precipitate of $CaSO_4$ has not once been found for the tissues analysed in Table 9, except for bone in which case it is advisable to conduct a dry ignition with subsequent hydrolysis in hydrochloric acid.

One would expect the thiocyanate method to have its limitations in the presence of many other substances. Thus, according to Walker (loc. cit.) no or unsatisfactory colour development is obtained in the presence of salts of silver, mercury, cobalt or large amounts of copper. Salts of these elements occur normally in such low concentrations in biological materials that the determination of their influence on the recovery of iron was deemed unnecessary. However, in cases of enzootic icterus, a jaundiced condition of the liver, exceptionally large amounts of copper accumulates in the bodily organs especially the liver. Values varying between 60 mg. per cent. and 600 mg. per cent. for dry sheep liver have been found at this Institute. In view of work that is in progress in this connection it was considered necessary to obtain recovery data on iron in the presence of various concentrations of copper and on iron added to these organs.

The apparently higher recoveries for iron is accounted for by the fact that copper reacts with thiocyanate giving a yellowish colour which is also extracted by the amyl alcohol. The choice of a suitable aliquot for the iron determination is thus governed by the copper content of the sample. In cases of enzootic icterus studied, copper has been found to constitute no trouble in

the determination of iron, especially as the iron content of the organs was found to exhibit the tendency to be much higher than under normal circumstances.

TABLE 8.

Copper interference on Iron Recovery.

Tube No.	Iron Present.	Copper Added.*	Transmission.	Per Cent. Iron Recovered.
	Mg.	Mg.	Mg.	
1	·025	0·0	48·0	100·0
2	·025	·005	48·0	100·0
3	·025	·01	48·1	99·8
4	·025	·02	48·1	99·8
5	·025	·03	48·0	100·0
6	·025	·04	47·5	101·0
7	·025	·05	47·0	102·0
8	·025	·10	46·0	104·0

* Copper added from an "electrolytic" copper standard solution.

TABLE 9.

Analyses of Healthy Animal Tissues (Bovine).

Tissue.	Copper Content.	Iron Content.	Iron Added.	Total Iron Calculated.	Total Iron Found.
	P.p.m.	P.p.m.	P.p.m.	P.p.m.	P.p.m.
Blood	1·2	510	100	610	594
Brain	7·5	233	50	283	278
Heart	16·0	378	50	428	420
Liver	90·0	555	100	655	654
Liver	280·0	354	100	454	458
Kidney	12·5	482	100	582	560
Spleen	6·8	11,500	200	11,700	12,000
Liver*	2100·0	3,300	100	3,400	3,300
Kidney*	230·0	230	150	380	372
Adrenal	11·7	315	50	365	366
Muscle	3·5	210	150	360	365
Thyroid	1·8	120	200	320	315
Bone marrow	0·85	23	100	123	120

* Sheep organs. A case of enzootic icterus.

DISCUSSION.

Except for a few modifications the procedure of digestion given in this paper is essentially the same as that given by Eden and Green (1940). Better results have repeatedly been obtained with more sulphuric acid than with the one ml. advocated by these authors. Furthermore the addition of a few drops of concentrated nitric acid prior to starting combusion is a decided advantage and very effective in shortening the time of digestion. The addition of 2 ml. sulphuric acid and 3 ml. perchloric acid, advocated to start

oxidation can be varied according to circumstances, depending on the final acid requirement or the bulk of the sample. The introduction of too much perchloric acid, however, constitutes a fair amount of danger and care should be taken not to exceed the ratio of $3HClO_4 : 1H_2SO_4$.

The use of an organic solvent such as iso-amyl alcohol is a satisfactory means of extracting the coloured complex, the colour intensity of which is dependent upon the degree of dissociation (K), from a solution with a relatively high di-electric constant.

If we consider the reaction :—

$$Fe_m\ (CNS)_n \rightleftarrows m\ Fe^{+++} + n\ CNS^{-}$$

where m and n are the charges on the complex, then the equilibrium reaction will be given by :—

$$K = \frac{(Fe^{+++})^m\ (CNS^{-})^n}{Fe_m\ (CNS)_n}$$

It is clear that the concentration of undissociated $Fe_m\ (CNS)_n$ molecules, and thus the colour intensity of the solution is inversely proportional to the value of K.

The proposed method has proved entirely satisfactory when applied to a wide variety of animal tissues (Table 9). For aliquots containing up to 25 micrograms of iron (30 micrograms are the limit for a suitable reading) recoveries of better than 0·5 microgram are possible. The greatest loss of iron usually occurs during the digestion procedure and great care should be exercised to avoid spluttering of the contents.

Summary and Conclusions.

1. A modified procedure of the thiocyanate method for the micro-determination of iron in biological materials is described.

2. Evidence is presented to show its efficiency in the presence of moderate quantities of copper and relatively large concentrations of phosphate and calcium.

3. Hydrochloric or sulphuric acid is preferable to nitric acid, which should rather be avoided.

4. For colour stability it is essential to avoid recovered iso-amyl alcohol previously used for either iron or copper determinations.

5. A detailed description of a wet combustion procedure is given.

6. Some data on the iron and copper contents of tissues are given.

7. It can be concluded that the proposed method not only satisfies the need for a time-saving and simple procedure, but also conforms to the requirements for accuracy and reliability, if the necessary precautionary measures are taken.

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