VIII

ЕВРОПЕЙСКИЙ КОНГРЕСС РАБОТНИКОВ НИИ МЯСНОЙ ПРОМЫШЛЕННОСТИ

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STUDIES ON THE MECHANISM OF FORMATION OF CURED MEATS GREEN PIGMENTS

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We have established in earlier works (1-4) that cured meats green pigments formation results from excess accumulation (500 γ% and more) of hydroxylamine in brines; hydroxylamine is formed basically during denitrification both in nitrate and nitrite cure. Denitrification occurs in weak-acid medium, which is usually observed in meats being cured, and in brines (5,6). Weak-acid medium, in its turn, promotes manifestation of reducing properties of hydroxylamine which reduces the ferric ions to ferrous ions. It is known, that quantitative ferric ions reduction by hydroxylamine occurs at pH from 3 to 6 (7), meanwhile such pH values favour the processes of meat myoglobin oxidation to metmyoglobin both prior to and during meat curing. Prior to curing, meat myoglobin is oxidized by atmospheric oxygen (8-10), during curing - by atmospheric oxygen solved in brines, and by free nitrates (11) and nitrites in brines (12,13); myoglobin oxidation by atmospheric oxygen is catalyzed by sodium chloride (8).

Literary data mentioned above permit to suggest that hydroxylamine accumulated in brines (4,14) and in meats being cured (15), takes part in reduction of metmyoglobin ferric ions to ferrous ones, necessary for nitrosomyoglobin formation

(16); the concentration of nitrosomyoglobin was found out by us to be in direct relation to myoglobin iron content in muscle tissue (17,18).

But along with a positive effect of hydroxylamine reducing properties in curing conditions, the same properties may have a negative effect on cured meats colour in the presence of atmospheric oxygen, nitrates and nitrites solved in brine.

The present study is aimed at the finding out of the effect of atmospheric oxygen solved in brines on the green pigment formation in the presence of excess hydroxylamine. In this connection we tried to find out ferric and ferrous ion contents (in %) in green, brown and grey hemoglobin solutions.

Instead of myoglobin there was used crystalline hemoglobin which simplified and facilitated the solution of our task greatly. This substitution is quite possible as oxidation-reduction properties of iron ions of heme pigments of blood and muscle tissue are similar (19). To obtain crystalline hemoglobin, we used Kanig's method (20). Hemoglobin so obtained, is dried as in the case of myoglobin solution, and brought to constant weight by the method that was described by us earlier (17,18).

To determine percentage contents of ferric and ferrous ions when they are both present in green, brown and grey hemoglobin solutions, a photocolorimetric method has been worked out, as the basis of which served Wong's (21) rhodanide method for determination of total iron content in blood. The advantage of this method over other colorimetric methods for blood

iron determination consists in the fact that in this method potassium rhodanate is used - an extremely sensitive reagent permitting to get coloured solutions in strong-acid mediums. This was successfully used by Wong for quantitative splitting of iron from hemoglobin and it facilitated our task of working out a method for determination of ferric and ferrous ions in hemoglobin solutions. Despite the above-mentioned advantage, Wong's method, however, has two essential shortcomings. First, addition of potassium persulfate saturated solution to oxidize ferrous ions causes fast formation of yellow substances which artificially increase extinction of iron rhodanide (7). Second, sodium tungstate is used as proteins precipitator; it is known to reduce ferric ions in acid medium thus decreasing extinction value of iron rhodanide. Besides, Wong's method yields to photocolorimetric one in accuracy due to its being based on visual determination of extinction. Below is described a photocolorimetric method, worked out by us and free of shortcomings, for ferric and ferrous ions determination in their joint presence in hemoglobin solutions.

Necessary reagents

Ammonium rhodanide solution (3 M). 23 gr of NH_4CNS (p.a.) are transferred to a 100 ml measuring flask and solved in 50-60 ml of bidistilled water and then 4 ml of acetone are added there. The obtained solution is permitted to stand until it reaches room temperature, after which the same water is added up to the volume. Then the solution is filtered through a paper filter into a dark ground stoppered

flask.

Potassium persulfate solution (1%). 1 gr of $K_2S_2O_8$ (p.a.) is solved in bidistilled water in a 100 ml measuring flask and then is filtered through a paper filter. If during storage there appear white flakes, it is necessary to prepare a fresh solution.

Concentrated sulfuric acid (sp. gr. = 1.835), p.a.

Sulfuric acid solution (9 N).

Sulfuric acid solution (10%).

Acetone, p.a., colourless.

Trichloro-acetic acid solution (20%).

Water for dilution and for other purposes must be bidistilled, by using glass vessels and Liebig's cooler. If iron traces are found, the bidistilled water are to be redistillated.

All the vessels and flasks used must be thoroughly treated with chromic acid mixture, flashed with tap water and then with bidistilled water twice or thrice.

Determination of ferrous ions concentration

Standard solution preparation

Solution I. 0.7 gr of recrystallized Mohr's salt /$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$/ are placed into a 1000 ml measuring flask and solved in 200-250 ml of boiled bidistilled water, acidified by 10 ml of 10% H_2SO_4, and then boiled bidistilled water is added up to the volume. 1 ml of such solution contains 100 γ of iron.

Solution II. 100 ml of Solution I are transferred into a 1000 ml flask containing 10 ml of 10% H_2SO_4; then boiled

bidistilled water is added up to the volume. 1 ml of such solution contains 10 γ of iron.

Solution III. 50 ml of Solution II are transferred into a 500 ml measuring flask with 5 ml of 10% H_2SO_4 in it. Then boiled bidistilled water is added up to the volume. 1 ml of such solution contains 1 γ of iron.

Solution IV. 25 ml of Solution III are transferred into a 250 ml measuring flask containing 2.5 ml of 10% H_2SO_4, and then boiled bidistilled water is added up to the volume. 1 ml of such solution contains 0.1 γ of iron.

Calibration curve plotting

To establish the starting point of the calibration curve, 1 ml of Solution IV is transferred into a 25 ml measuring flask and then in sequence 0.5 ml of bidistilled water, 5 ml of 9N H_2SO_4 and 2.5 ml of 1% $K_2S_2O_8$ are added. Then the flask is agitated, permitted to stand for 2-3 minutes, after which 1 ml of trichloroacetic acid and 12.5 ml of acetone are added; the mixture is cooled by running water and then 2.5 ml of ammonium rhodanide solution are added. The flask being stoppered, the solution is mixed and with a photocolorimeter its colour intensity is determined. 25 ml of this solution contain 0.1 γ of iron.

To find the next point on the curve 1 ml of Solution III is transferred to the 25 ml flask, and all the above-described sequential operations are repeated. 25 ml of such solution contain 1 γ of iron. Then to the 25 ml measuring flask 0.5 ml of Solution II are transferred and again all the sequential operations are repeated, except that 1 ml of

bidistilled water is added. 25 ml of such solution contain 5 γ of iron.

To find the next point on the calibration curve, to a 25 ml measuring flask 1 ml of Solution II is transferred and the sequential operations are repeated. 25 ml of such solution contain 10 γ of iron.

Above mentioned concentrations of H_2SO_4 in standard solutions of ferrous salts at room temperatures practically inhibit the oxidation of ferrous iron by atmospheric oxygen (7).

On the basis of the data concerning determination of the colour of solutions with known ferrous iron content, a calibration curve (see fig.) is plotted.

As is seen from the graph, linear relation between staining intensity and ferrous iron content is observed with the concentration of the latter within 0.1-10.0 γ in 25 ml of colorimetered solution. With higher iron concentration, solution colour is not subordinated to Lambert-Beer's law.

Determination of ferric ions concentration

For this purpose there were used the reagents of the same concentrations and volumes as when determining ferrous ions concentration, except that potassium persulfate - an oxidizer of ferrous ions - was not added.

Preliminary experiments with standard solution of ferric ammonium sulfates /$NH_4Fe(SO_4)_2 \cdot 12H_2O$/ revealed complete fitness of the worked out method for determination of both ferrous and ferric ions concentrations. Linear relations between the intensity of developing colour and ferric ions con-

tent in the solution are identical to those obtained for ferrous iron solutions, and they are observed, too, with iron ions concentrations in the range of 0.1-10 γ in 25 ml of the colorimetered solution.

In the case when both ferrous and ferric ions are present in the solution, it is necessary to determine ferric ions concentration first without adding $K_2S_2O_8$ and then to take another sample, to add there potassium persulfate and to determine a summary ferrous and ferric ions content in it. After that the concentration of ferrous ions is counted by subtracting the amount of ferric ions out of the total iron content.

The presence of $25 \cdot 10^{-6}$% NH_2OH in the medium analyzed does not prevent one from quantitative iron determination.

Determination of iron ions concentration in green, grey and brown hemoglobin solutions

2.5 ml of the solution under examination are transferred into a 30 ml centrifugal glass under a vaseline oil layer; then 2.5 ml of conc. H_2SO_4 are added, thoroughly mixed with a glass stick, and the solution is allowed to stand for 3-4 min. Then, under the vaseline oil layer 3 ml of oxygen-free bidist. water are added, followed by 2 ml of CCl_3COOH; all is mixed for 2-3 min. and allowed to stand for 3-4 min. The solution is cooled by tap water, then centrifuged at 5000 r.p.m. for 10 min.

Since the solution is heated during the addition of sulfuric acid which flavours the intensification of oxidizing ferrous ions by atmospheric oxygen (7), the solution is isolated from environment by means of no less than a 2 cm va-

seline oil layer to prevent the oxidation.

With lower concentrations of ferrous and ferric ions, the solution under analysis is not diluted. However, before sampling air is removed from the solution by energetic blasting of CO_2 for 10-15 minutes. Then, simultaneously with the cessation of CO_2 feed the solution surface is spread with about a 4 cm layer of vaseline oil. To prevent atmospheric oxygen from solving in the sample, first of all 2-2.5 ml of oil are pipetted into the solution, the sample is taken by slow suction.

With high ferrous and ferric ions concentrations, the solution, being analyzed, is pre-diluted by ten times by means of boiled bidistilled water and then, before sampling, the air is removed as described above. Sampling is done by the previously described method.

To determine ferric ions concentration, 5 ml of the supernatant are transferred into a 25 ml measuring flask, which is placed in melting ice to prevent the solution from heating during dilution. 8-10 minutes later, 5 ml of bidistilled water and 12.5 ml of acetone are added. The solution is mixed by agitating the flask and then is cooled for 7-8 minutes. After taking the flask out of the ice, 2.5 ml of rhodanide are added into the solution which is then immediately colorimetered. The light filter is green.

To find a summary ferric and ferrous ions content, they transfer 5 ml of supernatant into a 25 ml measuring flask and add there 2.5 ml of bidistilled water and 12.5 ml of acetone; the contents of the flask is agigated. After adding

2.5 ml of potassium persulfate the solution is reagitated and is permitted to stand. In 2-3 minutes 2.5 ml of rhodanide are added and the solution is immediately colorimetered.

The photocolorimeter is regulated in parallel with the basic experiments by means of the solutions prepared as described above, with the only exception that instead of ammonium rhodanide, the same amount of bidistilled water is added to them.

To solve our task, four types of hemoglobin solutions are prepared.

To prepare solution of type I, 0.15 gr of dry hemoglobin was placed into a 50 ml measuring flask, then 15-20 ml of bidistilled water and 10 ml of citrate-phosphate buffer solution with pH 5.2* were added sequentially. The flask contents was brought up to the volume with the same water. This solution served as control.

Solution of type II was prepared much in the same way as the previous one, but besides, 5 ml of 0.005% hydroxylamine were added.

Solution of type III was prepared like the previous one, but besides, 2.5 gr of 5% sodium chloride solution were added. This provided the sample with a limiting salt content usually occurring in cured meats (22). After agitating, each

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*) We stopped at pH 5.2 because, as our preliminary experiments with dilute defibrinated blood showed, in the presence of excess hydroxylamine there occurs the most intensive formation of heme pigments green colour. Green pigments formation occurs also at pH 5.8. At pH 4 heme pigments are stained brown and at pH 6.8 - dark-red. Citrate-phosphate buffer solutions were used in our experiments.

solution was transferred into a deep Petri dish and was permitted to stand at 15-17° without a cover.

Solution of type IV was prepared like the second one, but after bringing it up to the mark and mixing solved atmospheric oxygen was removed by CO_2. Then the solution surface was covered with vaseline oil and was permitted to stand at the same temperature as all the rest ones. Ferrous and ferric ions were quantitatively determined on the day when the solution in one of Petri dishes stained green.

The results of determinations are given in Table I.

Table I. Ferrous and ferric ions concentrations in green, brown and grey solutions of hemoglobin on the 7th day of keeping

Indices	With air admission			Without air admission
	without NH_2OH and NaCl (control)	with NH_2OH	with NH_2OH and NaCl	with NH_2OH
Colour	Brown	Green	Dark green	Grey
Fe^{++}, γ %	140.0	956.05	913.95	1020.08
Fe^{+++}, γ%	880.4	63.95	105.51	Traces
$Fe^{++}+Fe^{+++}$, γ%	1020.4	1020.0	1019.46	1020.08

Evaluation of total iron content in each hemoglobin solution in test portion of hemoglobin under analysis showed that the photocolorimetric method worked out by us is no less accurate than a known volumetric method, by means of which it was established that hemoglobin contained 0.34% of iron (19).

On the basis of the data of Table I percentage contents of ferrous and ferric ions in green, brown and grey hemoglobin solutions are counted; the results are given in Table 2.

Table 2. Percentage contents of ferrous and ferric ions in green, brown and grey hemoglobin solutions on the 7th day of keeping

Indices	With air admission			Without air admission
	without NH_2OH and NaCl (control)	with NH_2OH	with NH_2OH and NaCl	with NH_2OH
Colour	Brown	Green	Dark green	Grey
Fe^{++}, %	13.72	93.73	89.65	100.00
Fe^{+++}, %	86.28	6.27	10.35	Traces

The data of Tables 1 and 2 show, that iron ions of heme pigments with different valencies take part in the formation of cured meats green pigments. These data show also that ferrous ions number in green heme pigments greatly exceeds that of ferric ions, staining of hemoglobin solutions being dependent on percentage contents of ferrous and ferric ions in the solutions. It means that the formation of grey, green, dark green and brown pigments in cured meat products also depends on the content of heme pigments iron ions in them. The data in Tables 1 and 2 show, too, that at pH 5.2 of medium hydroxylamine, in the presence of solved atmospheric oxygen, reduces quantitatively ferric ions of methemoglobin or methe-

mochromogen to ferrous ions which are then partially oxidized forming green pigments. The number of oxidized ferrous ions in these pigments makes up 6.27%. When there is no solved atmospheric oxygen, but there is enough hydroxylamine, grey pigments are formed with ferrous ions in them. In the absence of hydroxylamine, however, but in the presence of solved atmospheric oxygen and at pH 5.2, there occurs intensive oxidation of ferrous ions of hemoglobin solution, resulting in the formation of brown pigments, which contain 86.28% of oxidized ferrous ions.

It is obvious, that hydroxylamine (which reduces ferric ions of heme pigments) being present in enough quantities, has an inhibitory effect on the oxidation of ferrous ions by atmospheric oxygen, solved in brines. It results in some stabilization of green colour.

Besides, the results obtained confirm Brook's data (8), on that sodium chloride ions catalyze the oxidation of ferrous ions of heme pigments by atmospheric oxygen and that this effect of sodium chloride with the excess hydroxylamine present is insignificant. In the presence of sodium chloride and hydroxylamine, hemoglobin solutions are stained dark green. The oxidized ferrous ions make up 10.35%.

CONCLUSIONS

1. A photocolorimetric method is worked out for determination of ferrous and ferric ions in their joint presence in hemoglobin solutions. The method is based on the colorimetration of iron rhodanide staining and allows to quantitatively

determine iron when its concentration in 25ml of the solution ranges between 10γ and 0.1γ. To determine ferrous ion concentration it is necessary to previously determine ferric ion concentration, prior to adding potassium persulfate, and then, having added it, to determine their summary content. Ferrous ions concentration is determined by subtraction the amount of ferric ions from the total iron ions content.

2. With excess hydroxylamine, green pigments are formed at pH 5.2-5.8; the most intensive staining occurs at pH 5.2. At pH 4 heme pigments are stained brown, and at pH 6.8 they are stained dark red.

3. Hydroxylamine in weak acid medium reduces ferric ions of heme pigments, and in particular, it reduced them quantitatively at medium pH 5.2.

4. Green pigments of cured meats are formed with excess hydroxylamine due to partial oxidation of ferrous ions of heme pigments under atmospheric oxygen solved in brines. Hydroxylamine reducing ferric ions, inhibits oxidation of ferrous ions of heme pigments by atmospheric oxygen, that is, it has an inhibitory effect on the oxidation reaction.

5. Disturbance of normal pink-red colour of cured meat products is mainly related with electrochemical state of iron ions of heme pigments. Depending on percentage contents of ferrous and ferric ions of these pigments in cured meats, there may be formed either grey or green, or dark green or brown pigments. In grey pigments ferrous ions prevail, whereas in green pigments oxidized ferrous ions make up 6.27% and

in dark green - 10.35%. In brown pigments oxidized ferrous ions amount to 86.28% already on the 7th day of keeping.

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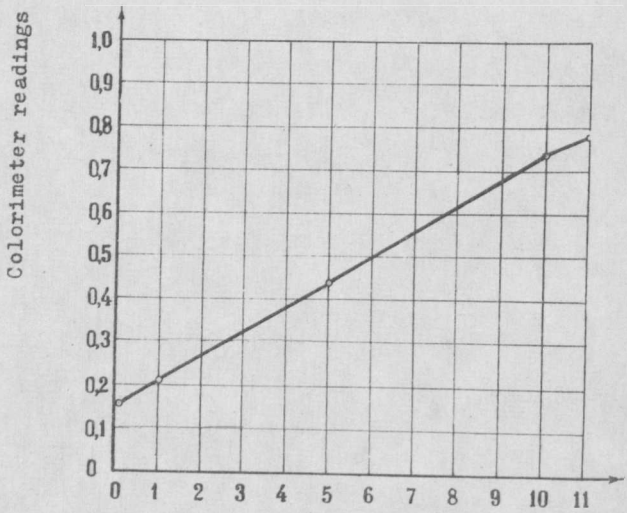

Iron concentration (in γ) per 25 ml of solution

A curve of determination of ferrous and ferric ion contents in solutions.

Зак.187. ВНИИМП


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