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Studies on Methods for the Determination of Lipoperoxides

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1. Various improvements and modifications of the colorimetric indophenol method for peroxide determinations are described. The method can be carried out as a "cold" method when a small amount of ferric chloride is added. The extinction produced by 0.001 milliequivalent of peroxide in 5 ml volume using benzene-ethanol or chloroform-ethanol as a solvent is about 1.00 at 520 mμ.

2. The bleaching effect of phospholipids in the thiocyanate method for peroxide determination is caused by the complex-binding of iron to the phosphoric acid groups of the phospholipids. It can be overcome by increasing the thiocyanate concentration.

3. Methods for the determination of peroxide groups in phospholipids and triglycerides containing high amounts of phospholipids are described.

4. The iodometric, the indophenol, and the thiocyanate methods for peroxide determination were compared. The latter method, carried out in the absence of oxygen, was found to be most suitable.

In a previous paper from this laboratory a new method, the colorimetric indophenol method, for the determination of the peroxides of fats and fatty acids (lipoperoxides) was described [1]. The method consists of heating the lipoperoxide-containing fat in a xylene-acetic acid solution with dichlorodihydroxydiphenylamine, the leuco-compound corresponding to the well-known redox indicator dichlorophenolindophenol. By oxidation of the leuco-compound, an amount of the indophenol equivalent to the quantity of lipoperoxide is formed. From the intensity of the red color of the indophenol the peroxide content is calculated.

The method has been used in this laboratory for several years and has been found sensitive and reproducible for the determination of lipoperoxides in several kinds of material. In the course of this work a number of improvements have been introduced in the method. The first part of this paper deals with our experiences with the colorimetric indophenol method, and at the same time two publications from other investigators [2,3], who have criticized the method, are discussed. Later parts of the paper contain a comparison of our method with other methods for the determination of peroxides as well as a study of the influence of phospholipids on the determination of peroxides.

A. THE COLORIMETRIC INDOPHENOL METHOD

Purification of solvents. All solvents used in work with lipoperoxides must be of a high degree of purity and free of peróxides as well as reducing substances and of heavy metal contamination. Peroxide-containing solvents can be purified by distillation under suitable conditions, for instance, water vapor distillation (toluene), or by filtering through a column of alumina (petroleum ether, benzene, toluene).

Several solvents (*e. g.*, petroleum ether and xylene) only remain peroxide-free for a very short time after they have been purified. On the other hand, benzene, chloroform, methanol and ethanol remain peroxide-free almost indefinitely, and good commercial qualities can be used without purification.

Preparation of color reagent. Dichlorodihydroxydiphenylamine, the leuco compound corresponding to dichlorophenolindophenol, is prepared by reduction of the latter by the following simplified method.

Two grams of 2,6-dichlorophenolindophenol (the product from British Drug House Company was found to be of a superior quality) are dissolved in 200 ml of hot distilled water. After filtration, the dye is reduced by the addition of about 1 g ascorbic acid. The precipitate of dichlorodihydroxydiphenylamine (in the following abbreviated "leuco") is filtered off on a glass filter and washed with redistilled water. When dissolved in a small quantity of absolute ethanol, some dark colored impurities are left on the filter. Further quantities of brownish impurities are removed by shaking the alcoholic solution with a small amount of activated carbon, followed by filtration. The leuco is precipitated again by the addition of redistilled water, filtered off, washed with redistilled water, and stored *in vacuo* over silica gel desiccant.

The leuco forms needles that are snow-white when freshly prepared but assume a bluish metallic appearance due to oxidation when stored for a long time. The use of redistilled water is essential, since the presence of small amounts of heavy metals generally occurring in ordinary distilled water markedly reduces the stability of leuco, especially in solution.

The color reagent is a 2 % solution of leuco in absolute ethanol. It keeps for several days when stored in a brown bottle, preferably in the ice-box. Two drops of the solution taken with a dropping pipette are used for an analysis. A blank containing 2 drops of leuco in 5 ml solvent should not produce an extinction exceeding 0.05 at 530 mμ (1 cm cuvette).

Determination of the extinction coefficient

In our first paper we reported that 0.001 milliequivalent of peroxide would give an extinction in 5 ml about 1.17. Hartmann and White [2], however, reported an extinction of 0.73, while Lea [3] recorded 0.83—0.84.

The extinction depends on the nature of the solvent. In the solvent mixture used in most of our experiments (benzene-ethanol-acetic acid, 45 : 50 : 5), careful determinations using several methods, as did Hartmann and White, gave an extinction of 1.00 at 520 mμ, and 1.02—1.03 at 530 mμ.

About the influence of the solvent the following can be said. If the extinction at the absorption maximum of a given concentration in ethanol is taken as unity, an extinction about 1.00 is also found with methanol, butanol, amyl alcohol, acetone, and ethyl acetate, but in solvents such as chloroform, benzene, and xylene, only about 0.60 is found (all solvents containing 2—5 % acetic acid). The presence of about 5 % ethanol in the nonpolar solvent will increase the extinction to about 0.8, and 15—30 % ethanol will bring it to about the same value as in ethanol. At the same time the addition of alcohol brings about a shift of the absorption maximum from about 500 mμ in xylene-acetic acid, to about 520 mμ, in the presence of 4 % ethanol, and to about 535 mμ at still higher alcohol concentrations. In our experiments we generally used absolute

ethanol for diluting our xylene-solutions to the mark in order to clarify them. This is the probable explanation of most of the discrepancy between our value and those found by the other workers, who use only 4 % butanol (0.2 ml color reagent) in their trials.

The presence of salts also may influence the extinction. When determinations are carried out in the presence of 10 % magnesium chloride, 0.001 milliequivalent peroxide will give an extinction of about 1.35.

The benzene-ethanol modification of the indophenol method

In our first paper we heated the xylene-acetic acid solution of leuco and fat in a boiling water bath for 10 minutes. Heating at 70° for 20 minutes is, however, sufficient to complete the oxidation of leuco by lipoperoxides. The greater part of the peroxide determinations in biological materials published by us were carried out using such a temperature and a solvent mixture consisting of ethanol-benzene-acetic acid 50 : 45 : 5.

The iron-catalyzed indophenol method

It is possible to carry out determinations at room temperature when a small amount of certain metals, for instance, iron or vanadium, is added. The determination can be carried out in the following way. The fat is dissolved in a mixture of equal parts of chloroform and alcohol to 5 ml volume. Two drops of the leuco reagent and one drop of a solution of 100 mg $FeCl_3$ in 100 ml of absolute alcohol are added, and the color compared with the blank after 10 minutes.

The indophenol method will give too high results when carried out in the presence of atmospheric oxygen. By the methods described above reproducible comparative results are obtained. In order to obtain correct values, however, it is necessary to carry out the determinations with the exclusion of oxygen. The iron-catalyzed indophenol method is especially easily carried out "anaerobically": The solution of fat + leuco is bubbled through with nitrogen for 10 minutes, a drop of ferric chloride is added, and the bubbling continued for 10 minutes more, after which the volume is adjusted and the colors are read in the photometer.

The presence of phospholipids causes an inhibition of the indophenol method. The inhibition can, in the case of the iron-catalyzed method, be overcome by the addition of magnesium chloride. Elevating its concentration results in acceleration of the color development. A method for the determination of peroxide groups in phospholipids by the indophenol method using the addition of 10 % magnesium chloride is reported in part C of this paper. The phospholipid inhibition of the iron-catalyzed indophenol method is probably due to complex-binding of iron to the phosphoric acid groups. A similar inhibition by phospholipids in the thiocyanate method for peroxide determination is probably also explained by complex-binding of the iron as will be reported in the following.

B. THE INFLUENCE OF PHOSPHATIDES ON THE THIOCYANATE METHOD

In a short communication Loftus Hills and Wilkinson [4] gave some observations on the influence of phosphatides on the determination of peroxide groups in fats by the ferric thiocyanate test. They added phosphatide in graded amounts to a series of solutions of 0.1 g of a slightly oxidized butterfat in 10 ml benzene-methanol. Milk phosphatide at a concentration of 0.05 % was found to interfere with the test, and in one of the experiments an amount of 0.5 % phosphatide completely inhibited color development.

These authors relate the effect of the phosphatides to the phosphoric acid group, pointing to the known interference by phosphoric acid in the thiocyanate test for ferric ions in aqueous solution. If this be true, *i.e.*, if complex formation between iron and the phosphoric acid groups of the phospholipids reduces the amount of red ferric thiocyanate compound, it is to be expected that the bleaching effect of phosphatide can be overcome by adding more thiocyanate or iron. This we found to be the case.

The first experiment, shown in Table 1, was carried out as described by Loftus Hills and Wilkinson. Graded amounts of phosphatide, freshly prepared from eggs, were added to a series of solutions of lard in 5 ml benzene-methanol (7 : 3). One drop of each of an ammonium thiocyanate and a ferric chloride solution, both prepared as described by Loftus Hills and Thiel [5], were added. The colors were read in the Beckman spectrophotometer at 500 mμ. As can be seen (Table 1), results almost identical with Loftus Hills and Wilkinson's with respect to the influence of phosphatide were observed. Next, 5 ml of a 6 % solution of ammonium thiocyanate in benzene-methanol was added to each test tube, and the colors read gain. The results as given in Table 1 are corrected for phosphatide blanks and adjusted to original volume of 5 ml. It is noted that even in the presence of 100 mg phosphatide, a large amount of ammonium thiocyanate could overcome most of the inhibition.

Table 2 shows the results of another experiment illustrating the effect of graded amounts of thiocyanate in overcoming the influence of a constant, high amount of phosphatide. A series of test-tubes were prepared containing

Table 1. The effect on the peroxide test of varying additions of phosphatide in the presence of small and large amounts of ammonium thiocyanate.

Weight of phosphatide added mg	Light absorption at 500 mμ	
	0.12 % NH_4SCN	3 % NH_4SCN
None	0.44	0.48
0.25	0.44	0.53
0.5	0.45	0.55
1	0.42	0.52
2.5	0.36	0.50
5	0.34	0.52
10	0.28	0.49
25	0.25	0.50
50	0.24	0.51
100	0.19	0.44

Table 2. The effect on the peroxide test of varying amounts of ammonium thiocyanate in the presence of a high amount of phosphatide.

Weight of ammonium thiocyanate added mg	Light absorption at 500 mμ
2.5	0.22
5	0.24
10	0.32
25	0.35
50	0.43
100	0.43
200	0.47
400	0.44

the same amount of a peroxide-containing lard as used in the previous experiment, 100 mg egg phosphatide, and the amount of ammonium thiocyanate given in the table, all in 5 ml benzene-methanol. One drop of the Loftus Hills-Thiel ferrous chloride solution was added, and the color read in the spectrophotometer.

Finally, an experiment is recorded in which the effect of increasing the iron concentration is demonstrated. The same solutions as in the first experiment — containing a constant amount of lard and graded amounts of phosphatide — were prepared in two series. One drop of the Loftus Hills ferrous chloride reagent per 10 ml solution was added to one series. One drop of a solution prepared in the same way and containing the same amount of hydrochloric acid, but twenty times as much iron, was added to the other series. The solutions were compared in the photometer with the corresponding blanks (Table 3).

The experiments recorded in Tables 1—3 quite well support the theory that the reason for the inhibiting effect of phosphatides in the thiocyanate test for peroxides is a complex-binding of ferric iron to phosphatides. This effect can be partly overcome by increasing the concentration of ferrous chloride added, or, more effectively, by increasing the thiocyanate concentration. In the Loftus Hills-Thiel method, the presence of phosphatide decreases the light absorption and also produces a shift in color from bright red to yellowish. This is possibly

Table 3. The effect on the peroxide test of low and high ferrous chloride concentration in the presence of varying amounts of phosphatides.

Weight of phosphatide added mg	Light absorption at 500 mμ	
	0.23 % $FeCl_2$ added	4.6 % $FeCl_2$ added
1.25	0.42	0.46
2.5	0.36	0.44
5	0.36	0.45
10	0.30	0.38
25	0.31	0.30
50	0.24	0.33
100	0.21	0.30

the reason that a still more complete inhibition is found when the colors are measured with a visual colorimeter of the type mostly used by Loftus Hills and his coworkers.

The experiments recorded in the tables were carried out in the same way as did Loftus Hills and Wilkinson, *i.e.*, without deaeration, but of course similar results are obtained when oxygen is excluded. The question whether the addition of high concentrations of ammonium thiocyanate can completely overcome the inhibiting effect of the phospholipids is discussed in the following part of this paper, which describes methods for the determination of peroxide groups in the presence of phospholipids.

C. THE DETERMINATION OF PEROXIDE GROUPS IN PHOSPHOLIPIDS AND PHOSPHOLIPID-CONTAINING FATS

Although it is well known that phospholipids usually contain a high amount of unsaturated fatty acids and are easily oxidized in the presence of atmospheric oxygen, it seems that the presence of peroxide groups in phospholipids has never been studied. Oxidation of the phospholipid has frequently been expressed simply by the decrease in iodine value.

Above we have reported our observations on the overcoming of the phospholipid inhibition in peroxide determination methods. The addition of magnesium chloride in the indophenol method, and of ammonium thiocyanate in the thiocyanate method, not only permit the determination of peroxide groups in triglycerides when phospholipids are present in large amounts, but also give reactions on oxidized samples of phospholipids containing very little triglyceride fat. The results obtained when the methods are employed on samples of phospholipids purified by repeated precipitation with acetone must be considered as indicative of the presence of peroxide groups in the phospholipids themselves.

Methods developed for the determination of peroxide in phospholipids and phospholipid-containing fats are outlined below.

Determination of peroxide groups in phospholipids by the indophenol method

A suitable amount of the lipid is dissolved in 5 ml of a mixture of equal parts of chloroform and 20 % $MgCl_2$, 6 H_2O in absolute ethanol. Two drops of leuco reagent and 1 drop of 0.1 % ferric chloride in ethanol are added. At the same time a blank containing 2 drops of leuco and 1 drop of ferric chloride solution in 5 ml chloroform-magnesium chloride-ethanol is prepared. After 10 minutes the colors are compared in the Beckman spectrophotometer at 530 mμ. The results are calculated on the basis of a linear relation between peroxide content and extinction of the amount of indophenol formed; 0.001 milliequivalent in 5 ml solution is assumed to give an extinction of about 1.35.

The test should preferably be carried out in the absence of oxygen. This can be done by passing oxygen-free nitrogen through the solution of lipid in magnesium chloride-containing solvent, and the corresponding blank for 10 minutes, then adding leuco and ferric chloride, and continuing to bubble with N_2 for 10 minutes. The deaeration can be carried out in the Beckman cuvettes using a volume of 3 ml and a glass capillary for the introduction of N_2.

Determination of peroxide groups in phospholipids by the thiocyanate method

A suitable amount of the lipid is dissolved in 5 ml of a solvent mixture containing 6 % ammonium thiocyanate in chloroform-ethanol (1 : 1). One drop of 0.22 % $FeCl_2$ prepared by Loftus Hills and Thiel's method is added. After a few minutes the solution is compared with a blank in the spectrophotometer at 500 mμ. The results are calculated on the supposition that 0.0003 milliequivalent peroxide in 5 ml solution gives an extinction about 1.0.

The test should be carried out in the absence of oxygen by passing purified N_2 through the solution for 10 minutes before ferrous chloride is added.

Results

A few typical results obtained by the two methods given above are presented in Table 4 which also contains the results found with the iodometric method. All methods were carried out in the absence of oxygen. The iodometric method used was the "cold" Lea modification [6], although this method has been worked out for triglycerides. The method is very unsuitable for phospholipids, since emulsions are formed when water is added before the titration, and at the same time a dark coloration of the oxidized phospholipids interferes with the exact observation of the disappearance of the iodine color. The results are, however, shown for comparison.

Table 4. Comparison of different methods for the determination of peroxide groups in phospholipids.

	Peroxide, milliequivalents/kg		
	Iodometric method	Indophenol method	Thiocyanate method
Egg phosphatides, freshly prepared	0	3.0	1.9
1 day old	0	5.1	5.0
3 days old	10	14.7	16.5
1 week »	12	20	23
1 month »	104	105	95
2 months old	250	320	340
Lecithin, *ex ovo*, Merck, about 10 years old	5	21	19

Discussion

From the reasons mentioned above, the iodometric method only permits very rough estimations. The results are probably too low, at least in freshly prepared phospholipids. On the other hand, with the two other methods, rather reproducible results are obtained that are almost identical, irrespective of the method.

In freshly prepared phospholipids generally higher values are found by the indophenol than by the thiocyanate method. The reason for this fact is not quite understood as yet. It might be that while in the indophenol method iron acts only as a catalyst, and so the final color development is independent of possible complex-binding of part of it to phosphoric acid groups, in the thio-

cyanate method partial binding of iron to phosphatides will lead to a decrease in color intensity. Such a difference between the functions of iron in the two methods could give a simple explanation of why too low values are found by the thiocyanate method in comparison with the, presumably, more stoichiometric values found by the indophenol method, when only small amounts of peroxide are present together with relatively much phospholipid. However, more work is needed to establish by which method the true peroxide content in the first stage of the oxidation of phospholipids is found. Until this problem is solved it cannot be definitely settled whether an upper limit exists for the amount of phosphatide that may safely be present in the thiocyanate method.

By means of the methods described we have been able to follow the formation of peroxides in freshly prepared phospholipids, and to demonstrate the presence of peroxides in certain phosphatides prepared from natural products. In a later paper methods for the separation of peroxides of phospholipids from peroxides of triglycerides will be described.

Since many fats and oils contain considerable amounts of phospholipids, Loftus Hills' modification of the thiocyanate method will yield too low values. When analyzing products like butter or soy-bean oil a higher concentration of thiocyanate should therefore be used, although perhaps not as high as in the case of products consisting almost exclusively of phospholipids.

Increasing the thiocyanate concentration up to a certain point gives a slightly higher light absorption of the ferric thiocyanate complex. A concentration of 2—5 % ammonium thiocyanate in the final solution will, in most cases, give maximal extinction.

As a solvent mixture we prefer a chloroform-ethanol solution. Although Loftus Hills and Thiel state that water has the same solubility in benzene-methanol as in chloroform-ethanol, we find that chloroform-ethanol (73 : 27) or chloroform-methanol will dissolve more water than the corresponding benzene-mixtures. Ethanol and, especially, methanol are good solvents for ammonium thiocyanate. Therefore, the use of aqueous solutions of this substance is not necessary.

As a result of these considerations we have arrived at the following modification of the thiocyanate method for fats containing moderate amounts of phospholipids. This modification can, of course, also be used for fats with low phospholipid content.

The fat is dissolved in chloroform-absolute ethanol (5 : 3) and brought to a volume of 8 ml. Two ml 30 % ammonium thiocyanate (analytical grade) in absolute methanol are added. After daeration for 10 minutes with purified nitrogen, 1 drop of the Loftus Hills-Thiel ferrous chloride reagent (or a reagent prepared in the same way but of double strength [7]) is added, and the colors are measured after a few minutes. In our hands, heating at 50° C was not necessary. Loftus Hills has come to the same conclusion (personal communication).

D. COMPARISON OF METHODS. GENERAL DISCUSSION

In this part of our paper we shall report typical results from experiments carried out in order to compare different methods for the determination of peroxides.

The iodometric method was carried out by the "cold" Lea method [6]. At the same time the substances were tested by the same method carried out "aerobically", that is, in exactly the same way but without bubbling through with nitrogen.

The thiocyanate method was used in the Loftus Hills and Thiel modification [5]. However, a double strength ferrous chloride solution was used [7], and the heating at 50° C was omitted. Also in this case two series of tests were carried out. In one series the solutions were deaerated with nitrogen for 15 minutes before the iron solution was added. In the case of benzoyl peroxide a higher concentration of ammonium thiocyanate (5 %) was used, since the concentration used by Hills and Thiel resulted in a very low reaction velocity.

Similarly, the same substances were tested with and without deaeration before the addition of ferric chloride, by the iron catalyzed indophenol method. The results are summarized in Table 5.

Table 5. Comparison of different methods for the determination of peroxide groups in fat, with and without the exclusion of atmospheric oxygen.

	Milliequivalents of peroxide per kg fat					
	Iodometric method		Thiocyanate method		Indophenol method	
	O_2 absent	O_2 present	O_2 absent	O_2 present	O_2 absent	O_2 present
Butterfat	0	0	0.2	0.25	0.3	0.46
»	0.6	1.2	1.25	1.45	1.1	1.75
»	1.07	2.2	1.85	2.1	1.75	2.45
Lard	4.3	4.7	5.8	7.6	4.0	7.4
»	280	344	395	450	320	570
Cod liver oil	5.4	6.4	6.6	7.1	4.8	9.6
» » »	28.5	36	42.5	49	39	52
Ethyl oleate hydroperoxide	840	940	970	1 230	840	1 140

It will be seen from Table 5 that all the methods give higher values in the presence of dissolved oxygen. In most cases the indophenol method showed the highest and the thiocyanate method the lowest degree of oxygen sensitivity.

The oxygen sensitivity of various methods has been studied by other investigators, *e.g.* Lea [3, 6, 9], who finds the iodometric method to be by far the least sensitive to oxygen. The reason for this discrepancy is, probably, smaller or larger differences in methods and purity of the chemicals. For example, Lea's studies on the influence of oxygen on the thiocyanate method were mainly carried out with a modification [9] rather different from ours.

It should be pointed out that we have found no considerable oxygen sensitivity in any of the methods when they were carried out on benzoyl or hydrogen peroxide. This also holds true for the original modification of the indophenol method first published [1]. Contrary to the criticism of Hartmann and White [2], our results suggest that atmospheric oxygen only interferes in the methods when a free radical is formed in the reaction which is stable enough to function as a carrier in a chain reaction.

When comparing the anaerobic results presented in Table 5, it is seen that, although in most cases the thiocyanate method gives the highest, the iodometric the lowest values, almost identical results are found, irrespective of the method.

The literature in this field is quite confusing, and direct comparisons between anaerobic methods for the determinations of peroxides in fats and oils are scarce. Lea [3], for instance, presents several tables comparing the aerobic colorimetric methods with the anaerobic iodometric method, but no figures concerning a direct comparison between the different anaerobic methods. He observed that ferric thiocyanate determinations with exclusion of atmospheric oxygen gave much too low results, but he presented no details that make it possible to find out why he was unable to obtain quantitative results with the thiocyanate method.

Kolthoff and Medalia [8] found lower results in certain oxidized fatty substances by the anaerobic thiocyanate method than by the iodometric method. However, they used a thiocyanate method very different from ours, with acetone as solvent.

Kolthoff and Medalia have proposed a mechanism for the thiocyanate reaction and possible by-reactions which explains the high results found by the influence of an organic solvent in the presence of oxygen, and low results due to decomposition by the solvent. They assume that the reaction between hydroperoxide and ferrous ions takes place through two one-electron transfers

$$ROOH + Fe^{++} \rightarrow RO^{\cdot} + OH^{-} + Fe^{+++};$$
$$RO^{\cdot} + Fe^{++} + H^{+} \rightarrow ROH + Fe^{+++}.$$

The free alkoxy radical RO $^{\cdot}$ is very reactive and may react with solvent molecules *etc.*, whereby the by-reactions are initiated.

The reactions are quite complex. Lea [3] can, however, scarcely be right in his rather pessimistic view that the complicated character *per se* of the reaction can prevent it from being made truly stoichiometric. The complicated character of the reaction so far cannot be used as an argument for the superiority of the iodometric method, especially since the chemical reactions involved in the iodometric method are probably equally complicated or quite analogous, with the intermediate formation of reactive alkoxy radicals.

Lea thinks that there is no advantage in carrying out the ferric thiocyanate determination with exclusion of atmospheric oxygen, since the method then loses much of its desirable simplicity, and also that the results with oxidized fats become much too low [3]. However, it seems that the thiocyanate method remains much simpler than the iodometric even though in both cases atmospheric oxygen is removed by bubbling with nitrogen. Lea [3] and G. Howard Smith [7] recommend the thiocyanate method in the presence of oxygen as a micromethod and think that by division with a conversion factor about 2, but slightly different for different fats, the values can be converted into the true values. Aerobic determinations carried out under uniform circumstance have proved to give relative values that are useful for many practical purposes. However, the physical foundation of the conversion factors involves so many single factors which can deviate from laboratory to laboratory and from trial to trial that their use becomes rather unsafe. Kolthoff and Medalia have

concluded, more justifiably, that the only sound basis for the development of satisfactory procedures for the thiocyanate reaction is complete exclusion of oxygen and the use of a solvent which entirely suppresses the induced decomposition of the peroxide.

When comparing the methods it should be borne in mind that the iodometric method is unsatisfactory for substances having a low peroxide content. The limit of sensitivity is about 0.05 ml 0.002 *N* thiosulfate, corresponding to 10^{-4} milliequivalent peroxide, probably a still higher value since a blank titration has to be subtracted. On the other hand, by the thiocyanate method, a difference in extinction of 0.05 can easily be observed, corresponding, in a 5 ml volume, to about 0.15×10^{-4} milliequivalent. For practical application, especially in work with foods, a high degree of sensitivity is the most important problem. In a product like butter, oxidative changes, giving a distinct off-flavor, are often observed by a peroxide content of about 0.2 milliequivalent per kg. Such a small quantity of peroxide can scarcely be determined by the iodometric method, whereas still lower values can quite well be determined by the colorimetric methods.

In nutritional work, *e.g.*, it is generally of little importance to know exactly the peroxide content of a product which has been definitely rancid for a long time, and there is thus little reason to use the rather troublesome iodometric method. Even though the colorimetric methods are only carried out with an error of about 5—10 %, this is, as a rule, sufficiently accurate for work with foods.

On the other hand, since a titration can be carried out very exactly, the iodometric method will allow much more exact determinations of higher peroxide contents. This is very important *e.g.*, in chemical work with peroxides, and the iodometric method must be preferred in such work.

A very sensitive method, based on the iodometric, is the thiofluorescein method, given by Dubouloz *et al.*[10]. However, while in the thiocyanate and the indophenol colorimetric methods, colors are measured that are proportional to the peroxide content, in the thiofluorescein method a decrease in color intensity is measured, so that the lowest contents of peroxide are least exactly determined. Furthermore, the thiofluorescein method is quite complicated in comparison with the other colorimetric methods.

Regarding the two colorimetric procedures, it must be concluded that the thiocyanate method is preferable to the indophenol method for general use. The reason for this conclusion is that although the very saturated indophenol color seems as intensive to the eye as the thiocyanate color, nevertheless a given amount of peroxide in the same volume will give a color intensity of only 30 % of the color intensity produced by the thiocyanate method with correspondingly lower sensitivity. For special purposes the indophenol method may still be useful, *e.g.*, in work with phospholipids, since in the first stage of the oxidation of such substances the indophenol method is possibly more truly stoichiometric.

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