A Study of the Determination of Peroxides in Fats*

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THE various methods for the determination of peroxides in fats are based on the same general principle of liberation of iodine from potassium iodide in an acid medium. Several different solvents are used by the numerous investigators. The details of the manipulations, such as timing, temperature, and use of inert atmosphere, also vary quite markedly.

Lea (1931) published a method in which a chloroform-acetic acid solvent mixture was used. The method gives reproducible results at a given sample weight, but it is inconvenient and even somewhat hazardous to use since the solvent mixture is heated to boiling. Wheeler (1932) published a greatly simplified method in which this same solvent mixture was used. The mixture was not heated, and the reaction time was only one minute. He suggested that the sample size and the normality of the thiosulfate should be varied if the samples differ greatly in peroxygen content. Lowen *et al.* (1937), using the method of Wheeler, showed that sample size strongly influenced the peroxide value obtained. Halibut and salmon oil samples ranging from 2 to 10 grams were used. A depression of 30-50% in peroxide value was observed as the sample size was increased. Liebhafsky and Sharkey (1940) used acetic acid as a reaction medium. They observed that the presence of water in the medium retarded the reactions of benzoyl peroxide and hydrogen peroxide with potassium iodide.

Stansby (1941) compared the Lea and Wheeler methods with his two variations of the peroxide determination. Chloroform or carbon tetrachloride was used as the solvent in his modified methods. An acetic acid-hydrochloric acid mixture was used in one procedure. This method was greatly simplified and did not require an elevated temperature. It gave moderate accuracy even with varying sample size. Stansby's other modification, using sulfuric acid, was a much more involved procedure but gave greater accuracy than the hydrochloric method. His comparison showed poor agreement among the Lea, Wheeler, and Stansby-H_2SO_4 methods. Both the Lea and Wheeler methods gave decreased peroxide values with increasing sample size.

Kokatnur and Jelling (1941) reported a method in which isopropanol was used as the solvent. A blank was unnecessary with this medium. The authors specified a two- to five-minute heating of the reaction mixture at incipient boiling. They also stated that the sample size and the normality of the standard thiosulfate should depend upon the peroxygen content of the sample. They recommended reheating or a longer heating period if iodine was liberated slowly. The liberated iodine was titrated in the hot solution to the disappearance of the yellow color. The separate aqueous phase necessary for a starch-iodine endpoint was thus eliminated.

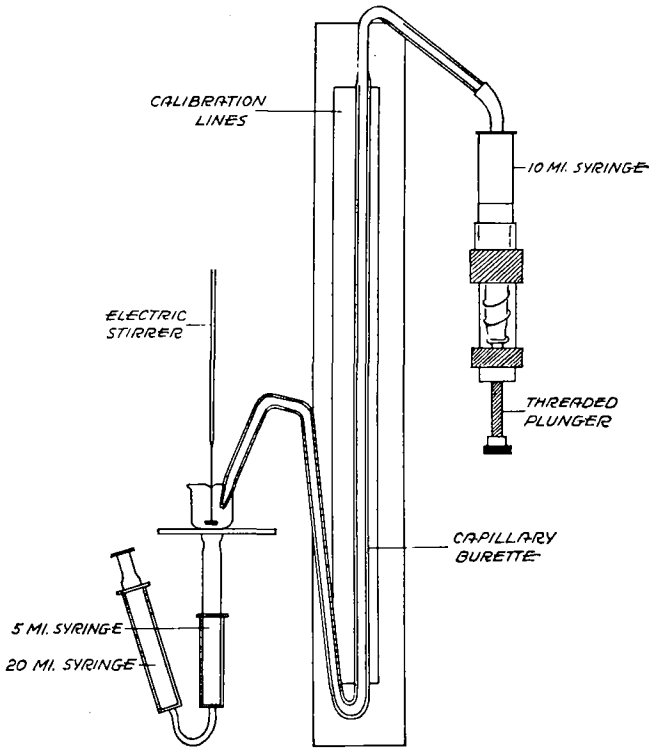

FIG. 1. Micro titration apparatus.

Lips, Chapman, and McFarlane (1943) made a comparison of the Lea (1931), Wheeler (1932), and Kokatnur and Jelling (1941) methods together with a colorimetric ferric thiocyanate method. The Kokatnur-Jelling method gave consistently lower results than the other methods.

The original Wheeler method was modified by Paschke and Wheeler (1944) to overcome some of its basic weaknesses. Sample weight was reduced to one gram, reaction time was increased to one hour, and a CO_2 atmosphere was used to minimize the further oxidation of the fat.

Lea (1946) has revised his earlier method and simplified the manipulative details. The method is essentially the same as the Paschke and Wheeler method since it employs a de-aeration procedure, the same solvent, and a one-hour reaction period. Lea made a thorough comparison of the Lea (1931), Wheeler (1932), and Paschke and Wheeler (1944) methods and the new Lea "cold" method.

Lea's modified method gave generally lower results than the other procedures. The earlier Lea method gave the highest values owing to further oxidation of the fat by dissolved oxygen. Lea stated that the low values obtained with the original Wheeler method resulted from cancellation of errors resulting from too short a reaction period and from further oxidation of the fat during the reaction period. He also

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stated that there was no direct evidence of loss of liberated iodine due to reabsorption.

In the light of the findings of these various investigators Lingenfelter (1945) selected the Kokatnur and Jelling method because of its many advantages. The reaction medium, isopropanol, is not only a good fat solvent but also mixed readily with water. The single phase system was desirable since Lingenfelter was interested in using a micro titration apparatus (Fig. 1) with which a 0.1 N thiosulfate standard could be used for all samples. This modified method has proved to be both simple and rapid. Kokatnur and Jelling did not indicate the limits that must be imposed on the temperature, the sample size, or the peroxygen content. The present report deals with these variables as they apply to the modified micro method.

Experimental

To determine the extent of these limitations an experiment was designed whereby several factors could be studied simultaneously. The factors included sample size (0.2, 0.5, 1.0 grams), temperature of the water bath (75°, 80°, 85°C.), the length of time the sample was held in the water bath (3, 5, 7 minutes), and the peroxide value of the sample (low, medium, high). The experiment was designated as a 3x3x3x3 factorial (Yates, 1937) so that the results might be analyzed statistically. Thus there were 81 treatments. The use of the factorial design and statistical analysis of the data rather than independent study of each variable has two principal advantages. It eliminates the necessity of running several hundred analyses without reducing the number of observations on any single variable. In addition, it permits the evaluation of interactions which could not otherwise be obtained.

Samples of rendered pork fat at three peroxide levels were dissolved in petroleum ether (b.p. 30-60°C.); hydroquinone was added to prevent further peroxide formation and the solutions were stored at —17.8°C.

The method as it was used in these comparative studies is as follows:

An aliquot of the fat extracted is pipetted into a 50-ml. beaker, warmed in the water bath, and then evaporated to dryness under a stream of nitrogen. The fat then is dissolved in 35 ml. of 99% isopropanol.[1] Into this solution is pipetted rapidly 1 ml. of 50% aqueous potassium iodide solution, followed by the addition of 1 ml. of glacial acetic acid. The solution is mixed and immersed in a hot water bath at a given temperature for the specified period of time, with occasional stirring. The beaker is then removed from the bath and 2 ml. of boiling distilled water are added. The solution is immediately titrated with 0.1 N $Na_2S_2O_3$ to the disappearance of the yellow color, using a micro-burette and with continuous electric stirring.

The treatments were randomized within a given temperature, that is, 27 analyses were completed daily for three days. The basic design of 81 treatments was then replicated. The average values for the two replications are plotted in the several curves shown in Figure 2.

Results of Factorial Design Experiment. An analysis of variance (Yates, 1937) was applied to the

[1] The isopropanol was allowed to stand with metallic sodium for a few hours and was then distilled before use.

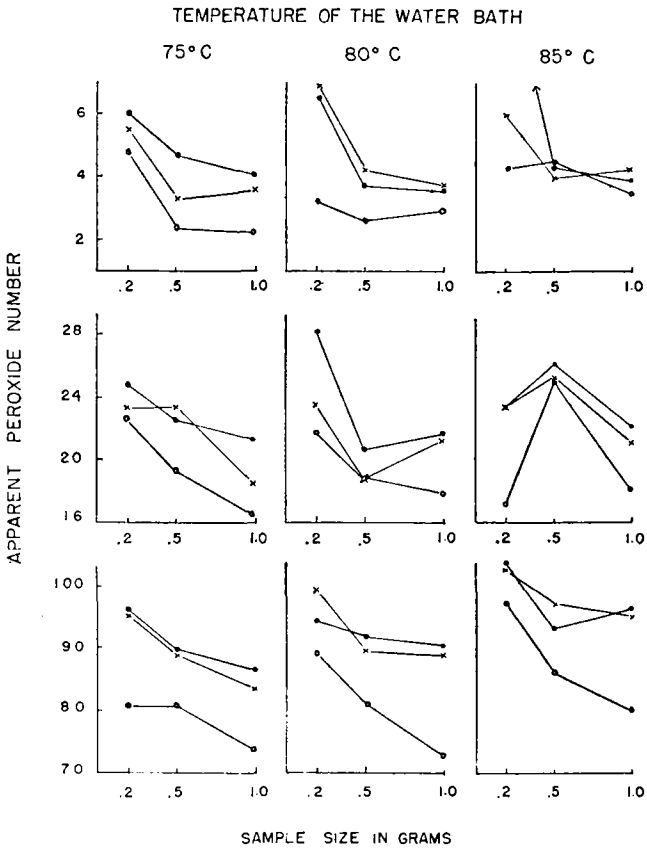

FIG. 2. The effects of time, temperature, and peroxygen content on the apparent peroxide numbers of 3 pork fats as determined by a modified Kokatnur-Jelling procedure.
○ = 3-minute reaction time, × = 5 minutes, ● = 7 minutes.

results presented graphically in Figure 2. By this analysis the effects of the temperature of the water bath, the length of time in the water bath, and the sample size were all shown to be highly significant at the 1% level. The highly significant differences between the peroxide values were part of the design of the experiment. The interaction of the peroxide value with each of the other three variables was also shown to be highly significant. There were no other significant interactions among the variables. In Table 2 are given the sources of variation, together with degrees of freedom, sums of squares, and F-values (ratio

TABLE 1

Analysis of Variance of Peroxide Number of Fats With Various Time-Temperature-Sample Size Relationships

Source of variation*	Degrees of freedom	Sum of squares	F-value
Total	80	111,673.98	.....
P	2	109,722.53	12,191.39**
T	2	492.20	54.69**
C	2	182.24	20.25**
S	2	376.43	41.83**
PC	4	176.20	9.79**
PT	4	313.50	17.42**
PS	4	169.83	9.43**
CS	4	16.37	.....
CT	4	1.91	.....
ST	4	18.97	1.05
PSC	8	71.15	1.97
CST	8	11.80	.....
PTS	8	37.71	1.05
PTC	8	11.06	.....
Error	16	72.08	.....

* P=peroxide value, C=temperature, °C., T=time, minutes, S=sample size, grams.
** Values highly significant at the 1% level (Snedecor, 1940).

TABLE 2

The Influence of Fat Sample Size and Peroxygen Content of Fat Aliquot on the Determination of Peroxide Value

High peroxide fat	Low peroxide fat	Total fat	Microequivalents of peroxygen in the fat sample‡	Peroxide number of total fat	
				Calculated‡	Found†
gm.	*gm.*	*gm.*			
.1	0	.1	10.3	103	103
.2	0	.2	20.6	103	100
.2	.1	.3	20.7	69	69
.2	.2	.4	20.8	52	52
.2	.4	.6	21.0	35	34
.2	.8	1.0	21.4	21	21
.2	1.0	1.2	21.6	18	16
.4	0	.4	41.2	103	104
.4	.1	.5	41.3	83	85
.4	.2	.6	41.4	69	64
.4	.4	.8	41.6	52	51
.4	.6	1.0	41.8	42	40
.4	.8	1.2	42.0	35	34
.8	.4	1.2	82.8	69	63
1.0	0	1.0	103.0	103	97
1.2	0	1.2	124.0	103	95

‡ Based on peroxygen contents (m. eq. per kg.) of 103 for high peroxide fat, 1 for low peroxide fat.

† Using procedure of Lingenfelter (80°C., five-minute reaction time).

of variance due to a given factor to variance due to error) used for testing the significance of the difference between variables.

In the light of these findings it seemed necessary to apply definite time, temperature, and sample size limits. The water bath temperature must not be allowed to fluctuate as much as 5°C. The water bath temperature should be standardized, preferably at 75° or 80°. Since a temperature of 85° is slightly higher than the boiling point of isopropanol, the reaction mixture approaches the boiling point as the reaction time progresses. This high temperature has the disadvantages of causing bumping and solvent evaporation with no added advantage.

The timing must be closer than the two-minute range used here. Three minutes is too brief a reaction period as is indicated in Fig. 2 in every comparison except the low peroxide fat at 85°C. The three-minute values were markedly lower than either the five- or seven-minute values.

The seven-minute reaction at 75° C. gave results similar to the five-minute reaction at 80°C. Although this laboratory has standardized the method for use at 80°C. and five minutes, other laboratories using the method may prefer to use the 75°C. and seven-minute specifications.

The sample size should be restricted to obtain consistent, reproducible results. The smallest sample size, 0.2 gram, gave high results as shown in 22 of the 27 comparisons in Fig. 2. Whether the peroxygen content alone or the quantity of fat or the combination of these two factors is responsible for the depression of the peroxide value in the larger samples was considered in the next experiment.

Results of Sample Size Experiment. The papers cited earlier indicated that the peroxygen content of the sample rather than the fat sample size itself was the factor which influenced the results in peroxide determinations. The following analyses were carried out to determine to what extent these two factors were influential.

Two pork fat extracts were prepared and stored as indicated earlier. The fat of one extract had a peroxide value of approximately 100, and the other had a value of 1. Aliquots of these two extracts were combined to give samples of increasing fat sample size without a corresponding change in the amount of peroxygen. They were also combined to give increasing amounts of peroxygen. The analyses were carried out according to the method as modified by Lingenfelter, using a five-minute reaction time at 80°C.

The results of these analyses are given in Table 2. Although the effects were not sharply defined, the general trend of these analyses indicated the following: the peroxygen content of the sample does have an influence, especially at the higher levels. The quantity of fat present did not appear to influence the results significantly.

These findings indicated that the sample size of high peroxide fats should be decreased so that no more than 50 microequivalents of peroxygen are contained in the aliquot to obtain a reliable peroxide value. Restriction of the peroxygen content will minimize the effects of interaction between the peroxide values and the other variables.

TABLE 3

Percentage Recovery of Benzoyl Peroxide by Three Methods

Milligrams of benzoyl peroxide	Method		
	Lingenfelter	Stansby	Lea (1931)
2.5	101.1	98.6	102.2
7.5	99.7	99.1	101.7
12.5	99.6	99.1	100.8
25.0	99.1	99.5	100.6
12.5+1 gm. fat	100.3	.....	.....
25.0+1 gm. fat	98.9	.....	.....

Comparison of Three Peroxide Methods. Lingenfelter's modification of the Kokatnur and Jelling method was compared with two other methods of peroxide determination. The two selected were Lea's (1931) because of its classical nature and Stansby's HCl method because of its simplicity. The standard reaction time set for the Lingenfelter method was five minutes and the water bath temperature was 80° ± 1°C. Benzoyl peroxide as well as three fat extracts were used as a basis of comparison. Samples of benzoyl peroxide added to a low peroxide fat were also included.

The results are given in Tables 3 and 4. The recovery of benzoyl peroxide was excellent by all three methods. Even in the presence of 1.0 gram of freshly rendered pork fat the recovery of the added peroxide by the micro-method was very good.

TABLE 4

The Peroxide Values of Three Fats as Determined by Three Different Methods

Type of fat	Grams of fat	Method		
		Lingenfelter	Stansby	Lea (1931)
Low peroxide	.2	3.6	.....	.....
	.5	3.3	4.0	.....
	1.0	2.8	3.1	4.8
Medium peroxide	.2	21.0	.....	.....
	.5	19.6	17.8	.....
	1.0	18.0	17.0	22.0
High peroxide	.2	108.7	104.8	142.3
	.5	107.3	100.3	112.7
	1.0	106.5	97.9	110.5

The peroxide values given in Table 4 indicate that Lea's original method gives higher results; this is probably due to the high temperature at which the reaction is carried out. The Stansby and Lingenfelter

procedures agree fairly well. Both exhibited a tendency toward depression of the peroxide value as peroxygen equivalents were increased.

Summary

The Kokatnur-Jelling method as modified by Lingenfelter for the determination of peroxides in animal fat has been found to agree satisfactorily with other methods now in use. The modified method involves a microtitration in a homogeneous medium (isopropanol) and has the additional advantages of being both simple and rapid.

A statistical analysis of the peroxide values shows that the reaction time and temperature and the sample size must be standardized to obtain reproducible and reliable results. Close adherence to reaction specifications such as five minutes at 80°C. or seven minutes at 75°C. is necessary. Three minutes is too short a reaction time. The individual fat sample should contain no more than 50 microequivalents of peroxygen to avoid depression of the peroxide value.

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The Fatty Acids of Dormant Tung Buds

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Introduction

IN studies (1, 2) of the prolongation of dormancy of tung buds (*Aleurites fordii* Hemsl.), it was found that blossoming was effectively delayed, although always with attendant injury, by alpha-naphthalenacetic acid and certain of its derivatives or by indole-3-acetic acid, when applied in Crisco [2] or lanolin emulsions. However, when various materials containing alpha-naphthalenacetic acid were applied as sprays, or when an aqueous solution of its potassium salt was injected into the buds, dormancy was not prolonged. The rudimentary blossom buds of tung are embedded in a sticky wax. One would expect that if the radicals of the higher fatty acids in this wax are similar to those of Crisco or lanolin, penetration of growth-regulating substance emulsified in these materials should be facilitated since, according to the solubility rule (3), similarity in structure among organic compounds is conducive to mutual solubility. In view of these facts it appeared desirable to identify the higher fatty acid constituents of the wax, and an investigation was undertaken for this purpose. The techniques used and the results obtained are presented in this paper.

EXPERIMENTAL WORK

Extraction of the Wax from Tung Buds

In December, 1941, 14.5 kg. of buds were collected from the trees in the Alachua Tung Oil Orchards near Gainesville, Florida. The buds were extracted with ethyl ether in a two and one-half gallon Soxhlet extraction unit for 24 hours. They were then dried in vacuo at 70°C. and 10 mm. pressure, ground to pass a 2-mm. mesh sieve, and re-extracted with fresh ethyl ether for an additional 48 hours. The ether was removed from the combined extracts by distillation and the residue finally dried in vacuo at 70°C. and 6-mm. pressure. The yield was 1.8 kg. of crude wax, of which 45% was unsaponifiable and 55% was fatty acids.

The wax (1.67 kg.) was saponified by refluxing for 4 hours with 270 gm. of potassium hydroxide in 3 liters of 95% ethanol. After diluting the solution with 3 liters of water, it was extracted in a liquid-liquid extraction unit with petroleum ether for 24 hours to remove the unsaponifiable material. The aqueous-ethanol solution containing the potassium salts of the fatty acids was acidified with hydrochloric acid, and the liberated organic acids were extracted with ethyl ether. The fatty acids remaining after distillation of the ether and final drying in vacuo at 70°C. and 6-mm. pressure weighed 975.3 gm. No fatty acids of low molecular weight were detected in the distillate.

Preparation of the Methyl Esters of the Fatty Acids

The fatty acids (963.0 gm.) were converted to the methyl esters by refluxing for 3 hours in 2.5 liters of absolute methanol containing 5% sulfuric acid (by weight). The solution was neutralized with anhydrous sodium carbonate and then diluted with 2.5 liters of water. The methyl esters of the fatty acids were extracted with ether and dried over anhydrous sodium sulfate for about 3 days. The ether was removed by distillation, the last traces being removed in vacuo at 70°C. and 6-mm. pressure. A yield of 975 gm. of crude methyl esters was obtained.

Fractionation of the Methyl Esters of the Fatty Acids

a) *Description of Distilling Column.* A column packed with a spiral screen as described by Lecky and

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[2] The trade name for a partially hydrogenated vegetable fat.