Rapid Method for Determining Antioxidant Activity and Fat Stability[1]

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ABSTRACT

The acceleration of lipid oxidation by hemin was used to determine antioxidant activity and either fat or oil stability. The test system consisted of a fat or oil emulsion dispersed in phosphate buffer (pH 7.2). The temperature of the system was maintained at 45 ± 1 C. The antioxidant was added to the fat before emulsification. The rapid rate of oxygen uptake which occurred on the addition of hemin to the emulsion was measured with a Beckman oxygen analyzer and was recorded automatically. All fats and oils tested exhibited a typical induction period, which could be increased by the addition of an antioxidant. This increase in the induction period was related to antioxidant activity. The age and purity of the hemin solution, the concentration of hemin in the emulsion, the age and pH of the emulsion, the peroxide value of the fat, and the temperature affect the induction period and the rate of oxygen uptake. For a given set of conditions, the induction period was reproducible, although the rate of oxygen uptake was not. The activities of several antioxidants (butylated hydroxyanisole, propyl gallate, *tertiary*-butylhydroquinone, and Topanol) and synergists (ascorbic acid, citric acid, and ethylenediaminetetraacetic acid), determined by the hemin-catalyzed oxygen uptake method, are compared with those obtained by the active oxygen method.

INTRODUCTION

The conventional methods (1) of determining fat stability and antioxidant activity are, in most cases, time consuming. The active oxygen method (AOM) (2), the Schaal oven method (3), shelf-storage tests (1), and a number of oxygen absorption methods (1) give reliable results but require considerable time to complete. Although many industrial laboratories use AOM routinely for grading fats and oils, it seemed that a more rapid method would be highly desirable. An accelerated lipid oxidation technique appeared to offer the best solution to this problem.

Hamilton and Tappel (4) developed a polarographic, hemoglobin-catalyzed, oxygen uptake method for determining antioxidant activity. A method reported by Kendrick and Watts (5) employs heme compounds to catalyze lipid oxidation. The fatty acid-antioxidant systems investigated by either Hamilton and Tappel or Kendrick and Watts were different from those normally encountered in our laboratory. Animal fats, e.g. chicken, turkey, and lard, and both hydrogenated and unhydrogenated vegetable oils are of special interest to us. Therefore, we attempted to develop a rapid oxygen uptake technique based upon our own requirements.

EXPERIMENTAL PROCEDURES

The animal fats, either chicken, turkey, or lard, were obtained by a hot water rendering procedure, filtered through Johns-Manville Celite no. 560, and partially deodorized to reduce the peroxide value (PV) to a range of 0.1 or less. Antioxidants were added as per experimental design.

The vegetable oils were used as received, and antioxidants were added as indicated.

The Beckman oxygen analyzer model 777 was coupled to a Sargent recorder model SR with a chart speed of 0.5 in./min.

The hemin, either Hemin 3X crystalline or chorohemin (Teichmann's crystals, mol wt 651.9), was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. The hemin solution was prepared by dissolving 52 mg hemin in ca. 75 ml water to which was added just enough 10% KOH to bring about solution (usually 2-4 drops). The solution was diluted with water to a final volume of 100 ml.

The antioxidants butylated hydroxyanisole (BHA), propyl gallate (PG), and *tertiary*-butylhydroquinone (TBHQ), were obtained through the courtesy of Eastman Chemical

[1]Presented at the AOCS Fall Meeting, Atlantic City, 1971.

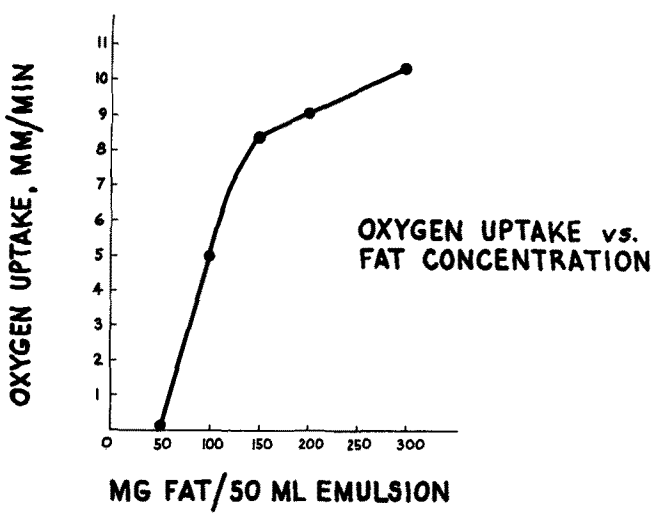

FIG. 1. Variation of oxygen uptake rate with changes in turkey fat concentration. 45 C, pH 7.2, 50 ml, 2.6 mg hemin.

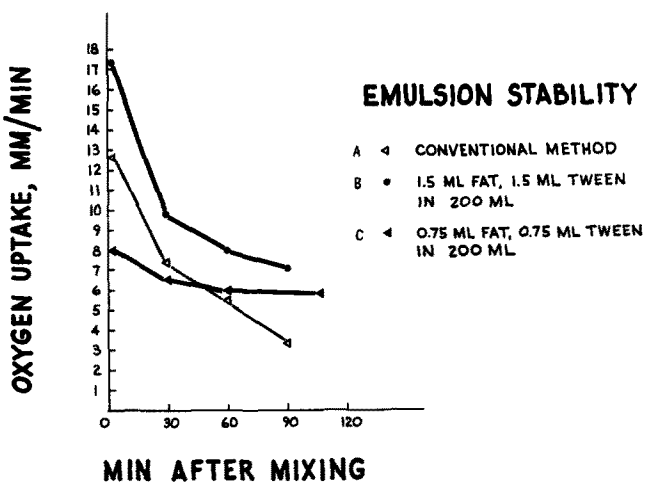

FIG. 2. Effect of emulsion preparation and stability upon oxygen uptake rate.

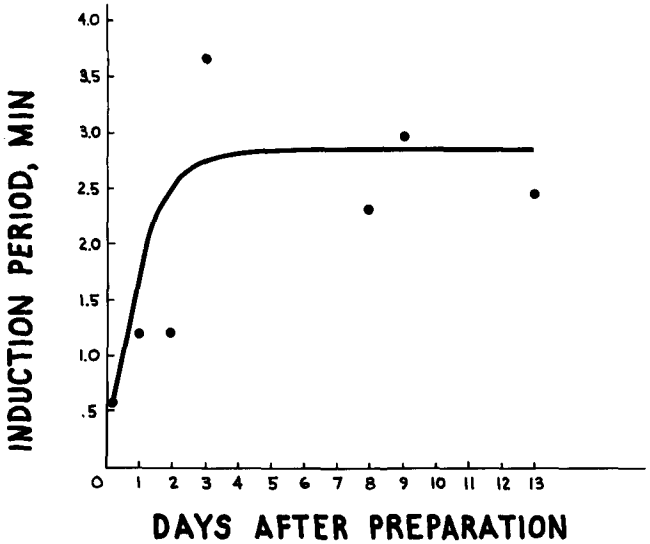

FIG. 3. Induction period obtained with 3X crystalline hemin.

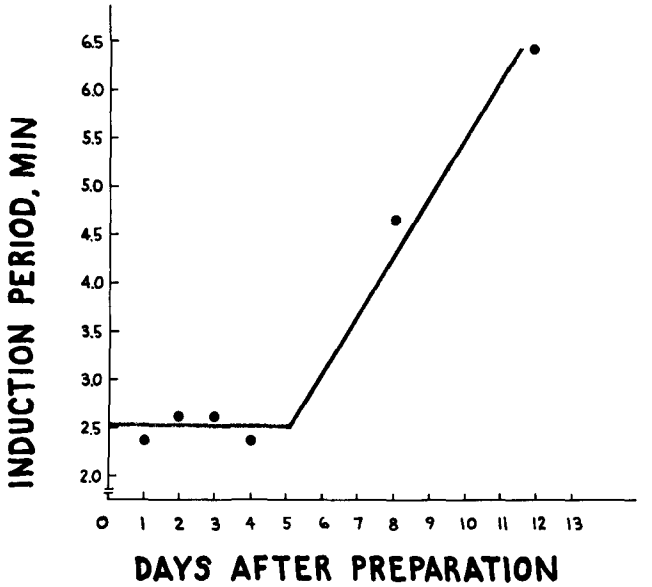

FIG. 4. Induction period obtained with chorohemin 1X crystalline.

Products, Rochester, N.Y. Topanol (3,5-di-*t*-butyl-4-hydroxy anisole), a molecular composite of BHA and butylated hydroxytoluene (BHT), was obtained through the courtesy of Imperial Chemical Industries Ltd., Billingham, England.

For the assay, the fat or oil emulsion was prepared by adding 6 ml 0.1 M pH 7.2 phosphate buffer at 45 C to 2.4 g Tween 20 and 1.5 g fat or oil, also maintained at 45 C. The emulsification was accomplished by passing the buffer-oil-Tween mixture through a hypodermic syringe 20 times. This operation also was carried out at 45 C. An aliquot of 1.2 ml emulsion was added immediately to 46.3 ml 0.05 M pH 7.2 phosphate buffer contained in a 50 ml Erlenmeyer flask, giving a final lipid concentration of 180 mg/50 ml. The contents of the flask were stirred continually and maintained at a temperature of 45 C by means of a combination hot plate-magnetic stirrer. Immediately after adding the emulsion, 2.5 ml hemin solution was added as rapidly as possible to the buffered emulsion, giving a final hemin concentration of 1.3 mg/50 ml. The oxygen electrode was inserted quickly into the neck of the Erlenmeyer flask, and the recorder was started. (For a 10 min period prior to the analysis, the lower end of the oxygen electrode was immersed to a depth of 3 cm in a 45 C water bath.) The rate of oxygen uptake was recorded automatically. A curve was obtained which resembled a typical plot of AOM time vs PV (2), i.e. there was an induction period, characterized by little or no oxygen uptake, followed by a rapid rate of oxygen uptake. The rate of oxygen uptake was usually on the order of 20 mm/min (equivalent to 20 mm of mercury), although rates ranging from 7 mm/min-83 mm/min were observed, depending upon the nature of the lipid sample.

In the course of this investigation, the effects of such variables as the lipid concentration, the age of the emulsion, the age of the hemin solution, the initial PV of the lipid sample, the lipid/hemin ratio, and the pH were studied.

RESULTS AND DISCUSSION

Initially, an attempt was made to carry out the lipid oxidation at room temperature, using a Tween 20-turkey fat emulsion and hemin as a catalyst. Over periods of 1-3 hr, no oxygen uptake was observed, even though several different molar ratios of hemin to fat, ranging from 9-1800, were tried. When the temperature of the emulsion was raised to 45 C, rapid oxygen uptake rates on the order of 5 mm/min were observed with the Tween 20-turkey fat emulsions. When an antioxidant was added to the system, the oxygen uptake rate decreased to 0.6 mm/min. The temperature of 45 C was chosen, because, at lower temperatures, the reaction either did not occur or the reaction rate was too slow to offer any time saving advantage. At temperatures above 50 C, the reaction rate was so rapid that no induction period was observed. At this point, we standardized on the procedure described previously and proceeded with the investigation.

The turkey fat concentration was varied from 50 mg-300 mg/50 ml, while the hemin concentration was kept constant at 2.6 mg/50 ml. The points on the curve in Figure 1 are averages of at least three determinations. An increase in the rate of oxygen uptake was observed as the turkey fat concentration was increased.

Tween 20-turkey fat emulsions were prepared in 2 different ways. In Figure 2, curve A was obtained using the method described under "Experimental Procedures." Curves B and C were obtained by using emulsions prepared by first mixing the fat with the Tween 20 and then dispersing this emulsion in 200 ml pH 7.2 phosphate buffer. This method was a variation of the procedure described by Surrey (6) and used by Kendrick and Watts (5). The rate of oxygen uptake was measured at 0, 30, 60, and 90 min after preparing the emulsion. Each point in Figure 2 is the average of three determinations. It appears that the emulsions broke down at a rapid rate. As the emulsion separated, there was a corresponding decrease in the oxygen uptake rate. It also appeared that the rate of oxygen uptake is dependent upon the method used for preparing the emulsion. During this phase of the investigation, a variation in the rate of oxygen uptake was noted, which was small with different amounts of the same fat in emulsions prepared in the same way (± 1-3 mm O_2/min) but which was large with different fats (± 10-15 mm O_2/min). The induction period, however, although different for different lipids, was reproducible for each particular lipid. Therefore, the induction period was used as an index of oxidative stability, rather than the rate of oxygen uptake. (As related to this work, the induction period was taken to be the time interval recorded beginning immediately after the immersion of the oxygen electrode in the emulsion-hemin mixture and ending just prior to the beginning of rapid oxygen uptake.) Blank determinations, using either the hemin alone or the lipid emulsion alone, resulted in no oxygen uptake.

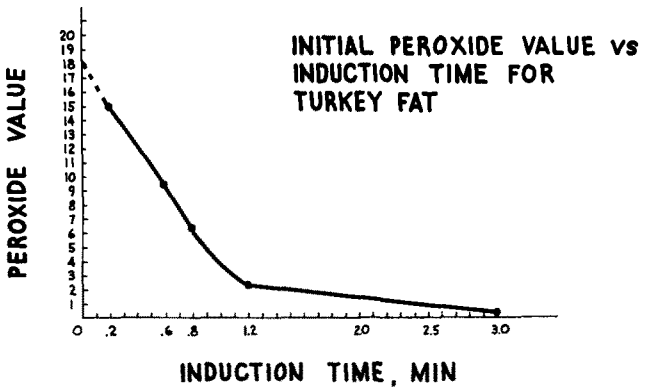

FIG. 5. Effect of peroxide value on induction period for turkey fat.

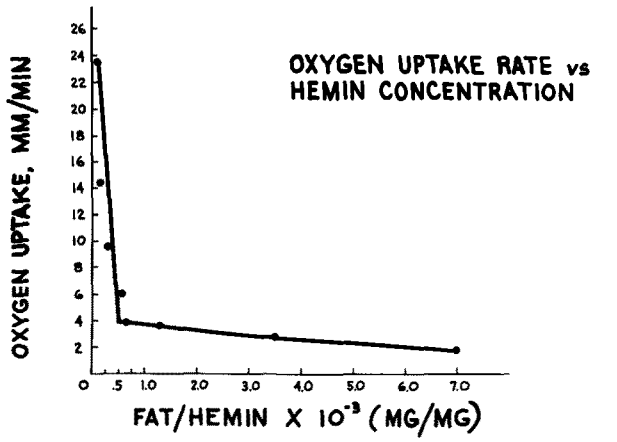

FIG. 6. Effect of fat/hemin ratio upon oxygen uptake rate. Turkey fat concentration constant at 180 mg/50 ml.

TABLE I

Induction Period and Oxygen Uptake Rate vs pH

Lipid	pH	Induction period, min	mm O_2/min
Turkey fat	3.0	3.0	18.8
Turkey fat	5.0	1.8	20.5
Turkey fat	7.0	3.2	22.5
Turkey fat	9.0	6.8	9.2
Linoleic acid	3.0	None	67.0
Linoleic acid	5.0	None	57.5
Linoleic acid	7.0	None	83.5
Linoleic acid	9.0	None	None
Methyl oleate	7.0	None	None
Linolenic acid	7.0	None	None

TABLE II

Antioxidant Activity by Active Oxygen Method vs Oxygen Uptake[a]

Antioxidant	Antioxidant index[b]	
	O_2 Uptake	Active oxygen method
0.02% BHA	13.6	8.3
0.02% PG	3.1	31.3
0.02% TBHQ	1.0	25.8
0.02% Topanol	16.2	39.6
0.02%/0.03% BHA/AA	44.5	44.2
0.02%/0.03% BHA/CA	19.6	12.5
0.02%/0.03% BHA/EDTA	15.4	9.7
0.05% 2-BHA	34.0	4.5
0.05% 3-BHA	50.0	6.8
0.05% Tenox-II	9.0	12.3

[a]BHA = butylated hydroxyanisole, PG = propyl gallate, TBHQ = *tertiary*-butylhydroquinone, AA = ascorbic acid, CA = citric acid, and EDTA = ethylenediaminetetraacetic acid.

[b]Based upon induction time by oxygen uptake and time to reach peroxide value 20 by active oxygen method. In all cases, lard was used.

TABLE III

Antioxidant Effectiveness[a] by Active Oxygen Method vs Oxygen Uptake

Oxygen uptake:	Topanol > BHA > PG > TBHQ BHA/AA > BHA/CA > BHA/EDTA
Active oxygen method:	Topanol > PG > TBHQ > BHA BHA/AA > BHA/CA > BHA/EDTA

[a]Determined in lard, using the antioxidants at 0.02% and synergists at 0.03%.

[b]BHA = butylated hydroxyanisole, PG = propyl gallate, TBHQ = *tertiary*-butylhydroquinone, AA = ascorbic acid, CA = citric acid, and EDTA = ethylenediaminetetraacetic acid.

The addition of 0.1% linoleic acid to the deodorized fat did not affect the induction period nor the rate of oxygen uptake. Emulsions containing only linoleic acid oxidized extremely rapidly, showing no induction period and an oxygen uptake rate of 83 mm O_2/min. Therefore, it appears that, while low levels of free fatty acids do not affect either the induction period nor the rate of oxygen uptake, unusually high levels of free fatty acids might be expected to shorten the induction period and increase the rate of oxygen uptake.

The next variable investigated was the effect of the age of the hemin solution itself upon oxygen uptake. As shown in Figure 3, the 3X crystalline hemin preparation gave a short induction period when used immediately. The induction time, however, increased during a 3 day period and then leveled off. The results tended to be erratic. After the initial 3 day period and over an additional 10 days, the induction period varied from 2.2-3.6 min with a standard deviation of ± 0.5 min.

In contrast to the hemin described in Figure 3, chorohemin, once crystallized hemin with a mol wt of 651.9, gave reproducible induction time during the 4 day period after preparation, as shown in Figure 4. During the 4 day period after preparation, the induction time varied from 2.8-2.4 min with a standard deviation of ± 0.1 min. After the 4 day period, the induction time increased rapidly from day to day. Because this preparation gave reproducible results and seemed stable during a 4 day period, the chorohemin was used in all subsequent analyses. After a 4 day period, a fresh solution was prepared.

Turkey fat with an initial PV of 0.1 was allowed to oxidize in an AOM apparatus until it reached PV of 2.6, 6.4, 9.5, and 14.9. An emulsion was prepared with the fat and the induction period noted. The results are shown in Figure 5. The higher the PV, the shorter the induction time. The data suggest that as the end of the induction period is reached, the PV approaches 18.20.

The turkey fat concentration was kept constant at 180 mg/50 ml. The hemin concentration was varied from 0.026-2.6 mg/50 ml. When the fat/hemin ratio (mg/mg) was varied between ca. 7000 and 500 (represented by the lower right-hand portion of the curve in Fig. 6), the rate of oxygen uptake was low, on the order of 3-4 mm/min or less. The induction period decreased from 5 min to 0 as the fat/hemin ratio was decreased from 7000 to 500. Then, as the fat/hemin ratio became less than 500, the rate of oxygen uptake increased dramatically from 4 to 23 mm/min (represented by the left-hand portion of the curve in Fig. 6). At the same time, an induction period was observed again, which remained in the range of 1.2-1.6 min as the fat/hemin ratio was varied from 500-50. Thus, it

TABLE IV

Antioxidant Activity of Eugenol

mg Percent[a] eugenol	Oxygen uptake	
	Induction period, min	mm O_2/min
0	2.2	23
0.05	2.2	23
0.25	4.0	8
0.50	5.8	5
1.00	14.0	1.5

[a]mg Percent of total assay mixture (50 ml), using chicken fat; initial PV < 0.1.

appears that at high fat/hemin ratios, insufficient hemin is present to bring about a rapid decomposition of lipid peroxides. As a result, the low oxygen uptake rate is observed. As the hemin concentration is increased, the induction period decreases, and the rate of oxygen uptake increases. Exactly why at higher hemin concentrations (fat/hemin < 500), an induction period should be observed is not obvious. Perhaps, as discussed by Kendrick and Watts (5), this could be attributed to heme complexes which temporarily interrupt the oxidative chain reaction mechanism.

Table I shows the effect of pH upon the induction period and oxygen uptake rate. As the pH of the turkey fat emulsions was increased from 3.0-9.0, the induction period reached a minimum at pH 5.0. At pH 9.0, the induction period was at its maximum, and the rate of oxygen uptake was at its lowest. In the case of linoleic acid, no induction period was ever observed. Only a rapid rate of oxygen uptake was observed, which increased with increasing pH. We believe that the reason no induction period or oxygen uptake was observed in the case of linolenic acid at pH 9 and in the case of methyl oleate and linolenic acid at pH 7 was that these substances were oxidized considerably during the emulsification process.

When individual antioxidants were compared, their effectiveness was markedly different, depending upon whether the AOM (2) or oxygen uptake method was used. In Table II, the indices obtained by the AOM are compared with those obtained by the oxygen uptake method. In the case of the AOM, the antioxidant index is expressed as a ratio of the time required for an antioxidant containing sample to reach a PV of 20 to the time required for a control (same fat or oil sample without antioxidant) to reach a PV of 20. Similarly, the antioxidant index determined by the oxygen uptake method is the ratio between sample induction time and control induction time. For antioxidants without synergists, there was a wide variation in antioxidant indices for certain antioxidants. For example, at a level of 0.02%, PG and TBHQ had indices of 3.1 and 1.0 by the oxygen uptake method, but 31.3 and 25.8 by the AOM, respectively. In the case of the antioxidant-synergist combinations, the agreement between the two methods is considerably better, especially with the BHA/ascorbic acid combination. By both methods, it appears that the 3 isomer of BHA is a better antioxidant than the 2 isomer. Table III summarizes in a general way the order of antioxidant effectiveness as determined by both the active oxygen and oxygen uptake methods.

As shown in Figure 7, with fat or oil samples, the correlation between AOM time (to PV 20) and induction time by the oxygen uptake method is not good. At present, we do not know how great a handicap this is. Other workers (7,8) have shown the AOM method to be inaccurate in estimating the potential shelf-life of shortenings and other products. Although we possibly could see a general

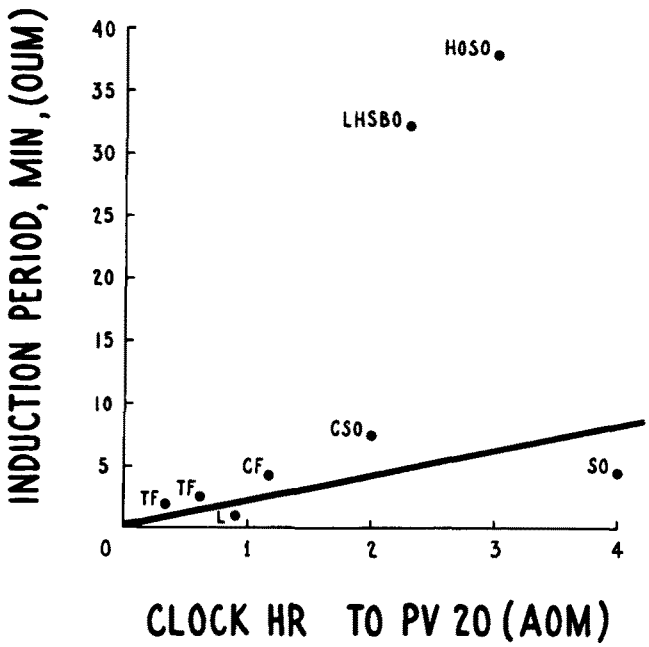

FIG. 7. Comparison of induction periods obtained by active oxygen method with induction periods obtained by hemin-catalyzed oxygen uptake method. TF = turkey fat, L = lard, CF = chicken fat, CSO = cottonseed oil, LHSBO = lightly hydrogenated soybean oil, HOSO = high oleic safflower oil, SO = sesame oil, and PV = peroxide value. Omitting the results for LHSBO and HOSO, a correlation coefficient of 0.62 was obtained.

trend of increased induction time with increased AOM time, this did not hold true in all cases. Thus, samples of lightly hydrogenated soybean oil and high oleic safflower oil had induction times as determined by the oxygen uptake method, which were ca. seven-eight times longer than would be expected from the AOM data. Attempts to correlate induction times with unsaturated fatty acid composition were also unsuccessful.

It would appear that the induction time is a composite of such variables as initial PV, natural antioxidant content, fatty acid composition, and possibly triglyceride composition. It is also possible that the mechanism operative during the induction period is different in the active oxygen and hemin-catalyzed oxygen uptake methods. In at least one instance, the physical state of the antioxidant-lipid mixture seems to be important in determining the effectiveness of the antioxidant. For example, Chipault, et al., (9) have shown that some spices (cassia, cinnamon, pepper, turmeric, and clove) are particularly effective in retarding oxygen absorption by aqueous lard emulsions but extremely poor in preventing the oxidation of lard alone under AOM conditions. As shown in Table IV, we have found that eugenol, a flavor constituent of cloves, is effective in prolonging the induction period and decreasing the rate of oxygen uptake in aqueous chicken fat emulsions. At comparable levels and at a level of 0.1% in chicken fat alone under AOM conditions, eugenol showed no antioxidant activity. Therefore, it would appear that the hemin-catalyzed oxygen uptake method can provide a better index of antioxidant potency in certain instances. It is evident that more work is needed with this method to evaluate its usefulness in correlating antioxidant activity with the stability of lipid bearing products under certain conditions of storage, for example, at room temperature and below.

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