Comparison of Antioxidants for Fats on an Equivalent Molar Basis[1]

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IN THE LITERATURE concerning antioxidants for fats the different investigators usually have compared various antioxidants on an equal weight percentage basis (1, 2, 3, 4, 5). Although valuable information has been obtained for a large number of compounds, quantitative comparisons of effectiveness are difficult or impossible to make. Morris and Riemenschneider compared various compounds on an equivalent molar basis within each series but expressed the over-all data on an equal weight percentage basis (6). In a study of antioxidants for carotene in mineral oil (7) Bickoff compared more than 100 phenolic compounds on an equivalent molar basis.

In attempts to provide a quantitative expression for comparison of antioxidants for fats the terms "protection factor" and "antioxidant index" have been used. Both are defined as the ratio of induction period of treated sample to that of untreated substrate. Riemenschneider *et al.* have presented data which indicate that comparisons and correlations based on these figures are valid only if the data are obtained with precisely the same substrate (5). This restriction seriously limits the value of the data in the literature.

In the present paper evaluations were made on an equivalent molar basis, and results have been expressed in terms of "catechol index." The catechol index is defined as the ratio of the antioxidant activity of a test compound to that of an equivalent molar concentration of catechol. This index is thus a pure number which simultaneously involves all the considerations of concentration of antioxidant, induction period of treated sample, induction period of untreated substrate, and, at least to some extent, the substrate on which the assay was made. Evidence is presented which indicates that, for a given antioxidant, this equivalent will remain nearly constant with different lots of a substrate prepared over a period of several years. These evaluations were made with an oven-incubation method modified to give a degree of reproducibility higher than that reported for other methods (8, 9, 10).

Experimental

Materials and Methods

Oven. A standard laboratory air-oven was equipped with an efficient air agitator and thermostat. The range of variations in temperature, 100 ±1.5°C., was determined from readings made at all the possible sample positions in the oven.

Glassware. The only items of special equipment used were small glass containers for the experimental samples. Microbeakers[4] (inside diameter 11.2 ±0.05 mm. and height approximately 1 cm.) were placed in Petri dishes (9 cm. in diameter). The procedure used in cleaning glassware closely resembled that outlined by Fore *et al.* (4), except that the microbeakers were given an additional treatment with hot conc. H_2SO_4-conc. HNO_3 mixture before rinsing. The glassware has been in use for several months without noticeable loss of precision or accuracy in the tests.

Antioxidants. The compounds tested were the best quality available from the manufacturer, and many were used without further purification. Those which were recrystallized (Table 3) showed little change in activity with purification.

Substrate. Bleached and deodorized moisture-free lard served as the substrate for all the tests reported here. The lard, as received, contained 0.7–1.0% moisture which was removed as the benzene azeotrope by heating the lard, in batches of 1.5 kg., with 250 ml. of benzene at 145°C. for about 2 hrs. or at 100°C. for 6 hrs. After the excess benzene was removed by distillation, the lard was cooled to about 50°C., transferred to 500-ml. glass-stoppered flasks, and stored at −20°C. During the drying and cooling the lard was stirred vigorously in a stream of nitrogen. Successive batches were designated as Lards A, B, C, and D.

Procedure for Testing Antioxidants. An appropriate amount of the antioxidant (500 micromols of catechol or 250 micromols of dimeric-type compounds, such as NDGA) was dissolved in freshly distilled ethanol, and the solution was made up to 50.0 ml.

A 5.0-ml. portion of this solution was added to 50.0 g. of the lard which had been melted and weighed into a 125-ml. Erlenmeyer flask. After thorough mixing the ethanol was removed under reduced pressure by holding the mixture at 80°C. for 10 min. with frequent shaking. An automatic pipette, standardized for the purpose, was used to transfer 0.2 g. of the antioxidant-substrate mixture, containing 1 micromol of the antioxidant per gram of substrate, to each of about 20 microbeakers. In an alternate procedure transfers were made with a modified Mohr pipette which had been heated at the 0.3-ml. mark, drawn out and cut to deliver 0.2 g. of fat. Deliveries with this pipette were quite reproducible (*ca.* 2.5% error). The microbeakers were placed in a covered Petri dish in the air oven at 100 ±1.5°C. To follow the course of the autoxidation reaction, at hourly intervals one or more microbeakers were removed without taking the Petri dish from the oven. The peroxide value (expressed in millimoles of peroxide oxygen per kilogram of fat) was determined by the method of Wheeler (11) as adapted to small samples by Riemenschneider *et al.* (12) and further modified in this laboratory. For this determination the 0.2-g. sample in the microbeaker was placed in a 50-ml. Erlenmeyer flask; 3 ml. of glacial acetic acid and freshly redistilled chloroform (3:2) and 1 ml. of saturated KI solution were added. After 1 minute 10 ml. of distilled water and 4 drops of 1.0% starch solution were added, and the liberated I_2 was titrated with

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0.01 N $Na_2S_2O_3$. After the final stage of the induction period was reached (peroxide value 15 or more), three microbeakers were removed for each hourly reading to provide an indication of the reliability of the peroxide values obtained.

To minimize pro-oxidant activity of the antioxidants and to avoid inconveniently long induction periods, the more active antioxidants were tested at concentrations of 0.25 or 0.5 micromol per gram of substrate. The relationship between concentration of antioxidant and the corresponding increase in induction period was a straight line for NDGA, catechol, and hydroquinone up to 3 micromols per gram of lard (less than 0.05% by weight). Although all of the more active compounds were tested at lower concentrations, some of the inactive compounds were tested at a level of 5 micromols per gram of lard.

Calculations. All results were expressed in terms of the "catechol index." Catechol was selected as the standard for reference because of its reproducibility as an antioxidant, its stability on the shelf, and the availability of good quality commercial samples.

$$\text{Catechol index} = \frac{\frac{I_a - I_o}{M}}{I_c - I_o}$$

In this equation I_o is the induction period of lard alone, I_c is the induction period of the lard which contains 1 micromol of catechol per gram, I_a is the induction period of the lard containing the antioxidant to be tested, and M is the concentration of the antioxidant in micromolar equivalents per gram of lard. The induction period of a sample is taken as the point, on a peroxide value *vs.* time graph, at which a peroxide value of 20 intersects a regression line fitted to peroxide values between 15 and 80. The point can be read from the graph or, more precisely, calculated from the equation

$$x = \frac{\bar{y} + b\bar{x} - 20}{n}, \text{ where } \bar{x} = \frac{Sx}{n} \text{ and } \bar{y} = \frac{Sy}{n};$$

x is the time in hours, y the peroxide value, b the regression coefficient or average deviation in y with respect to x.

Observations Concerning the Method. The procedure used initially for testing antioxidants involved the incubation of a 10-g. sample of antioxidant-substrate mixture, from which a 0.2-g. aliquot was taken periodically and the peroxide value was determined. The intermittent removal of the sample from the oven to take aliquots during the induction period, even for periods as short as 15 seconds once each hour, was found to lengthen the induction period materially and erratically, probably because of the resultant cooling. The use of a number of small samples made possible the removal of only enough material for a test, without disturbing the remainder. Since the inside diameter of the microbeaker was soon observed to affect the rate of peroxide development, the tolerance in beaker sizes was restricted as stated above.

The use of dried lard gave far better reproducibility in the results than the use of moist lard as received. During drying the peroxide value of the lard fell from about 2 to 0. The induction period of the dried lard alone was slightly longer than that of the moist lard, and the induction periods of the dried lard-antioxidant mixtures were considerably longer than those obtained with the corresponding moist lard-antioxidant mixtures. When water was added to the dried lard-antioxidant mixtures, the induction periods were erratic. These results were in agreement with the work done with dry and moist air with the active oxygen method by Nagy *et al.* (13).

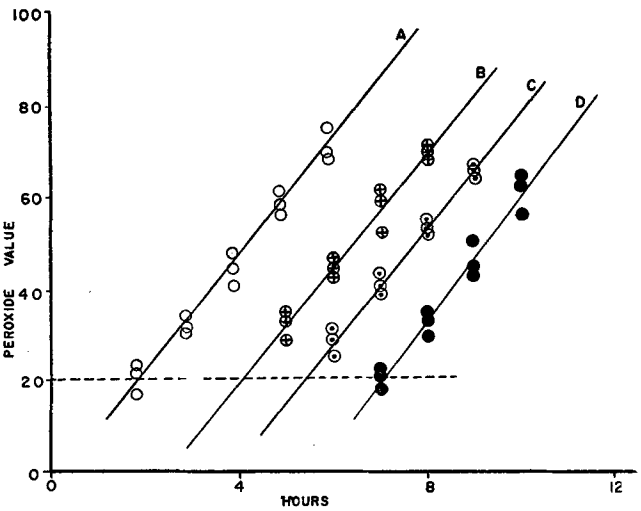

FIG. 1. Regression curves showing replicate data with different antioxidants.
A. Lard alone.
B. Hydroquinone (1.0 micromol per gram of lard).
C. Catechol (1.0 micromol per gram of lard).
D. NDGA (0.5 micromol per gram of lard).

Results and Discussion

Figure 1 shows some typical results obtained with this method. The regression lines were plotted to show the order of agreement between slopes of different stability curves. Of 48 regression lines calculated, the slopes of two samples differed highly significantly from the average of the other slopes obtained that day, as shown by an analysis of variance. Since the divergent slopes were taken to be an indication of a competing reaction and hence of contamination, the data for these samples were disregarded.

The reproducibility of the method is illustrated in Table I. The over-all average variation was 2.07%. Table II illustrates the calculation of the catechol index and shows the results for NDGA from the data obtained with four different batches of lard. The agreement between these data (1.30, 1.39, 1.47, 1.42) indicates the validity of comparisons of catechol indexes determined with different lards. Table III lists the indexes calculated for 28 compounds.

TABLE I

Variation in Induction Periods Between Replicates
(Analyses made on different days)

Lard	Antioxidant	Conc.[a]	Induction periods	Average variation [b]
			(hours)	%
A	None	....	2.18, 2.10	1.9
B	None	....	2.19, 2.01, 2.25	4.2
C	None	....	1.88, 2.06	4.6
D	None	....	1.90, 1.95, 1.90	2.0
A	NDGA	0.5	9.04, 8.99, 9.29	1.3
B	NDGA	0.5	9.96, 9.78	0.9
C	NDGA	0.5	9.95, 10.20, 9.85	1.3
D	NDGA	0.5	7.00, 7.14, 7.01	0.8
A	Catechol	1.0	7.57, 7.48	0.5
D	Catechol	1.0	5.47, 5.60, 5.53	0.7
D	Hydroquinone	1.0	4.54, 4.66, 4.16	4.5

[a] Micromols per gram of lard.
[b] Average variation from the mean value, expressed as percentage of the mean value.

TABLE II
Comparison of Stabilizing Effects of Catechol and NDGA on Four Different Lards

Lard	Antioxidant	Conc.[a]	Induction periods		Catechol index[b]
			Observed	I_a-I_o	
			(hours)	*(hours)*	
A	None	....	2.14	.....	.....
	NDGA	0.5	9.11	6.97	1.30
	Catechol	1.0	7.52	5.38	1.00
B	None	....	2.15	.....	.....
	NDGA	0.5	9.87	7.72	1.39
	Catechol	1.0	7.74	5.59	1.00
C	None	....	1.97	.....	.....
	NDGA	0.5	10.00	8.03	1.47
	Catechol	1.0	7.43	5.46	1.00
D	None	....	1.90	.....	.....
	NDGA	0.5	7.05	5.15	1.42
	Catechol	1.0	5.52	3.62	1.00

[a] Micromols per gram of lard.
[b] Ratio of activity to that of an equivalent amount of catechol.
I_o—induction period of lard alone.
I_a—induction period of lard with antioxidant.

The use of a calibrated Mohr pipet instead of an automatic pipet had no statistical effect on the catechol indexes but did decrease the spread in peroxide values within the three samples tested each hour. Drying the lard at 100°C. for 6 hrs. instead of 145°C. for 2 hrs. also had no statistical effect on the indexes, but the lower temperature was used to insure a minimum of degradation or polymerization.

Relationships between structure and antioxidant activity were shown in several cases (Table III). The monohydroxybenzenes, with the exception of the di-*tert*-butyl-*p*-cresols, were inactive. The meta-, di-, and trihydroxybenzenes showed little or no activity. The *ortho* and *para* dihydroxybenzenes, taken as a whole, had about equal activities. Within the *ortho* trihydroxy series the ester grouping reduced the activity more than the free carboxyl group as compared to the parent compound. The effect probably resulted from the synergistic action of the free carboxyl group (4), which would tend to mitigate the deactivating effect.

TABLE III
Comparison of 28 Compounds Tested as Antioxidants for Lard

	Catechol index
Monohydroxy compounds	
Phenol	0.0
Salicylic acid	0.0
m-Hydroxybenzoic acid	0.0
p-Hydroxybenzoic acid	0.0
p-tert-Butylphenol*	0.0
4,6-Di-*tert*-butyl-*m*-cresol	0.31
2,6-Di-*tert*-butyl-*p*-cresol*	2.35
o-Dihydroxy compounds	
Catechol*	1.00
Protocatechuic acid	1.00
Pyrocatechuic acid	0.0
p-tert-Butylcatechol*	1.56
NDGA	1.41[a]
m-Dihydroxy compounds	
Resorcinol	0.0
α-Resorcylic acid	0.0
β-Resorcylic acid	0.0
γ-Resorcylic acid	0.16
p-Dihydroxy compounds	
Hydroquinone*	0.78
Gentisic acid*	1.00
Toluhydroquinone*	0.94
2,5-Di-*tert*-butylhydroquinone*	0.31
α-Tocopherol	0.31
Butylated hydroxyanisole	1.25
Polyhydroxy compounds	
Pyrogallol	3.91
Gallic acid*	2.97
Propyl gallate	1.87
Phloroglucinol	0.16
Hydroxyhydroquinone*	2.66
Hexahydroxybenzene	0.0

* Recrystallized before testing.
[a] Calculated for 0.5 micromol per gram of lard.

The comparative activities of hydroquinone, 2,5-di-*tert*-butylhydroquinone, catechol, and *p-tert*-butylcatechol suggested that a bulky alkyl group activated the compound if *para* to the *ortho* active groups but deactivated if *ortho* to *para* active groups, perhaps by a steric effect. Morris and Riemenschneider also found that alkylated catechol was more effective than catechol itself (6).

The activating effect of *tert* butyl groups was again demonstrated in the butylated cresols although in these compounds the deactivating effect was not apparent. Butylated hydroxy-anisole and α-tocopherol, while special cases, seemed to conform to the observed generalizations.

Hexahydroxybenzene and pyrocatechuic (2,3-dihydroxybenzoic) acid, the inactivities of which are not readily explained on the basis of structure, were both insoluble in lard and hence inactive (17). The slight activities exhibited by two of the *meta* compounds were considered to be the result of impurities or experimental error, or both.

Summary

An oven incubation method for determining the relative effectiveness of antioxidants is described, which has yielded results with an over-all average variation of 2.07%. The antioxidants were compared on an equivalent molar basis, and potencies are expressed as a "catechol index."

The catechol index of a given antioxidant is defined as the ratio of its antioxidant activity to that of an equivalent molar concentration of catechol. It provides a quantitative measure of the relative effectiveness of various antioxidants, which takes into consideration the concentration of antioxidant, the induction periods of the stabilized sample and control substrate, and, at least to some extent, the substrate used.

Catechol indexes of 28 phenolic compounds are given and discussed, and several relationships between structure and antioxygenic activity are pointed out.

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