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A New Efficacy Test of Antioxidants Based on Air-Oxidation of Linoleic Acid[1)]

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Though many methods are available for the efficacy testing of antioxidants, a satisfactory method has not been established yet, because the conventional methods require a long experimental time, considerable technical skill, or large and expensive apparatus.

We have established a new method based on the air-oxidation of linoleic acid, one of the essential fatty acids, by air bubbling (2 h incubation at 60 °C with 500 ml/min air flow rate). Good reproducibility and low variation were obtained by this method. Six tested antioxidants (ascorbic acid, ascorbyl stearate, butyl hydroxyanisole, dibutyl hydroxytoluene, propyl gallate, α-tocopherol) inhibited the oxidation of linoleic acid proportionally to their added concentration. The relative efficacies of the six antioxidants were broadly in agreement with expection.

Keywords——antioxidant; antioxidant efficacy test; air-oxidation; linoleic acid; peroxide value; thiobarbituric acid value

Oxidation of fats and oils produces lipid peroxide, which imparts an unpleasant taste to foods and can induce pathological conditions such as liver dysfunction, vasculitis, hypoplasia and others.[2)] In practice, many natural or synthetic antioxidants have been used widely in oils and fats to prevent such oxidation.

It is well known that the fat-stability tests commonly employed for the evaluation of antioxidant activities of test substances have several disadvantages.[3)] For example, they require expensive apparatus, technical skill, a long experimental time, and a large sample size, and the results show considerable variability. Furthermore, as oils and fats differ form one another in the degree of unsaturation and composition of constituent fatty acids, as well as in constituent glycerides and content of natural antioxidants such as α-tocopherol,[3)] the apparent antioxidant activities of test substances may vary with different lot numbers of the oil or fat.

This paper presents a new and simple method for the determination of the antioxidant activities of various substance. Our method is based on the utilization of linoleic acid instead of oils or fats. Linoleic acid is one of the essential fatty acids and is oxidized easily by air. Our method also gives good reproducibility and low variation, and requires only a short experimental time and simple apparatus.

Experimental

Reagents——Linoleic acid was obtained from Wako Pure Chemical Industries Co., Ltd., and was kept in a freezer until use. Butyl hydroxyanisole (BHA), dibutyl hydroxytoluene (BHT), propyl gallate and ascorbic acid were obtained from Wako Pure Chemical Industries Co., Ltd., and α-tocopherol and ascorbyl stearate were obtained from Tokyo Kasei Kogyo Co., Ltd. Other reagents were of special grade.

Oxidation of Linoleic Acid——Six test tube (25 mm in diameter and 200 mm in length) and an air pump (NS-1, Nippon Jisei Sangyo Co., Ltd.) were joined in series. As the order of the six test tubes did not affect the oxidation of linoleic acid, 5 ml of linoleic acid with or without an antioxidant was added randomly to each test tube. Then the test tubes were put into an isothermal bath (T-80, Tokyo Rikakikai Co., Ltd.) and incubated at a definite temperature and air flow rate for a definite time. After the oxidation, the peroxide value (POV) and thiobarbituric acid value

(TBAV) of the linoleic acid in the test tubes were determined.

POV Determination of Linoleic Acid[4]——A sample of about 1 g was weighed out exactly and dissolved in a mixture of acetic acid–chloroform (3:2, v/v). Saturated KI solution was added to it. The mixture was allowed to stand in the dark for 10 min, then 30 ml of distilled water was added. The whole was titrated with 0.1 N $Na_2S_2O_3$ standard solution using 1 ml of 0.5% starch solution as an indicator.

TBAV Determination of Linoleic Acid[5]——A sample of about 5 mg was weighed out exactly into a test tube. Then, 0.5 ml of 3% sodium dodecyl sulfate (SDS) solution and 1.5 ml of 2.0 M acetic acid buffer solution (pH 3.6) were added with stirring, followed by 1.5 ml of 0.8% TBA aqueous solution and 0.5 ml of distilled water. Then, the mixture was heated for 75 min in a boiling water bath. After that, the solution was chilled for 5 min with tap water, and 1.0 ml of 0.2 N HCl solution and 5.0 ml of a mixture of *n*-butanol–pyridine (15:1, v/v) were added. The whole was shaken vigorously, and the absorbancy of the *n*-butanol layer was measured at 532 nm with a spectrophotometer (Type 124 double-beam spectrophotometer, Hitachi).

Calculation of the Efficacy of Antioxidants——The inhibitory ratio (I.R.) was calculated with the following equation.

$$\text{I.R.} = \frac{A-B}{A-C} \times 100\ (\%)$$

A: POV (TBAV) of linoleic acid after incubation.
B: POV (TBAV) of linoleic acid after incubation with an antioxidant.
C: POV (TBAV) of linoleic acid before incubation.

Results and Discussion

I. Optimum Conditions for the Oxidation of Linoleic Acid

As shown in Fig. 1, a linear relationship between air flow rate (250—750 ml/min) and POV (TBAV) in the oxidation of linoleic acid at 60 °C for 2 h was observed. The POV (TBAV) increased with increase of the air flow rate. The same relation was obtained between incubation time (0.5—5 h) and POV (TBAV) under the conditions of 500 ml/min air flow rate at 60 °C (Fig. 2). Next, the effect of incubation temperature (50—90 °C) under the conditions of 500 ml/min air flow rate for 2 h was examined. The results are shown in Fig. 3. The maximum POV was obtained at 75 °C and TBAV at 80 °C. That is, there was an optimum temperature for oxidation of linoleic acid, though there was a little difference between POV and TBAV.

Further, the effect of incubation time at higher temperature on the oxidation of linoleic acid was examined under the condition of 500 ml/min air flow rate. As shown in Fig. 4, the increase of POV became smaller with elevation of the temperature. The same tendency was observed when TBAV was used as the index of oxidation. The maximal POV was reached in

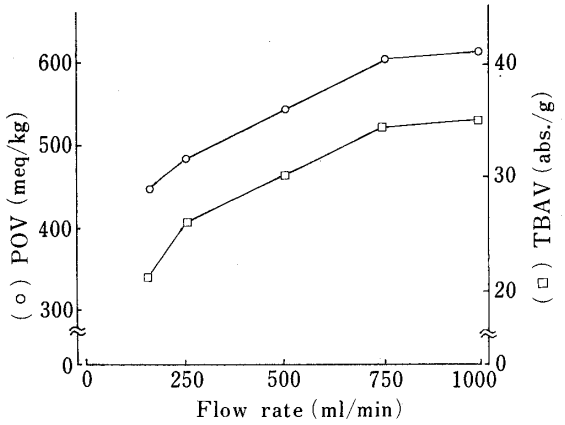

Fig. 1. Effect of Air Flow Rate on the Oxidation of Linoleic Acid

Linoleic acid was incubated for 2 h at 60 °C.

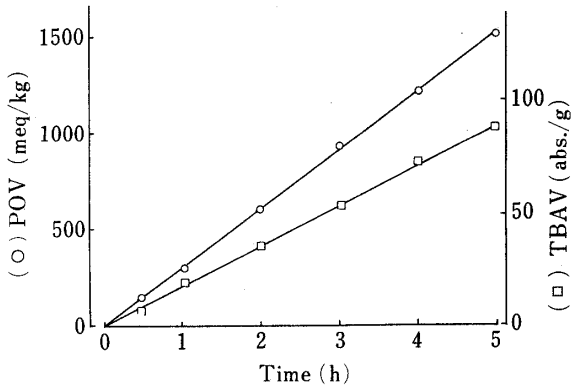

Fig. 2. Effect of Incubation Time on the Oxidation of Linoleic Acid

Linoleic acid was incubated at 60 °C with 500 ml/min air flow rate.

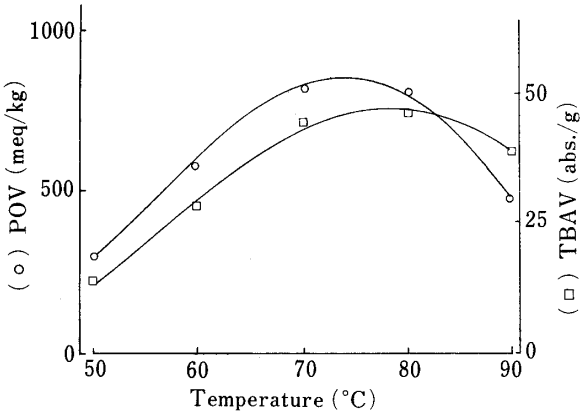

Fig. 3. Effect of Incubation Temperature on the Oxidation of Linoleic Acid

Linoleic acid was incubated for 2 h with 500 ml/min air flow rate.

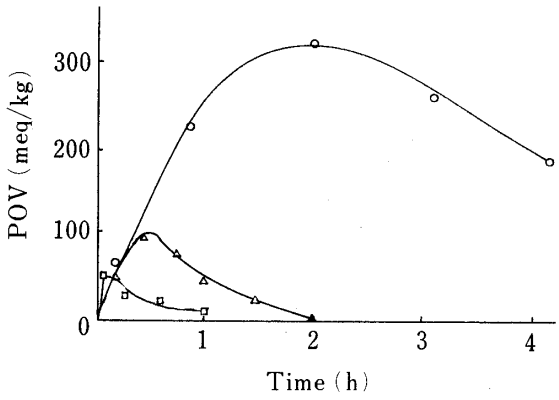

Fig. 4. Time–Course of POV in the Oxidation of Linoleic Acid at High Temperature

Linoleic acid was incubated at 110 °C (○—○), 140 °C (△—△) and 180 °C (□—□) with 500 ml/min air flow rate.

TABLE I. POV and TBAV of Linoleic Acid after Oxidation[a)]

Exp. No.[b)]	POV		TBAV	
	Mean ± S.D. (meq/kg)	C.V. (%)	Mean ± S.D. (abs./g)	C.V. (%)
1	523.8 ± 2.9	0.5	38.6 ± 1.5	3.8
2	525.7 ± 3.7	0.7	38.8 ± 1.4	3.5
3	526.4 ± 3.1	0.6	38.7 ± 1.1	2.9

a) Linoleic acid was incubated for 2 h at 60 °C with 500 ml/min air flow rate.
b) Each experiment consisted of 6 samples.

5 min at 180 °C (45 meq/kg), 30 min at 140 °C (100 meq/kg) and 120 min at 110 °C (310 meq/kg). However, these POV were fairly small compared to that obtained under the conditions of 120 min at 60 °C (544 meq/kg).

From these results, it was concluded that an incubation temperature below 80 °C was preferable to oxidize the linoleic acid by air bubbling. Therefore, we decided to examined the efficacy of antioxidants under the conditions that linoleic acid was incubated for 2 h at 60 °C with 500 ml/min air flow rate. The results of three experiments performed under these conditions are shown in Table I. No significant difference among experiments was observed. The coefficients of variation were 0.5—0.7% for POV and 2.9—3.8% for TBAV. The excellent reproducibility and low variation of our method seems to be superior to those of known methods for the efficacy testing of antioxidants. Further, the experimental time of 2 h seems to be extremely attractive in comparison with AOM[6)] or the oven test.[7)]

II. Application of the Test Method

Six antioxidants, ascorbic acid, ascorbyl stearate, BHA, BHT, propyl gallate and α-tocopherol, which are approved for use in Japan, were tested. The six antioxidants were examined first to determine whether they interfered with the measurements of POV and TBAV of oxidized linoleic acid samples at the concentrations used (10^{-4}—1%). They did not affect the determinations. Next, the antioxidants were tested under the optimum conditions described above (60 °C, 2 h, air at 500 ml/min). As shown in Fig. 5 (POV) and Fig. 6 (TBAV),

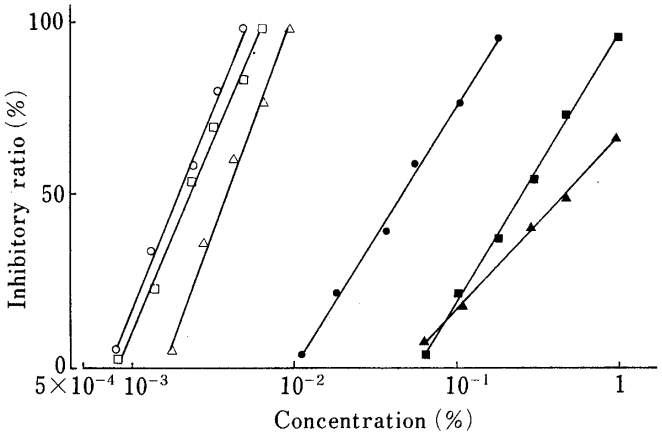

Fig. 5. Efficacies of Antioxidants on the Oxidation of Linoleic Acid (POV)

Each plot represents the mean of 5 samples.
○—○, propyl gallate; □—□, BHT; △—△, BHA; ●—●, ascorbyl stearate; ■—■, α-tocopherol; ▲—▲, ascorbic acid.

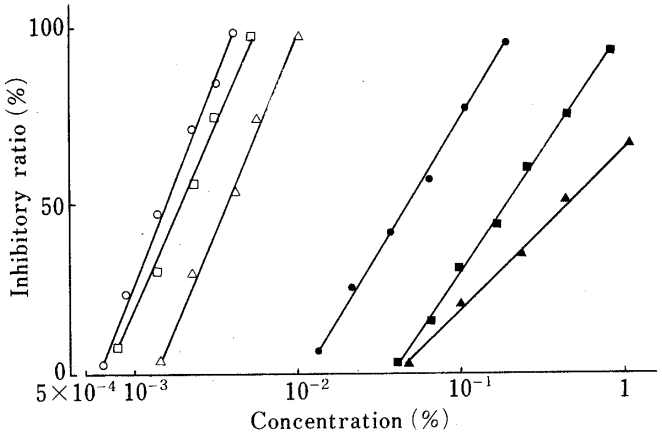

Fig. 6. Efficacies of Antioxidants on the Oxidation of Linoleic Acid (TBAV)

Each plot represents the mean of 5 samples.
○—○, propyl gallate; □—□, BHT; △—△, BHA; ●—●, ascorbyl stearate; ■—■, α-tocopherol; ▲—▲, ascorbic acid.

TABLE II. The 50% Inhibitory Concentration (IC_{50}) Values of Antioxidants

Antioxidant	IC_{50} (%)	
	POV	TBAV
Propyl gallate	1.99×10^{-3}	1.59×10^{-3}
BHT	2.23×10^{-3}	1.91×10^{-3}
BHA	3.73×10^{-3}	3.69×10^{-3}
Ascorbyl stearate	4.49×10^{-2}	4.72×10^{-2}
α-Tocopherol	2.48×10^{-1}	1.94×10^{-1}
Ascorbic acid	4.47×10^{-1}	4.60×10^{-1}

all these antioxidants inhibited the oxidation of linoleic acid proportionally to their added concentration, and linear relationships between antioxidant concentration and inhibition of oxidation were obtained when both POV and TBAV were used as indices of the oxidation.

The 50% inhibitory concentration (IC_{50}) values for the six antioxidants are shown in Table II. The efficacies of the antioxidants used were as follows: propyl gallate > BHT > BHA > ascorbyl stearate > α-tocopherol > ascorbic acid. This order, except for ascorbic acid and ascorbyl stearate, agreed approximately with the order[8] obtained by AOM using a cotton seed oil, a hydrogenated cotton seed oil and a lard. The efficacy of ascorbyl stearate was said to be 40% of that of ascorbic acid.[9] However, ascorbyl stearate was more effective than ascorbic acid in this experiment. This may be because of the low solubility of ascorbic acid in the linoleic acid solution. Further work is necessary on this point.

The maximum concentrations[10] permitted for addition to fats or butters are below 0.02% (w/w) in BHA and BHT, and below 0.01% (w/w) in propyl gallate. Therefore, it was demonstrated in these experiments that the oxidation of linoleic acid was sufficiently inhibited by the addition of antioxidants at lower concentrations than those allowed for food additives.

Based on these results, our method should be useful as simple and reliable procedure for determining the efficacy of antioxidants.

References and Notes

1) A part of this study was presented at the 101st Annual Meeting of Pharmaceutical Society of Japan, Kumamoto, April 1981.
2) H. Kaneda, *Eiyo to Shokuryo*, **30**, 71 (1977).
3) G. Kajimoto, H. Yoshida, A. Shibahara and S. Yamashoji, *Eiyo to Shokuryo*, **34**, 349 (1981).
4) Pharmaceutical Society of Japan, "Hanyo Eisei Shikenho to Kaisetsu," Nanzando, Tokyo, 1976, p. 61.
5) H. Tanizawa, Y. Sazuka, and Y. Takino, *Chem. Pharm. Bull.*, **29**, 2910 (1981).
6) D. H. Wheeler, *Oil Soap*, **9**, 89 (1932).
7) P. O. Pool, *J. Oil Fat Ind.*, **8**, 331 (1931).
8) R. N. Moore and W. G. Bickford, *J. Am. Oil Chem. Soc.*, **29**, 1 (1952).
9) I. Kawashiro, S. Fujii and M. Yoshida, "Food Additives," 3rd ed., Koseikan, Tokyo, 1979, p. 67.
10) Shokuhin Tenkabutsu Koteisho Kaisetsusho Henshuiinkai, "Shokuhin Tenkabutsu Kotei-Kaisetsusho," 4th ed., Hirokawa Shoten, Tokyo, 1979.