# A Rapid Method for Evaluation of Antioxidants

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## Abstract

A rapid, sensitive method for evaluating antioxidants is described. The antioxidant comparisons are based on minimizing β-carotene loss in an emulsified, aqueous, coupled oxidation of linoleic acid and β-carotene. The effects of linoleic acid levels were observed. Attempts to replace β-carotene with vitamin A or linoleic acid with ergosterol gave undesired results. The quantitative applications of the method are discussed.

## Introduction

A DESIRABLE METHOD for evaluating the antioxidant activity of a compound should be rapid, reproducible, should require small amounts of chemical, and should not be influenced by the physical properties of the compound.

Various approaches have been used in measuring antioxidant activity. Thompson (1) determined the antioxidant activity by measuring the extent of carotene preservation in heated alfalfa samples. Olcott and Einset (2) measured the increase of weight of heated oils in the presence of antioxidants, which inhibited the oxygen uptake by the oil. Bickoff et al. (3,4,5) measured the disappearance of carotene dissolved in refined mineral oil in a thin layer in the presence of various antioxidants. Palmateer et al. (6) absorbed fats and antioxidants on Celite for large surface area and measured the development of oxidative rancidity with the 2-thiobarbituric acid reaction. In all these cases, the test required days or weeks until a measurable value was obtained for ranking antioxidants. Clark and Kitchen (7) dissolved the antioxidants in anhydrous lanolin and bubbled preheated air through the solutions followed by peroxide determination. While good curves were obtained in 3 hr, oil insoluble antioxidants required homogenization into the system. The methyl linoleate uptake of oxygen in a Warburg apparatus was found to be a rapid method for evaluating antioxidants (8). Autoxidation in aqueous β-carotene/linoleate systems has been studied in the presence or absence of lipoxidase, but antioxidants were not added to the system (9,10). Blain and Shearer (11,12) incorporated β-carotene, methyl linoleate, and antioxidants in agar gels and measured carotene losses colorimetrically. Even with hemoglobin as a catalyst, up to 100 hr were required for 50% loss of carotene in the presence of effective antioxidants.

This paper describes the development of a rapid, sensitive method for ranking antioxidant activity based upon minimizing β-carotene loss in the coupled oxidation of linoleic acid and β-carotene using an emulsified, aqueous system. The quantitative application of the method is also discussed.

## Experimental

### Apparatus

Emulsifiable concentrates were prepared in 50 ml round-bottomed flasks. Chloroform was evaporated from the concentrates on a rotary evaporator using a water aspirator.

Oxidation reactions were performed in large (25 mm OD by 200 mm length) test tubes fitted with standard taper 24/25 female glass joints. The reaction temperature was maintained by placing the tubes in a constant temperature water bath.

A Coleman Model 14 Universal Colorimeter was used for spectrophotometric measurements.

### Reagents

Crystalline β-carotene (Nutritional Biochemicals Corporation, Cleveland, Ohio).

Purified linoleic acid (solidifying when held at −15C for 12 to 16 hr). (Various sources of highly purified linoleic acid have been used. Melting point was the most useful property in judging the suitability of a particular lot. The acid melts at −9.5C. Any lot that remained an oil after 12 to 16 hr of storage at −15C invariably produced erratic results and overly rapid oxidation rates.)

Tween 40 surfactant (Atlas Chemical Industries, Inc., Wilmington, Delaware).

Vitamin A ester concentrate, 200,000 USP units/g (Nutritional Biochemicals Corporation, Cleveland, Ohio).

Glycerol dichlorohydrin (Eastman Chemical Company, Rochester, N.Y.).

Ergosterol (Sigma Chemical Company, St. Louis, Mo.).

pH 9.0 borate-KCl 0.5 M buffer (13).

pH 7.0 phosphate 0.067 M buffer (13).

Butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), and 2,4,5-trihydroxybutyrophenone (Tennessee Eastman Chemical Products, Inc., Kingsport, Tenn.).

Propyl gallate (Nutritional Biochemicals Corporation, Cleveland, Ohio).

3,3′-Thiodipropionic acid and 3,3′-dilaurylthiodipropionate (Halby Products Company, Inc., Wilmington, Delaware).

Ascorbic acid (Distillation Products Industries, Eastman Organic Chemicals Department, Rochester, N.Y.).

Santoquin® (Monsanto Company, St. Louis, Mo.)—also known as Ethoxyquin or 1,2-dihydro-2,2,4-trimethylquinoline.

### Procedure

*Emulsion Preparation.* Six milligrams of crystalline β-carotene, 1.0 g linoleic acid, and 2.0 ml Tween 40 were dissolved in 20 ml reagent grade chloroform. Due to its viscosity, the Tween 40 was warmed in a water bath at 50C before pipetting. The chloroform was removed at 40C under vacuum using a rotary evaporator. The resulting viscous red oil was immediately diluted with triple distilled water (single distilled water passed through a mixed-bed ion exchange resin column also may be used) to 25 ml in a volumetric flask and was thoroughly mixed. This emulsifiable concentrate can be held in the dark at room temperature as long as 1.5 hr.

Pure oxygen was bubbled through triple-distilled water (or the desired buffer) for 0.5 hr. The 25 ml emulsifiable concentrate was poured into 500 ml of

the oxygenated media using vigorous magnetic stirring. This diluted emulsion must be used immediately.

*Antioxidant Evaluation.* Solutions of antioxidants were prepared in absolute ethanol at a 7.5 μg/ml concentration. At such a low concentration, solubility of antioxidant compounds was rarely a problem. For less soluble materials, 7.5 mg compound was heated in 1.0 ml absolute ethanol directly in a 100 ml volumetric flask and immediately brought to volume with room temperature absolute ethanol. A 0.1 dilution was made immediately using absolute ethanol. A 2.0 ml aliquot of antioxidant solution was placed in the large test tubes to provide 15 μg of antioxidant per tube. The control tube contained 2.0 ml absolute ethanol. Fifty milliliters of the diluted, oxygenated emulsion were added to each tube, glass stoppers were attached, and the tubes were inverted several times for thorough mixing. The stoppers were removed and the tubes immediately placed in racks in a 50C water bath. At desired intervals (10 or 15 min were normally satisfactory), 2.0 ml aliquots from each tube were pipetted directly into the colorimetric cuvettes containing 10.0 ml 95% ethanol. Readings were made against a 95% ethanol blank with the colorimeter set at 450 mμ using a PC-4 filter. A zero-time reading was taken when the diluted emulsion was pipetted into the large test tubes.

*Replace β-carotene with vitamin A.* Solutions of vitamin A ester concentrate (200 μg/ml) and linoleic acid (20 and 40 mg/ml) were prepared in toluene. Ten milliliters of the respective solutions were added to 90 ml toluene saturated with oxygen. Two-milliliter aliquots of an absolute ethanolic Santoquin solution (7.5 μg/ml) were added to large test tubes. Control tubes contained 2 ml of absolute ethanol. Fifty milliliters of the oxygenated toluene solution containing the desired vitamin A/linoleic acid combination were added. The tubes were placed in a 50C water bath. Vitamin A concentrations were measured at 15 min intervals by the glycerol dichlorohydrin method of Sobel and Werbin (14,15). Optical density readings were made on the colorimeter set at 555 mμ using a PC-4 filter.

## Results and Discussion

Since the primary purpose of the method was to evaluate and rank antioxidants, a reasonably long induction time was desired. A long-extended induction time would accentuate the differences between good and poor antioxidants. However, a sufficiently rapid procedure was required for completing a series of comparisons in an 8-hr period. Several parameters were investigated in defining the desired conditions of the test.

*Level of Linoleic Acid.* It has been reported that increasing levels of methyl esters from cottonseed oil acids increased the rate of oxidation of carotene dissolved in a paraffin (16). The linoleic acid level could be a means for controlling induction time if similar results were obtained in the aqueous, emulsion system.

The two linoleic acid concentrations tested were 1.0 and 0.5 g in the 25-ml emulsifiable concentrate solution. The results, in the presence and absence of 5 μg Santoquin/tube, are shown in Fig. 1. Curves A and C, representing the highest level of linoleic acid, show a faster oxidation rate than the lowest level (Curves B and D). Curves C and D show the extension of the induction time by addition of Santoquin.

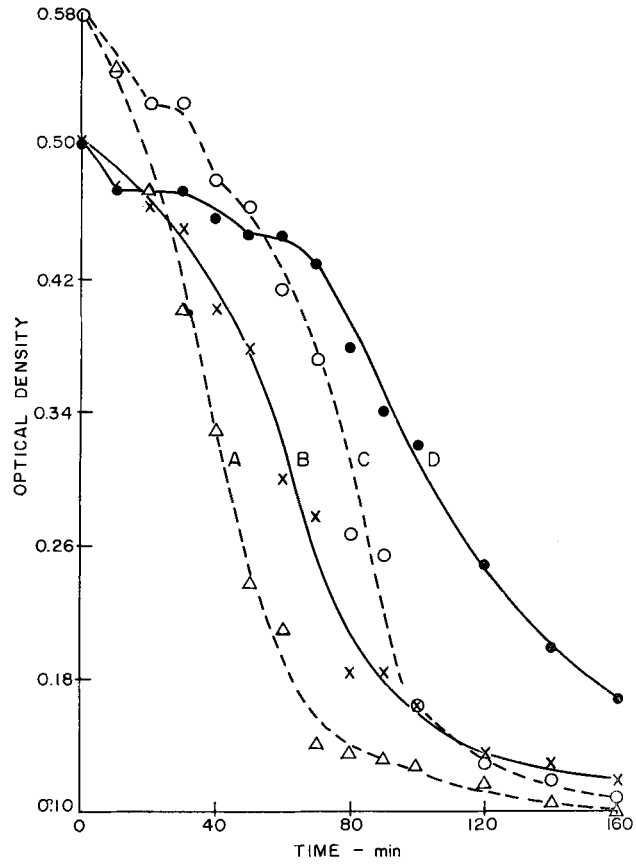


FIG. 1. Effect of linoleic acid level upon oxidation rate. A, no Santoquin and 1.0 g linoleic acid in 25 ml emulsifiable concentrate; B, no Santoquin and 0.5 g linoleic acid in 25 ml emulsifiable concentrate; C, 5 μg Santoquin/reaction tube and 1.0 g linoleic acid in 25 ml emulsifiable concentrate; and D, 5 μg Santoquin/reaction tube and 0.5 g linoleic acid in 25 ml emulsifiable concentrate.

Antioxidants perform their inhibition by various mechanisms, such as peroxide decomposition or chain stopping. If Santoquin is operating by a single mechanism, there should simply be a smooth, horizontal extension of induction time when depicted graphically as in Fig. 1. If the antioxidant has no effect on the autoxidative events, the control and antioxidant curves should be parallel but displaced in time at a particular concentration of linoleic acid. However, the curves in Fig. 1 are not quite parallel and the induction portion shows inflections in the presence of Santoquin (Curves C and D).

Shelton (17) has discussed the differences in oxidation events and rates in the presence and absence of antioxidants, which could explain the nonparallel curve effect. The inflections in the Santoquin curves would suggest that Santoquin performs as an antioxidant by several mechanisms. The 1.0-g linoleic acid level proved suitable for the antioxidant evaluations.

Biological samples, such as feeds and tissues, where antioxidant application was desired normally contain fats. Therefore, no attempt was made to study the aqueous, emulsion system in the absence of linoleic acid.

*Replace Linoleic Acid with Ergosterol.* A consistently good supply of linoleic acid can be difficult to obtain. Since some sterols can be easily oxidized to cyclic hydroperoxides by oxygen, linoleic acid was replaced by ergosterol in the preparation of the emulsion. However, the ergosterol oxidized so rapidly

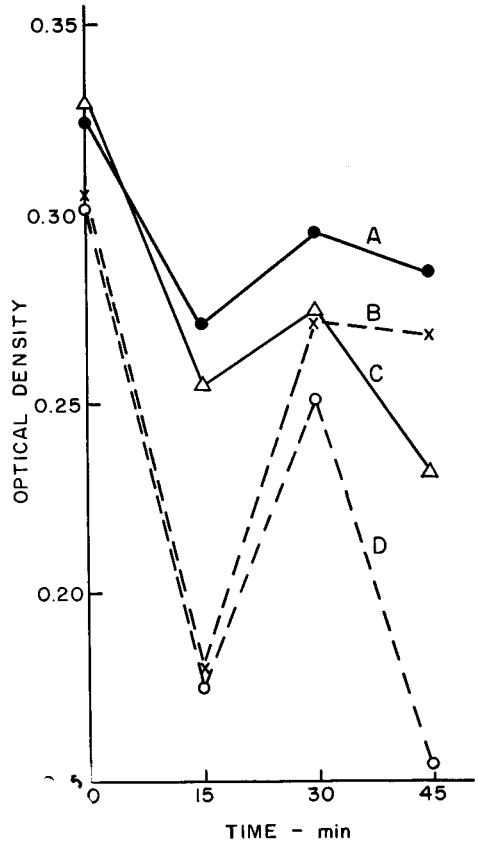


FIG. 2. Replacement of β-carotene by vitamin A. Concentrations are expressed for the final reaction mixture with vitamin A present at 19 μg/ml in all reactions. A, 0.29 μg/ml Santoquin and 1.9 mg/ml linoleic acid; B, no Santoquin and 1.9 mg/ml linoleic acid; C, 0.29 μg/ml Santoquin and 3.9 mg/ml linoleic acid; D, no Santoquin and 3.9 mg/ml linoleic acid.

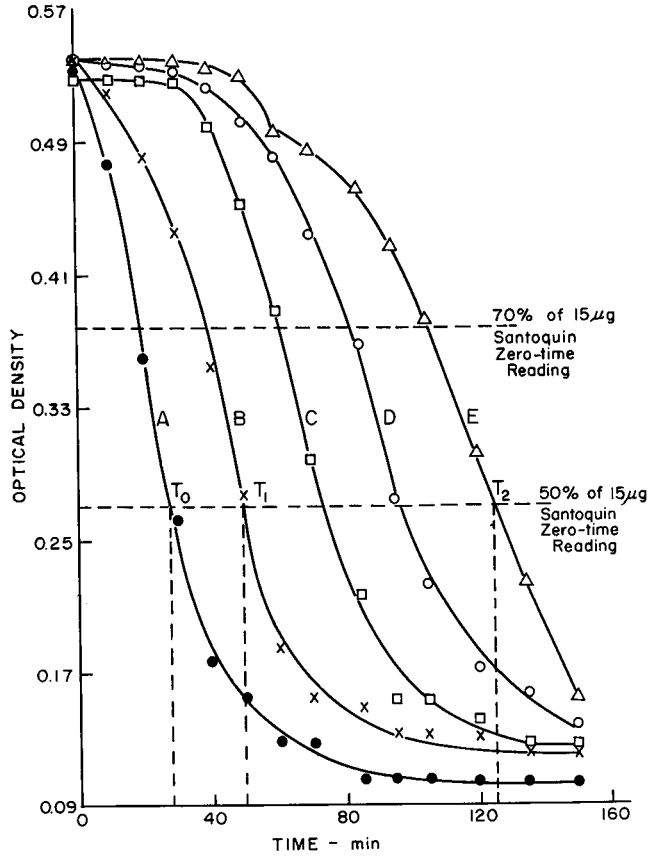


FIG. 3. Effect of antioxidant concentration on oxidation rate. Amount of antioxidants is the total in each reaction tube. A, no antioxidant; B, 15 μg BHT; C, 5 μg Santoquin; D, 10 μg Santoquin, and E, 15 μg Santoquin.

that the zero-time optical density was 0.105 compared to 0.500 to 0.600 normally obtained with linoleic acid. The reading of control tubes at the end of an experiment was 0.05 to 0.10. This rapid oxidation of ergosterol made it an unsuitable pro-oxidant for this test.

*Replace β-carotene with vitamin A.* While the rate of β-carotene oxidation was affected by cottonseed fatty acid levels, Budowski and Bondi (16) showed the induction period rather than oxidation rate to be affected in the oxidation of vitamin A. They used a dehydration method for analyzing vitamin A and claimed no interferences by oxidation products in the vitamin A measurements. However, the method was too time-consuming for the rapid procedure desired. The glycerol dichlorohydrin method of Sobel and Werbin (14,15) was chosen for its simplicity. Since anhydrous conditions were required in the color development, the coupled linoleic acid/vitamin

TABLE I

Effect of pH on Antioxidant Evaluation

| Antioxidant[a] | Antioxidant Response Value (Per cent of Santoquin)[b] | | |
|---|---|---|---|
| | Buffered | | Distilled water |
| | pH 9.0 | pH 7.0 | pH 7.0 |
| BHA | 48.5 | 13.2 | 28.2 |
| 2,4,5-Trihydroxybutyrophenone | — 6.0[c] | 3.8 | 6.7 |
| Propyl gallate | 0.4 | 10.4 | 11.0 |
| 3,3'-Dilaurylthiodipropionate | 2.3 | 0.9 | 9.4 |
| 3,3'-Thiodipropionic acid | — 2.3[c] | 1.9 | 10.1 |

[a] Tested at 15 μg/tube.
[b] Santoquin Antioxidant Response Value equals 100% by definition.
[c] A negative value is interpreted as pro-oxidation.

TABLE II

Reproducibility of Antioxidant Evaluations

| Antioxidant[a] | Antioxidant Response Value (Per cent of Santoquin)[b] | | | |
|---|---|---|---|---|
| | I | II | III | Mean ± SD |
| BHA | 28.2 | 26.7 | 29.2 | 28.0 ± 1.5 |
| Propyl gallate | 11.4 | 13.3 | 14.7 | 13.1 ± 1.3 |
| 2,4,5-Trihydroxybutyrophenone | 6.7 | 3.3 | 3.2 | 4.4 ± 2.1 |
| 3,3'-Dilaurylthiodipropionate | 9.4 | 13.3 | 9.7 | 10.8 ± 2.3 |
| 3,3'-Thiodipropionic Acid | 10.1 | 13.3 | 10.5 | 11.3 ± 1.9 |
| Ascorbic Acid | 4.3 | 4.5 | 8.9 | 5.9 ± 2.7 |
| 2,5-Ditert. butylhydroquinone | 8.7 | 12.2 | 10.5 | 10.5 ± 2.1 |

[a] Tested at 15 μg/tube.
[b] Santoquin Antioxidant Response Value equals 100% by definition.

A reaction was performed in toluene. This eliminated the need for extraction of an aqueous emulsion.

Results of the coupled oxidation in the presence and absence of Santoquin are shown in Fig. 2. The vitamin A concentration was 19 μg/ml in the final reaction mixtures. Santoquin was used at 0.29 μg/ml (Curves A and B) and linoleic acid at two levels, 1.9 mg/ml (Curves A and C) and 3.9 mg/ml (Curves B and D) in the final reaction mixtures. All curves show a decrease in vitamin A after 15 min, followed by an increase in optical density at 30 min. Since vitamin A should continue to be oxidized, the increase in color development must be owing to oxidation products. The highest level of linoleic acid (Curves B and D) showed the greatest relative increase at 30 min, suggesting linoleic acid oxidation products as the major source of the interfering color. Because of the interferences, this system was not studied further.

*Level of Antioxidant.* The β-carotene/linoleic acid emulsion was prepared in oxygenated pH 9.0 buffer,

and three levels (5, 10, and 15 μg/tube) of Santoquin were used. For comparison, BHT was evaluated at the 15-μg level. Results are shown in Fig. 3. The three levels of Santoquin (5, 10, and 15 μg) are shown in Curves C, D, and E, respectively. Upon comparison with control Curve A, Curve E (15 μg) was found most satisfactory, providing a rapid test (2 to 3 hr) with an extended induction period sufficiently beyond the control for ranking antioxidants weaker than Santoquin. The 15-μg antioxidant level/tube was selected for testing.

The method used in determining antioxidant activity is also shown in Fig. 3. Curve B was that obtained with 15 μg of BHT. At the 50% of 15 μg Santoquin zero-time carotene reading, three induction times ($T_0$, $T_1$, and $T_2$) were obtained for the control, BHT, and Santoquin, respectively. The following calculation was used for ranking BHT to Santoquin.

$$(T_1-T_0)/(T_2-T_0) \times 100 = \text{Antioxidant Response (Per cent of Santoquin)}$$

$$(49-28)/(125-28) \times 100 = 21.6\%$$

Since the curves are not parallel, a similar calculation was made for the 70% of 15 μg Santoquin zero-time carotene stage. The two values were averaged for the final Antioxidant Response Value for BHT of 22.3% of Santoquin.

To simplify the drawing of the curves, the readings between 10% and 90% of zero-time carotene can be plotted on logit graph paper (No. 31.450 Logistic Ruling, Codex Company, Inc., Norwood, Mass.). The sigmoid curve is straightened (18,19), eliminating the need for a French curve. Use of logit paper is the preferred method for graphing the results.

*Effect of pH.* While pH should not affect a purely free radical system, it could affect the state of the antioxidant. For acidic or basic antioxidants, salt formation could cause greater water solubility, making the chemical less available to the emulsified carotene/linoleic acid globule. The results of pH on several antioxidants are shown in Table I. The evaluations are expressed as "per cent of Santoquin," with Santoquin arbitrarily given 100% Antioxidant Response Value.

The pH has a marked effect on the antioxidant properties for the compounds in Table I. Not only are the rankings altered, but two compounds (3,3′-thiodipropionic acid and 2,4,5-trihydroxy butyrophenone), at pH 9.0, also act as pro-oxidants. That is, their oxidation rates were more rapid than control, leading to a negative Antioxidant Response Value. At pH 7.0, differences are noted between buffer and distilled water. The salts in the buffer may be altering the partitioning of the antioxidant into the emulsified carotene/linoleic acid globule. To eliminate any pH or salt effect, the distilled water system was selected for antioxidant evaluations.

*Test Reproducibility.* A series of antioxidants was compared to Santoquin on three separate days with emulsions and antioxidant solutions freshly prepared each day. Results are shown in Table II. All evaluations were compared at the 15 μg/tube level using distilled water in the final emulsion. Antioxidant Response Values are the averages of the 50% and 70% of Santoquin zero-time carotene stages. The standard deviations are small. At the 95% confidence level, antioxidants can be ranked with Antioxidant Response Value differences of 5% to 10% of Santoquin. While

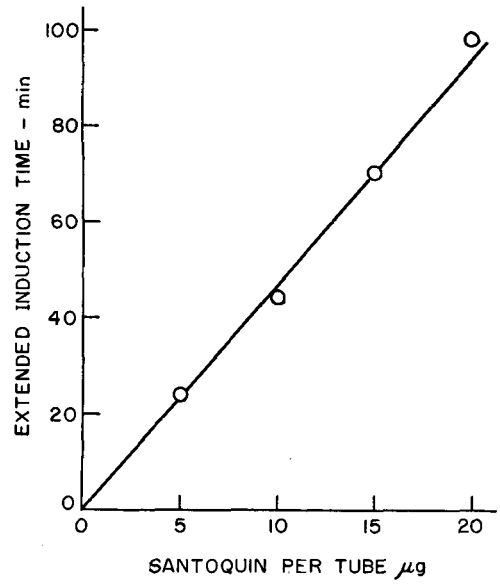


FIG. 4. Santoquin standard curve. Extended induction time versus Santoquin content/reaction tube.

the antioxidants were compared on an equivalent weight basis, they may also be compared on an equivalent molar basis. Since the amount of antioxidant may affect oxidation rates, the equimolar comparisons should be made experimentally rather than by mathematical conversion of the data in Table II.

*Analytical Application.* For several levels of an antioxidant, the extended induction period over control can be graphed versus the concentration. The graph may be used for calculating the content of antioxidant in an unknown.

The extention of induction period over control was determined for four Santoquin levels (5, 10, 15, and 20 μg/tube) at the 50% and 70% of zero carotene stages and were averaged for the extended induction time values at each level of Santoquin. The graph of extended induction time versus Santoquin level/tube is shown in Fig. 4. Each point represents the average of the extended induction time for two tubes/Santoquin level, with differences of less than 5% between each duplicate.

A Santoquin emulsifiable concentrate containing 70.0% Santoquin had been diluted 50 times with distilled water and stored, exposed to air and at room temperature, for six days. An aliquot, representing 8.4 μg of the Santoquin originally added, gave an extended induction time of 36 min over control for a value of 7.7 μg Santoquin, using the graph of Fig. 4. After six days exposed to air and at room temperature, less than 10% of the Santoquin was lost.

While this test can be used as a quantitative measure of antioxidant content of unknown versus Santoquin as a standard, it is not specific for Santoquin. In fact, any antioxidant could be used as a standard for comparison. This method permits the evaluation of antioxidant activity residing in commercial, animal, or plant samples as compared to any desired antioxidant.

This procedure should also be useful in assessing the oxidative state of a fat or oil compared to a standard such as pure linoleic acid. By setting a fixed level of antioxidant, a series of curves should be obtained with the most oxidized fat producing the shortest extended induction time.

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