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A Simple and Convenient Method to Determine the Activities of Antioxidants Using α-Methylindole Reagent and High-Performance Thin-Layer Chromatography[1)]

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A convenient method to determine the antioxidative activities of chemicals by using α-methylindole (MI) reagent in conjunction with high-performance thin-layer chromatography (HPTLC) is described. One μl of a mixture of 2% linolenic acid and test sample in ethanol solution was applied to a thin-layer chromatographic (TLC) plate and peroxidized at 80 °C for 20 min. The lipid peroxides which were produced from the linolenic acid on TLC were detected as pink spots by spraying MI reagent and were measured at 557 nm with a TLC densitometer.

When the test samples showed intrinsic colors interfering with this measurement, they were separated by developing the TLC plate after spraying MI reagent. The antioxidative activity of the test sample is expressed as the amount required to inhibit the peroxidation of linolenic acid by 50%.

This method is very simple and rapid. It may be useful when the test samples are available only in small amounts.

Keywords——linolenic acid peroxide; antioxidant; antioxidative activity; HPTLC; α-methylindole

Lipid peroxides (LPO) are known to be produced from lipids containing unsaturated fatty acids and are closely related to the deterioration of the quality of oil and fatty foods.[2–4)]

To evaluate antioxidative potentials, various testing methods have been developed. They include the active oxygen method (AOM),[5,6)] oven test,[7)] weighing method,[8)] ASTM bomb method,[9)] and differential scanning calorimetric (DSC) method.[10)] Other tests which utilize a polarographic oxygen analyzer,[11,12)] colorimetric/fluorometric analyzer and other approaches for peroxide determination[4,13,14)] have also been employed.

All the above methods, however, require a fairly large quantity of oil and test samples and several hours or days are necessary for the evaluation of antioxidative potential. They may be inconvenient for testing valuable or unstable chemicals and for handling a large number of samples.

In the present study, we report a very simple and convenient method for the determination of the antioxidative effect of chemicals using α-methylindole (MI) reagent in conjunction with high-performance thin-layer chromatography (HPTLC).

Experimental

Chemicals——MI was obtained from Wako Pure Chemicals (Tokyo) and purified by recrystallization from EtOH–H_2O. Linolenic acid and dibutyl hydroxytoluene (BHT) were obtained from Tokyo Kasei Co., Ltd. (Tokyo). Other chemicals used were of reagent grade quality. Preparation of linolenic acid hydroperoxide (LNHPO) was carried out as follows: linolenic acid was autoxidized by bubbling air at a flow rate of 2 l/min at 30 °C for 40 h.

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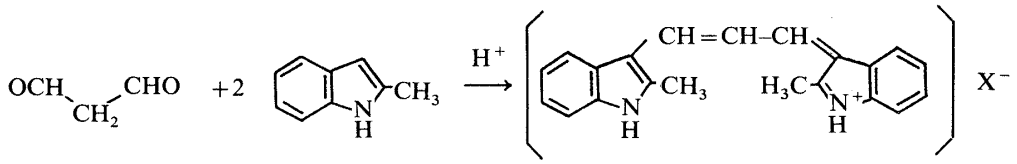
Peroxidized oil was applied to a column of silica-gel, and eluted with benzene.[15] HPTLC plates (Silica-gel 60 without fluorescence indicator, 10 × 10 cm) were obtained from Merck Co., Ltd. The optical densities of the colored spots were determined with a high-speed TLC densitometer (model CS-920, Shimadzu, Kyoto, Japan). A glass sprayer (Camag, Muttenz, Switzerland) connected with an air pump (model UP-2, Nippon Rikagaku Kikai Co., Ltd., Tokyo) was used for spraying MI reagent at the rate of 15—18 l/min.

Procedure——One-tenth ml of test sample at 5 different concentrations in EtOH was mixed with the same volume of 2% linolenic acid (in EtOH) in an ice bath and allowed to stand for 5 min. One μl of the above mixture was spotted at 10 mm intervals on the TLC plate by using a 10 μl microsyringe (Hamilton, Reno, Nev., U.S.A.). One μl of a 1 : 1 mixture of EtOH and 2% linolenic acid–EtOH was spotted as a reference (Ref. A) on the TLC plate. All these spots were dried under a stream of dry nitrogen. The plate was then heated at 80 °C for 20 min on a dry block bath (model AL-50, Scinics, Tokyo). After cooling of the plate to room temperature, one μl of the 1 : 1 mixture of EtOH and linolenic acid–EtOH was spotted on the right side of the plate as another reference (Ref. B). BHT–EtOH solution (2%) was then sprayed all over the plate to inhibit further oxidation of lipid. After spraying of 50% formic acid and 0.5% MI-EtOH solution in that order, the plate was heated at 100 °C for 60 s on a hot plate (model TPH-45, Toyo Kagaku, Tokyo). Pink color spots that developed were scanned with the TLC densitometer at 557 nm. When the test sample showed intrinsic color, *e.g.* plant samples, the plate was developed for an appropriate time to separate the pink color from the original pigment in the test samples. The solvent systems of *n*-hexane : ether : AcOH = 50 : 40 : 4 containing 0.1% BHT or ethyl acetate: isopropyl alcohol = 45 : 35 containing 0.1% BHT were useful for this purpose.

Calculation——The antioxidative potential of a test sample was calculated by subtracting the value of Ref. B from those of samples and Ref. A. The percentage inhibition and the concentrations of the test samples giving 50% inhibition were determined from these curves.

Results and Discussion

MI reacts with malondialdehyde under acidic conditions to produce a pink pigment.[16]



malondialdehyde α-methylindole pink pigment

As the pigment formed in this reaction was found to be more stable than that formed from malondialdehyde with TBA (thiobarbituric acid), MI was employed for the color development in this study, and the optimum conditions for this reaction were examined.

As shown in Fig. 1, the highest color development was observed by spraying either 50% formic acid or 15% trichloroacetic acid (TCA) followed by MI. The spraying of TCA, however, resulted in the coloration of the whole HPTLC plate with the passage of time and was not appropriate. Figure 2A shows the effect of temperature on the color development. The color development reached a maximum at 100 °C and its intensity decreased over 100 °C. A sufficient heating time for the plate was 1 min. Figure 2B shows the effects of heating time and temperature on the autoxidation of linolenic acid on the HPTLC plate; the autoxidation was maximum at 70—80 °C for 20 min. Regular TLC plates could be used instead of HPTLC plates but the latter gave higher color development with minimum deviation as compared to the regular TLC plates in this experiment.

From these results, the procedure for testing the activities of antioxidants was chosen as described in Fig. 3. The mean value of the color development in 8 tests using 1 μl of 2% linolenic acid was 2827.5 ± 129.6 and the coefficient of variation was 4.6%.

This method was successfully applied to measure the antioxidative activities of known antioxidants as well as unknown components contained in plant samples, though the pink color spots needed to be separated from the original pigments in the case of the plant samples. The pink color remained at the origin with the solvent system of *n*-hexane : ether : AcOH =

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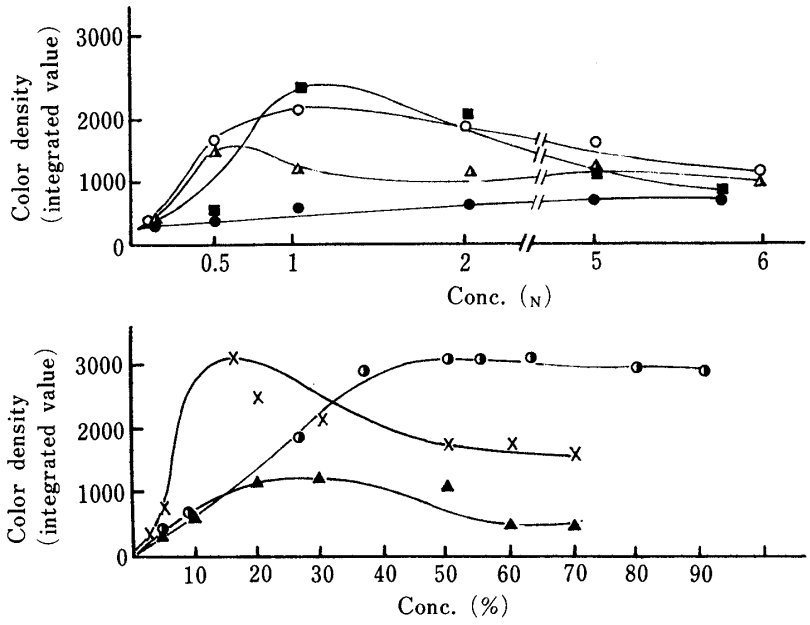

Fig. 1. Effects of Various Acids on Color Development in the Reaction of LNHPO with MI Reagent

CH_3COOH (—●—), HCl (—○—), H_2SO_4 (—△—), HNO_3 (—■—), HCOOH (—◑—), trichloroacetic acid (—×—), phosphotungstic acid (—▲—). One μl of the 1% LNHPO was spotted on the HPTLC plate. The plate was sprayed with 2% BHT, 0.5% MI and various acids, followed by heating at 100 °C for 1 min.

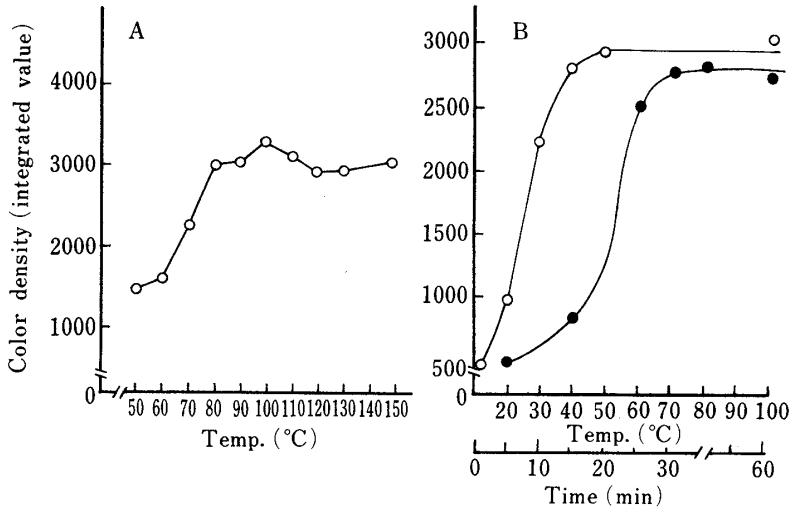

Fig. 2A. Effects of Temperature on Color Development in the Reaction of LNHPO with MI Reagent

One μl of LNHPO was spotted on the HPTLC plate. The plate was sprayed with 2% BHT, 0.5% MI and 50% formic acid, followed by heating at the indicated temperature.

Fig. 2B. Effects of Temperature and Heating Time on the Autoxidation of Linolenic Acid

One μl of linolenic acid was spotted on the HPTLC plate. The plate was then heated at the indicated temperature for 20 min (—●—) or heated at 80 °C for the indicated time periods (—○—). The color was developed and measured as described in Experimental.

50 : 40 : 4, while it moved slightly (*Rf* 0.06) with the solvent system of ethyl acetate : isopropyl alcohol = 45 : 35.

Figure 4 shows an example of the dose–response curve for the activities of ethyl, propyl and butyl gallates. The activities of various antioxidants and plant extracts determined in this

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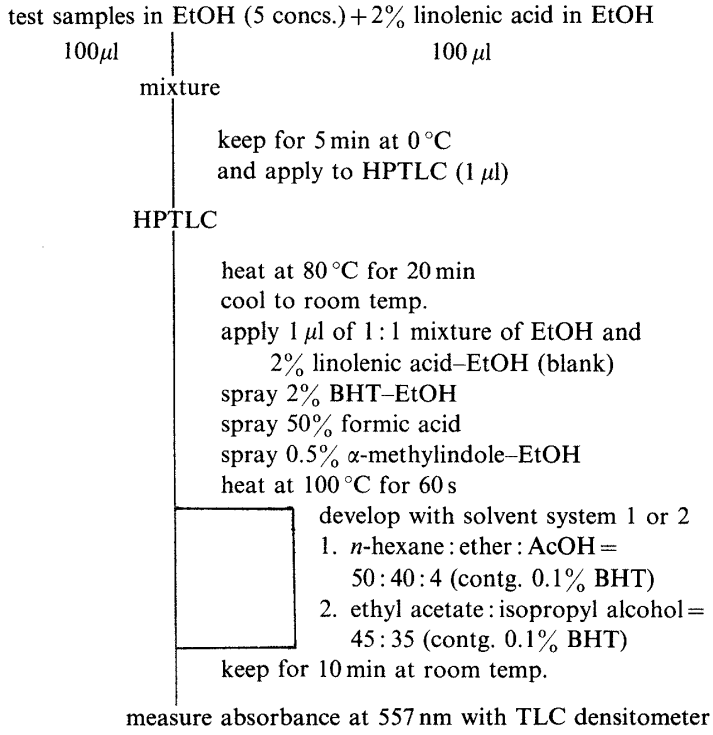

Fig. 3. Testing Procedure for the Determination of Antioxidative Activity using α-Methylindole Reagent and HPTLC

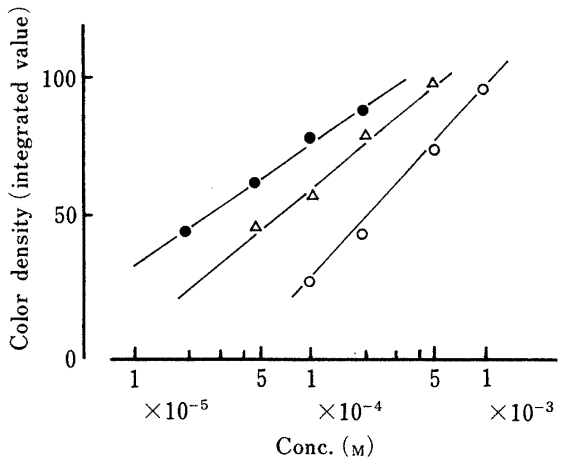

Fig. 4. A Dose–Response Curve for the Inhibitory Effects of Antioxidants on the Autoxidation of Linolenic Acid

Ethyl gallate (—●—), propyl gallate (—△—), butyl gallate (—○—). Data represent the mean of three individual experiments. The assay was carried out as described in Experimental.

study are summarized in Table I.

It was found that ethyl gallate has the highest antioxidative effect among 11 antioxidants used. *dl*-α-Tocopherol was less effective, as shown in Table I, which is in accord with the results reported by other authors,[12,17,18] though it shows a significant effect in *in vivo* lipid peroxidation.[19]

The water extracts of tea leaves (green tea, black tea and Pu-Ehr tea) showed fairly high antioxidative effects in this test system. The effects may be due to polyphenol compounds, including tannins. Tannins are known to inhibit lipid peroxidation in rat liver mitochondria and microsomes stimulated by adenosine diphosphate with ascorbic acid or reduced nicotinamide adenine dinucleotide phosphate.[20]

In conclusion, a new method using MI-HPTLC has been developed for the determination of antioxidative activities. This method is simple and convenient. It should be useful for the initial screening of large numbers of samples for antioxidative activities.

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TABLE I. Effects of Antioxidants on Autoxidation of Linolenic Acid Measured by MI-HPTLC

Antioxidants	Conc. for 50% inhibition (M)	Plant samples[a]	Conc. for 50% inhibition (g/ml)
Ethyl gallate	2.2×10^{-5}	Astrag. Rad.	3.0×10^{-3}
Methyl gallate	3.3×10^{-5}	Rhei Rhizoma	7.0×10^{-3}
BHT	3.4×10^{-5}	Cins. Rad.	1.0×10^{-2}
Ethyl protocatechuic acid	3.5×10^{-5}	Platycod Rad.	2.7×10^{-1}
BHA	3.6×10^{-5}		
Nordihydroguaiaretic acid	4.9×10^{-5}	Pu-Erh tea	3.1×10^{-4}
n-Propyl gallate	5.4×10^{-5}	Green tea	4.7×10^{-4}
Cetyl gallate	9.3×10^{-5}	Black tea	5.8×10^{-4}
Lauryl gallate	1.1×10^{-4}		
n-Butyl gallate	2.2×10^{-4}		
dl-α-Tocopherol	8.6×10^{-3}		

a) Plant samples except tea were prepared by extracting chopped small pieces dried plants (10 g) with 500 ml of 70% MeOH. In the case of tea, 5 g of dried tea leaves was extracted with 250 ml of boiling water for 5 min. All the extracts were filtered, lyophilized and dissolved in EtOH for determination of their antioxidative activities. All data plant samples were obtained after developing the TLC plate with the solvent mixture of ethyl acetate : isopropyl alcohol = 45 : 35 containing 0.1% BHT.

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