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Re-evaluation of Malondialdehyde and Thiobarbituric Acid-Reactive Substances as Indices of Autoxidation Based on Oxygen Consumption

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Soybean oil was oxidized at 170 and 40 °C in a closed vessel to measure the oxygen consumption, which is assumed to be the most reliable index for the evaluation of autoxidation. It was demonstrated that the formation of malondialdehyde (MDA) and thiobarbituric acid (TBA)-reactive substances correlated linearly with the consumed oxygen until the latter value reached 500 μmol/L at 170 °C. When the oxygen consumption exceeded the value, MDA and TBA-reactive substances did not increase but rather tended to decrease. On the other hand, in the autoxidation of the oil at 40 °C using 2,2′-azobis(2,4-dimethylvaleronitrile) as an initiator, MDA and TBA-reactive substances increased linearly with the oxygen consumption at least until the latter reached 1500 μmol/L.

INTRODUCTION

The TBA method, which determines spectroscopically malondialdehyde (MDA) by the reaction with 2-thiobarbituric acid (TBA), is the most common assay in lipid peroxidation studies (Buerge and Aust, 1978; Esterbauer and Cheeseman, 1990; Draper and Hadley, 1990). However, this method encounters some serious problems. One is the lack of specificity; i.e., TBA reacts with products of lipid peroxidation such as hydroperoxides and aldehydes to generate substances which absorb light at 535 nm, similar to the adduct of MDA and TBA. The second problem exists in the fact that the limit of the method has not been defined quantitatively and the yield of TBA-reactive substances (TBA-RS) based on the total radical reactions has not been determined yet. Furthermore, it is not established whether MDA itself even measured specifically can be used as a chemically reliable index to estimate the amount of radical reactions.

To shed more light on these fundamental aspects of lipid peroxidation, we determined for the first time the yield of MDA based on the oxygen consumption, which is a chemically reliable parameter to evaluate the degree of peroxidation of the lipid, utilizing soybean oil as a model substrate. MDA determination was made specifically by the HPLC method following conversion to the pyrimidine derivative recently developed by us (Kishida et al., 1990). We also measured the formation of TBA-RS to compare with the true MDA. On the basis of these results, we discuss the efficiency and the limit of TBA-RS and MDA measurements in evaluating the extent of lipid peroxidation stoichiometrically on a molar basis.

MATERIALS AND METHODS

Materials. Soybean oil, malondialdehyde bis(dimethylacetal), and 2,2′-azobis(2,4-dimethylvaleronitrile) were obtained from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Authentic samples of tocopherols were purchased from Eisai Co. Ltd. (Tokyo, Japan).

Measurement of Oxygen Consumption. Oxygen consumption was measured in an apparatus shown in Figure 1. Soybean oil (5–10 mL) was placed in a 50-mL glass cylinder (made of 50-mL graduate cut at the top line of 50 mL) or a 100-mL glass reactor (similarly made of 100-mL graduate). The vessel was tightly sealed with a stopper of silicon rubber fitted with an oxygen sensor oxygen meter (Model UC-12-SOL, made by Central Kagaku Co. Ltd., Tokyo, Japan). The oxygen concentration of the air space above the oil phase in the reaction vessel was monitored at room temperature before and after the reaction. Oxygen consumption during the reaction was calculated on the basis of the difference of these values and expressed as the concentration of oxygen changed in the volume of the oil.

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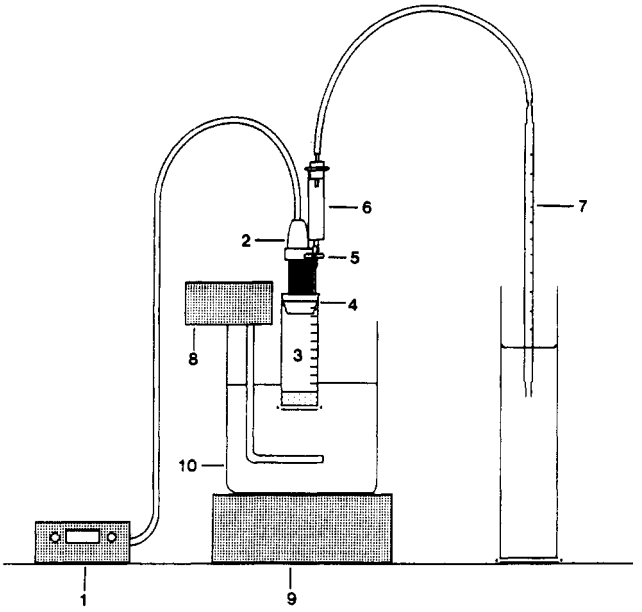

Figure 1. Schematic diagram of apparatus for autoxidation of oil and determination of oxygen consumption. Parts (indicated by number): 1, oxygen meter; 2, oxygen sensor; 3, 50- or 100-mL glass cylinder; 4, silicon rubber stopper; 5, three-way valve; 6, syringe; 7, calibrated glass pipet; 8, heater; 9, magnetic stirrer; 10, silicon oil bath.

As a reference of the measurement with the oxygen meter, the other determination was also made at the same time using a manometric method. The apparatus is also shown in Figure 1. The rubber stopper into which an oxygen sensor was perforated was fitted with a stainless steel needle whose pointed end reached the gas phase of the reaction vessel. The needle was connected to a syringe with a three-way valve which was closed to the direction of the reactor during the reaction. The syringe was connected with a thin silicon rubber tube to a calibrated glass pipet (5 or 10 mL depending on the consumed oxygen), which was immersed perpendicularly into water in a glass beaker as shown in Figure 1. After the determination of the consumed oxygen with the oxygen meter, the three-way valve was opened to connect the reaction vessel with the pipet. By this procedure, the water level inside the pipet rose depending on the volume of consumed oxygen during the autoxidation reaction. The value of the oxygen consumption determined by the oxygen meter correlated closely with that measured by the pressure loss using the simple manometric method. The correlation coefficient of these values was 0.98, and the former value was adopted as the consumed oxygen.

It must be noted that in the present experiments the change in the volume of the reactor (50 or 100 mL) did not affect the relationships among oxygen consumption, TBA-RS, MDA, and tocopherols. Therefore, the results are shown as totally combined.

Oxidation of Soybean Oil at 170 °C. Soybean oil was oxidized at 170 °C in the sealed vessel as described above. The heating was carried out by immersing only the portion of the reaction vessel containing the oil (to protect the oxygen sensor from the high temperature) into a silicon oil bath, which was stirred with a magnetic stirrer and kept at 170 °C. The reaction was stopped by cooling to room temperature. After the determination of the consumed oxygen by the two different methods as described above, TBA-RS, MDA, and tocopherols were measured.

Oxidation of Soybean Oil at 40 °C. Soybean oil and an appropriate amount of the initiator [2,2′-azobis(2,4-dimethylvaleronitrile] were placed in the reaction vessel as described above, and the oxidation was made at 40 °C. At maximum, 90 mg of the initiator was added to 5 mL of soybean oil. When the oxygen consumption ceased, the reaction vessel was taken out from the heating bath and cooled to room temperature. The following procedures were made similarly to the reaction at 170 °C as described above.

TBA Method. TBA-RS were determined according to the TBA method (Buege and Aust, 1978). To the oxidized oil (100 μL) were added TBA reagent (2 mL) and a 5–20% ethanol solution (10 μL) of butylated hydroxytoluene. The mixture was heated in boiling water for 15 min, and the absorbance at 535 nm was recorded after cooling and centrifugation (2000*g*, 5 min).

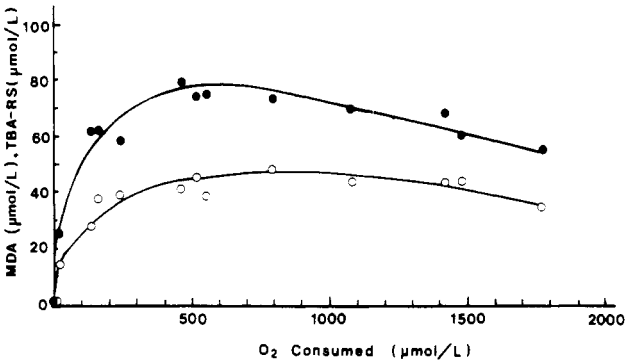

Figure 2. TBA-RS and MDA formations during autoxidation of soybean oil at 170 °C based on oxygen consumption. (○) MDA; (●) TBA-RS.

Determination of MDA. After the measurement of the oxygen consumption, a 400-μL aliquot was taken and added to a mixture of 400 μL of hexane and 400 μL of hydrochloric acid (0.1 M). After vortexing and centrifugation at 2000*g* for 5 min, 100 μL of the aqueous layer was subjected to the determination of MDA according to the literature (Kishida et al., 1990).

Determination of Tocopherols. The hexane layer obtained in the procedures of MDA determination described above was applied to HPLC analysis. Tocopherols were separated on a column (Wako 5 Sil, 4.6 × 150 mm) using a mixture of hexane and 2-propanol (99.3:0.7 v/v, 1 mL/min) as the mobile phase. The quantity of tocopherol isomers separated was determined with a fluorescence monitor (type RF-535, manufactured by Shimadzu Co. Ltd., Kyoto, Japan) using excitation at 295 nm and emission at 325 nm.

RESULTS AND DISCUSSION

Relationship among TBA-RS, MDA, and Consumed Oxygen in Autoxidation of Soybean Oil at 170 °C. Since activated oxygen species such as peroxy, alkoxy, and hydroxyl radicals carry radical chains in the autoxidation of lipids to form oxygenated products, the consumption of oxygen is a chemically reliable indicator for the evaluation of total radical reactions. The formation of TBA-RS and MDA was determined as given under Materials and Methods and plotted with the consumed oxygen as shown in Figure 2.

The formation of TBA-RS, expressed as the equimolar concentration of MDA, increased as the oxygen consumption was raised. When the consumed oxygen was low (less than ca. 200 μmol/L), TBA-RS was about 40% of the oxygen consumption on a simple molar basis. When the amount of oxygen consumed was about 500 μmol/L, the TBA-RS level reached a maximum. It took about 1.5 h to consume 500 μmol/L oxygen. Thereafter TBA-RS did not increase and retained a constant level or rather declined in spite of the progress of autoxidation as evidenced by a further increase in oxygen consumption. The reaction was followed up to 1700 μmol/L of oxygen consumption (Figure 2). These results demonstrated that TBA-RS are a good indicator of lipid peroxidation at the early stage of the reaction, where the oxygen consumption was less than 500 μmol/L, which was postulated to be a limitation of the TBA method.

The level of MDA was specifically determined in the oxidation of soybean oil at 170 °C utilizing the method reported by us (Kishida et al., 1990). The results are also included in Figure 2. The quantity of MDA was about 60–65% of TBA-RS and amounted to nearly 20% of

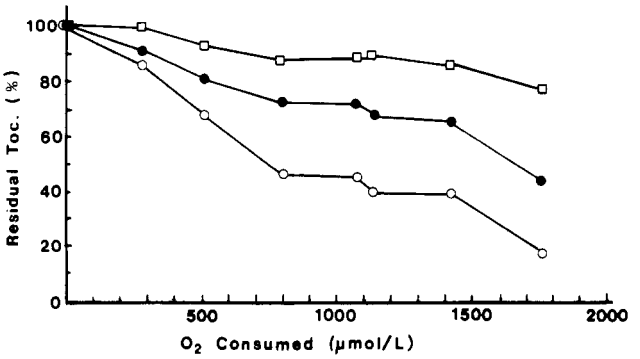

Figure 3. Residual percent of tocopherol isomers during autoxidation of soybean oil at 170 °C. (○) α-Tocopherol; (●) γ-tocopherol; (□) δ-tocopherol.

consumed oxygen at maximum (at consumed oxygen of 796 μM). This was an unexpectedly high value, because MDA was one end product and assumed to be formed only in a small amount as a result of extensive reactions, even if the compound served as an indicator of peroxidation. It is conceivable that the high reaction temperature (170 °C) is favorable for the formation of MDA via thermal decomposition of hydroperoxides, peresters, and other oxidized precursors. This may be supported by comparison with the results obtained in the experiments under lower temperature (described later).

The change in MDA level resembled that in TBA-RS (Figure 2). When the oxygen consumption was less than 500 μmol/L, MDA increased as TBA-RS. However, when the decrease of oxygen exceeded 500 μmol/L, the MDA level remained almost unchanged as shown in Figure 2. This observation may be explained on the grounds that the disappearance of MDA by condensation and radical reactions is accelerated under the high temperature, since the level of MDA is determined by the balance between the formation rate (promoted as described above) and the disappearance rate of MDA which has two reactive aldehyde groups.

These results demonstrate that the determination of MDA had a similar limitation to TBA-RS as a parameter in the evaluation of lipid peroxidation of soybean oil at 170 °C.

Change of Tocopherols in the Oxidation of Soybean Oil at 170 °C. Since tocopherols are well-known and naturally occurring antioxidants and are destroyed in the course of radical reactions, we followed the level of tocopherols in soybean oil during the autoxidation at 170 °C. The relationship between the consumed oxygen and the residual percent of tocopherol isomers is shown in Figure 3. The initial concentrations of α-, γ-, and δ-tocopherols in the soybean oil were 108.3, 307.1 and 182.9 μg/g of oil, respectively. All tocopherol isomers decreased with oxygen consumption up to 1800 μmol/L. This result indicated that the decrease of tocopherols reflected the reaction course better than TBA-RS and MDA in a wide range of the concentration of consumed oxygen, and thus tocopherols might be used as a valuable and convenient parameter in the estimation of lipid peroxidation at high temperature such as those used in food processing.

Among tocopherols, the α-isomer decreased most rapidly, followed by the γ-isomer. This observation was consistent with the relative antioxidative activity of tocopherols in the oxidation of biomembranes reported by Niki et al. (1986).

Relationship among TBA-RS, MDA, and Consumed Oxygen in the Autoxidation of Soybean Oil at 40 °C Using a Radical Initiator. The autoxidation of the oil

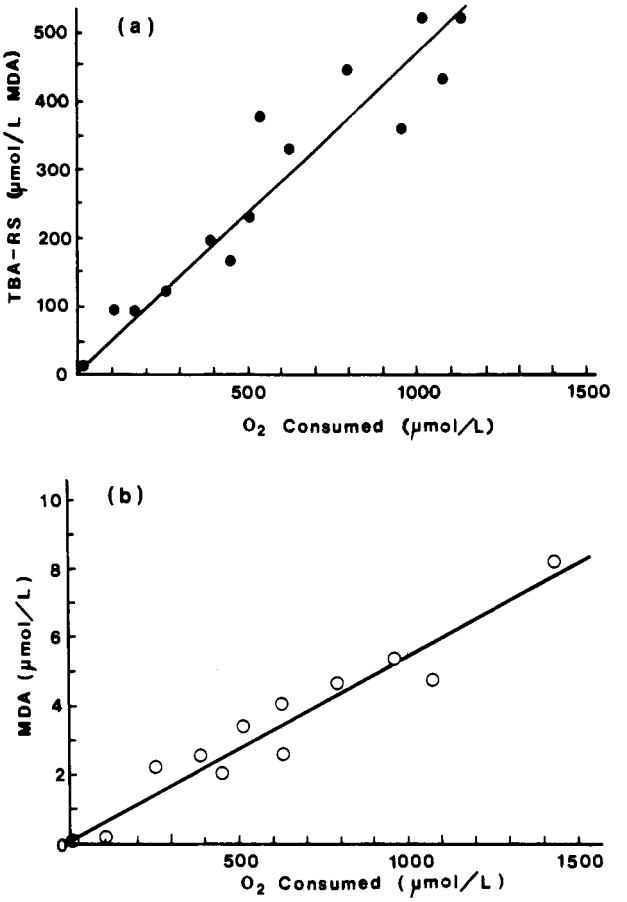

Figure 4. TBA-RS and free MDA formed during autoxidation of soybean oil at 40 °C using a radical initiator. (a) TBA-RS; (b) MDA.

was made at a lower temperature, 40 °C, to investigate the effect of temperature and also the possible application to the analysis of radical reactions taking place in biological systems. At 40 °C, the rate of autoxidation was very low and 2,2′-azobis(2,4-dimethylvaleronitrile) was used as a radical initiator. The quantity of oxygen consumption was controlled by varying the amount of the initiator. In the measurement of TBA-RS, butylated hydroxytoluene was added to prevent sufficiently the additional reaction caused by the possibly remaining initiator during the TBA reaction. To this end, preliminary experiments were made. An oil sample containing the initial concentration of the initiator was prepared and directly subjected to TBA reaction in the presence of variable amounts of butylated hydroxytoluene. The amount of butylated hydroxytoluene was determined so that it prevented the effect of the added initiator on the TBA value.

Under our conditions, the measurement of TBA-RS saturated at about 0.7 of absorbance at 535 nm i.e., those samples which gave absorbance greater than 0.7, did not afford a linear relationship between the sample volume and the TBA-RS level. For example, the TBA-RS did not decrease to the half-value when the half-volume (50 μL) of the oil was used, in contrast to the case where the TBA-RS value was less than ca. 0.7. Therefore, for the sample which showed absorbance greater than 0.6, a smaller amount of the oil (e.g., 20 μL instead of the usual 100 μL) was subjected to the TBA reaction.

The results are shown in Figure 4. TBA-RS correlated linearly with the consumed oxygen for a wide range between 0 and 1200 μmol/L. In sharp contrast to the reaction at 170 °C, the saturation phenomenon was not observed under these conditions. The yield of TBA-RS

on the basis of the reacted oxygen amounted to nearly 40%, which is greater than the maximal value obtained in the reaction at the higher temperature. MDA was also determined, and the results are included in Figure 4. MDA also increased linearly with the consumed oxygen, similar to TBA-RS. The yield of MDA was ca. 0.5 and 1% based on oxygen consumption and TBA-RS, respectively. These values were much lower than those observed in the oxidation at 170 °C. This result may support the above discussion that the formation of MDA was accelerated under the elevated temperature, giving a higher yield based on the consumed oxygen, and the disappearance of MDA was slow at the low temperature, affording a linear relationship between MDA level and oxygen consumption.

Both parameters (TBA-RS and MDA) were found to reflect closely the course of the autoxidation and may serve as reliable indices of peroxidation at 40 °C.

In the reaction using the initiator, the consumption of tocopherols preceded the oxidation of the lipid and the tocopherol levels tended to be decreased rapidly by the initiator. The decrease of tocopherols may be caused by facile reactions with radicals generated by the initiator as analyzed in detail (Liebler et al., 1990). Thus, the change in the concentration of tocopherols was not suitable as an indicator to evaluate oxidation in the reaction taking place at 40 °C in the presence of the initiator. The reason for the large temperature effect remains to be explored.

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