

Comparison of the Formation of Malondialdehyde and Thiobarbituric Acid-Reactive Substances from Autoxidized Fatty Acids Based on Oxygen Consumption

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Fatty acid having 18 carbon atoms was oxidized at 170 °C in a closed vessel to measure the oxygen consumption, which was assumed to be a chemically reliable index for the autoxidation. The yields of malondialdehyde (MDA) and 2-thiobarbituric acid-reactive substances (TBA-RS) were compared with the data for oxygen consumption. In autoxidation of oleic acid, the formation of MDA and TBA-RS correlated with the consumed oxygen up to the value of 700 $\mu\text{mol/L}$. When the oxygen consumption exceeded the value, MDA did not increase and TBA-RS tended to decrease. In autoxidation of linoleic acid, TBA-RS linearly increased with the oxygen consumption up to 2000 $\mu\text{mol/L}$. MDA also increased with oxygen consumption up to 700 $\mu\text{mol/L}$ but thereafter reached a plateau. The yield of MDA from linoleic acid was similar to that from oleic acid. In autoxidation of linolenic acid, the formation of MDA and TBA-RS correlated with the oxygen consumption until the value reached 1150 $\mu\text{mol/L}$. When the oxygen consumption exceeded that value, MDA and TBA-RS decreased. Neither MDA nor TBA-RS was detected from stearic acid in spite of the increase in oxygen consumption up to 2000 $\mu\text{mol/L}$.

INTRODUCTION

Aldehydes are produced when lipids break down in biological systems and foods, and it is of interest to measure these compounds as indices of the degree of lipid peroxidation. Among them, malondialdehyde (MDA) is considered to be the most abundant individual aldehyde resulting from lipid peroxidation (Esterbauer and Cheeseman, 1990). Its determination by the thiobarbituric acid method, which determines colorimetrically 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, the validity of the method has been questioned in the evaluation of radical reactions (Draper and Hadley, 1990). Furthermore, it is not established whether MDA itself, even if measured specifically, can be used as a chemically reliable index to estimate the extent of lipid peroxidation.

To shed more light on chemically reliable indices of lipid peroxidation, we reported for the first time the determination of the yield of MDA based on oxygen consumption, which is the most reliable indicator for the evaluation of lipid autoxidation, using soybean oil as a model substrate (Kishida et al., 1993). MDA determination was specifically made by the HPLC method after the conversion to the pyrimidine derivative developed by us (Kishida et al., 1990). It was shown that the formation of MDA and TBA-reactive substances (TBA-RS) in autoxidation of soybean oil correlated linearly with the consumed oxygen until the latter value reached 500 $\mu\text{mol/L}$ at 170 °C. When the oxygen consumption exceeded the level, MDA and TBA-RS did not increase but rather tended to decrease. These results demonstrated that MDA and TBA-RS had limitations as a parameter of lipid autoxidation of soybean oil at 170 °C.

As a next extension to clarify the relationship between the nature of the fatty acid and the formation of MDA and TBA-RS in lipid peroxidation, we report in this paper the comparison of the amount of free MDA and TBA-RS formed during autoxidation of a series of 18-carbon fatty acids having different numbers of double bonds. This paper is also concerned with the efficiency of these indices on the evaluation of lipid peroxidation based on oxygen consumption.

MATERIALS AND METHODS

Materials. Oleic, linoleic, linolenic, and stearic acids were used as fatty acid with 18 carbons. The fatty acids and malondialdehyde bis(dimethylacetal) were obtained from Wako Pure Chemical Co. Ltd. (Osaka). Among fatty acids, linoleic and linolenic acids were distilled under reduced pressure before use.

Oxidation of Fatty Acid and Measurement of Oxygen Consumption. Fatty acid was oxidized at 170 °C in a closed glass vessel to measure the oxygen consumption by the method described in detail previously (Kishida et al., 1993). Briefly, fatty acid (5–10 mL) was placed in a glass reactor. The vessel was tightly sealed with a silicon rubber stopper with an oxygen sensor of oxygen meter (Model UC-12-SOL, Central Kagaku Co. Ltd., Tokyo). The oxygen concentration of the air space above the fatty acid 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 fatty acid. After the determination of the consumed oxygen, TBA-RS and MDA were measured.

In the reaction of stearic acid, the fatty acid was melted by heating at 70 °C and the melted sample (10 mL) was taken into the reaction vessel. The reaction was carried out at 170 °C as described above. Before and after the reaction, the oxygen concentration of the air space above the oil phase was determined. During the measurement, the fatty acid was kept melted by immersing only the portion of the reactor containing the sample into a silicon oil bath maintained at 70 °C. After the determination of the consumed oxygen, TBA-RS and MDA were measured.

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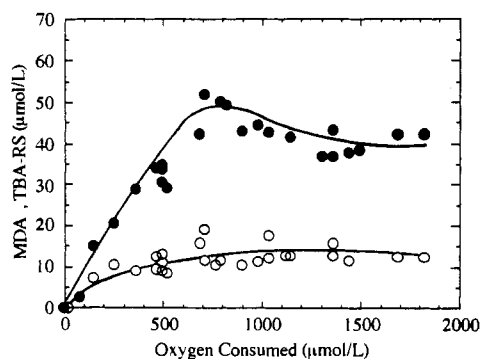


Figure 1. TBA-RS and MDA formation during autoxidation of oleic acid based on oxygen consumption. (●) TBA-RS; (○) MDA.

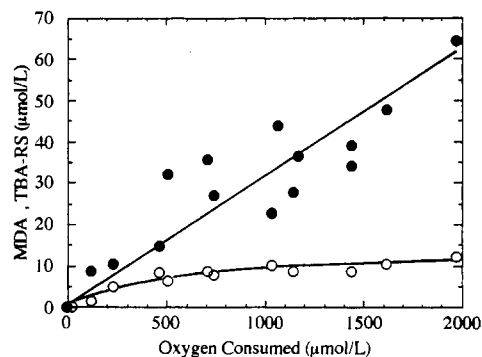


Figure 2. TBA-RS and MDA formation during autoxidation of linoleic acid based on oxygen consumption. (●) TBA-RS; (○) MDA.

TBA Method. TBA-RS were determined according to the TBA method (Buege and Aust, 1978). To the oxidized fatty acid (20–100 μL) were added TBA reagent (2 mL) and ethanol solution (10 μL) containing 5% butylated hydroxytoluene. In the case of linolenic acid, 20% butylated hydroxytoluene instead of 5% was used to prevent oxidation during the TBA reaction. The mixture was heated in boiling water for 15 min. After cooling, 1-butanol (2 mL) was added to the reaction mixture. The absorbance of the butanol layer was recorded at 535 nm after extraction and centrifugation. The formation of TBA-RS was expressed as the equimolar concentration of MDA.

Determination of MDA. After the measurement of the oxygen consumption, fatty acid (400 μL) was taken and added to a mixture of 400 μL of hexane and 400 μL of hydrochloric acid (0.1 mol/L). After vortexing and centrifugation at 2000g for 5 min, 100 μL of the aqueous layer was mixed with 100 μL of an ethanol solution of *N*- α -benzoyl-L-arginine ethyl ester hydrochloride (0.4 M). After the addition of 2 mL of an ethanol solution of hydrogen chloride (10 M), the reaction mixture was allowed to react at 25 $^{\circ}\text{C}$ for 12 h. After neutralization and extraction of the resulting pyrimidine compound with chloroform, HPLC analysis was made. Details were published in the literature (Kishida et al., 1990).

RESULTS AND DISCUSSION

The consumption of oxygen is the most chemically reliable indicator for the evaluation of total radical reactions. However, the determination of the reacted oxygen is not always possible. Therefore, some parameters that reflect oxygen consumption are required. In this study, the amounts of TBA-RS and MDA were investigated to determine whether these indices serve as such a parameter. TBA-RS and MDA were determined as given under Materials and Methods and plotted against the data for oxygen consumption. These results are presented in Figures 1–3.

Autoxidation of Oleic Acid. In the initial stage of autoxidation of oleic acid at 170 $^{\circ}\text{C}$, TBA-RS increased

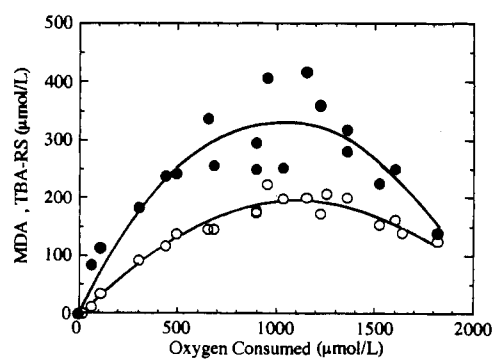


Figure 3. TBA-RS and MDA formation during autoxidation of linolenic acid based on oxygen consumption. (●) TBA-RS; (○) MDA.

linearly with oxygen consumption as shown in Figure 1. In the linear range, TBA-RS was about 8% of the consumed oxygen on a simple molar basis. When the amount of used oxygen was about 700 $\mu\text{mol/L}$, the TBA-RS level reached a maximum. Thereafter, TBA-RS declined and retained a constant level in spite of a further increase in reacted oxygen. The reaction was followed until 1822 $\mu\text{mol/L}$ of oxygen disappeared. These results suggested that TBA-RS are a good indicator of lipid autoxidation in the early phase of the reaction, where the oxygen consumed was less than 700 $\mu\text{mol/L}$, which was postulated to be a limitation of the TBA method.

The amount of MDA was specifically determined by the method reported by us (Kishida et al., 1990). The results are also included in Figure 1. Formation of MDA correlated again with the amount of the consumed oxygen up to 700 $\mu\text{mol/L}$. The yield of MDA in the linear region was about 23% of TBA-RS (at consumed oxygen of 788 $\mu\text{mol/L}$). This result demonstrated that the specificity of the TBA method in determining MDA at this point was 23%. The low specificity was consistent with the literature which investigated MDA formation in the autoxidation of soybean oil (Kishida et al., 1993).

When the oxygen consumption exceeded 700 $\mu\text{mol/L}$, MDA reached a plateau and did not increase. This observation may be explained on the grounds that the disappearance of MDA by condensation and radical reactions takes place under high temperature, since the level of MDA is determined by the balance between the formation rate and the disappearance rate of the compound which has two reactive aldehyde groups.

These results demonstrate that the determination of MDA has a limit similar to that of TBA-RS as a parameter in the evaluation of autoxidation of oleic acid at 170 $^{\circ}\text{C}$.

The consumption of oxygen proceeded in roughly linear fashion with reaction time (correlation coefficient $r = 0.90$), and the rate was about 30 $\mu\text{M}/\text{min}$.

Autoxidation of Linoleic Acid. As shown in Figure 2, TBA-RS correlated linearly with the consumed oxygen in a wide range up to ca. 2000 $\mu\text{mol/L}$ in the autoxidation of linoleic acid at 170 $^{\circ}\text{C}$. The yield of TBA-RS on the basis of the reacted oxygen amounted to about 3%, which was less than that in the case of oleic acid in the linear range. This result suggests that the yield of TBA-RS does not necessarily depend on the number of allylic hydrogens which may be attacked preferentially, leading to chain reactions affording TBA-RS. Until the oxygen consumption reached 700 $\mu\text{mol/L}$, MDA also gradually increased. However, after the oxygen loss exceeded 1000 $\mu\text{mol/L}$, the MDA level remained almost unchanged. At the stage of 1033 $\mu\text{mol/L}$ of reacted oxygen, the yield of MDA was ca. 1% based on the oxygen consumption. The dependence

of the MDA formation from linoleic acid on the consumed oxygen resembled that of oleic acid.

These results indicated that TBA-RS reflected the reaction course better than MDA in a wide range of the concentration of consumed oxygen in the autoxidation of linoleic acid.

The consumption of oxygen also proceeded in nearly linear manner ($r = 0.95$) with reaction time, and the rate was about $50 \mu\text{M}/\text{min}$, which was 1.7-fold that of oleic acid. This acceleration may be due to the presence of labile bis(allylic) hydrogens in linoleic acid. However, the difference in the reactivity between the two fatty acids was very small, considering the study (Nagaoka et al., 1990) which described the rate constant of hydrogen abstraction from methyl linoleate by tocopheryl radical at 25°C as being higher than that from methyl oleate by 3 orders of magnitude. The large discrepancy in the reactivity of the two fatty acids between the present and the reported results may be explained by two reasons. One is the higher reaction temperature applied in our experiment, because high temperature usually lowers the difference in reaction rate exponentially as is well established. The other may be ascribed to the nature of the attacking radical. In our reaction, radical chain reaction is supposed to be propagated by alkylperoxy and alkoxy radicals which are assumed to be much more reactive than tocopheryl radical, which is stabilized and has low reactivity. Since highly reactive species normally react less selectively, the difference in reaction rate by substrate change becomes small.

Autoxidation of Linolenic Acid. As shown in Figure 3, the formation of TBA-RS in the oxidation of linolenic acid at 170°C correlated linearly with the consumed oxygen, which was less than ca. $600 \mu\text{mol}/\text{L}$. In the linear region, the yield of TBA-RS was as high as ca. 50% based on the reacted oxygen. Thereafter, TBA-RS decreased, while oxygen consumption increased.

MDA was also determined, and the results are included in Figure 3. The MDA level as well as that of TBA-RS elevated initially with the consumed oxygen. The quantity of MDA was about 48–60% of TBA-RS. The yield of MDA on the basis of disappeared oxygen amounted to nearly 23% at maximum (at consumed oxygen of $952 \mu\text{mol}/\text{L}$), which was the greatest among fatty acids used in this study. This observation may be explained on the grounds that linolenic acid has the most double bonds in the fatty acids investigated in the present study and contains eight allylic hydrogens and two methylene-interrupted double bonds, which was suggested to be a preferable condition for MDA formation (Esterbauer et al., 1991). However, when the consumption of oxygen exceeded $1000 \mu\text{mol}/\text{L}$, the MDA level declined but the degree of the decrease was less than that of TBA-RS.

In the oxidation of linolenic acid, oxygen was also consumed in a roughly linear manner with the reaction period ($r = 0.90$) and the rate was ca. $54 \mu\text{M}/\text{min}$, which is about 1.1-fold that of linoleic acid. This may be reasonable because the rate constant of hydrogen abstraction from methyl linolenate by tocopheryl radical was reported to be only 2.1-fold of that from methyl linoleate (Nagaoka et al., 1990), and the discussion above on the distinction between the present and the reported (Nagaoka et al., 1990) experiments concerning the relative reactivity of linoleic and oleic acids may also be applicable in this case.

Although the rate of oxygen consumption of linolenic acid resembles that of linoleic acid, the profiles of the formation of MDA and TBA-RS are clearly distinct

between these fatty acids. The difference in the reaction mechanism of product formation remains to be explored.

Autoxidation of Stearic Acid. The autoxidation of stearic acid, which contains no double bond, was made at 170°C . The reaction was followed up to $2200 \mu\text{mol}/\text{L}$ of oxygen consumption. However, neither TBA-RS nor MDA was detected from the oxidized stearic acid over the wide range of oxygen consumption (figure not shown). The reaction proceeded very slowly, and it took around 5 h to consume $2000 \mu\text{mol}/\text{L}$ of oxygen. The low reactivity of stearic acid was consistent with the literature (Nagaoka et al., 1990).

Condition of MDA Formation. It is generally accepted that linoleic acid is a poor precursor of MDA, which is formed preferentially from polyunsaturated fatty acids with more than two methylene-interrupted double bonds (Esterbauer et al., 1991). In this study, the low yield of MDA from linoleic acid was consistent with the proposal. However, we found that a significant amount of free MDA was generated by the autoxidation of even oleic acid at 170°C , and the yield was ca. 1% based on the consumed oxygen, which was comparable to that from the reaction of linoleic acid. On the other hand, no MDA was detected in an oxidized saturated fatty acid, stearic acid in our study. These results indicated that at least one double bond is required for the significant formation of MDA as well as TBA-RS from fatty acids.

TBA-RS and MDA as a Parameter of Lipid Peroxidation. Both TBA-RS and MDA reflected linearly the oxygen consumption up to around $600\text{--}700 \mu\text{mol}/\text{L}$ in the peroxidation of all unsaturated fatty acids used in the present study. This was also observed in the autoxidation of soybean oil, while the linear range was $500 \mu\text{mol}/\text{L}$ of the consumed oxygen (Kishida et al., 1993). These results indicate that TBA-RS and MDA are reliable indices in the limited early stage of lipid peroxidation at 170°C .

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