Comparing Methylene Blue–Photosensitized Oxidation of Methyl-Conjugated Linoleate and Methyl Linoleate

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Comparing Methylene Blue-Photosensitized Oxidation of Methyl-Conjugated Linoleate and Methyl Linoleate

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The photooxidations of methyl-conjugated linoleate (MCL) and methyl linoleate (ML) were compared using methylene blue as a sensitizer in ethanol solutions. MCL was lost to a lower extent and yielded less hydroperoxide but bleached methylene blue at a higher rate than ML. The different isomers of MCL were not equally lost, and the photooxidation of MCL was accompanied by interisomerization. The results suggested that different mechanisms were involved in the photooxidations of MCL and ML

Keywords: *Conjugated linoleic acid; methyl-conjugated linoleate; methyl linoleate; methylene blue; photooxidation*

INTRODUCTION

Conjugated linoleic acid (CLA) is a generic name for a mixture of isomers of linoleic acid (LA) with conjugated double bonds at positions 9 and 11 or 10 and 12 (Ha et al., 1987). CLA is present mainly in animal foods such as dairy and meat products (Chin et al., 1992; Banni et al., 1994; Jiang et al., 1996). It has also been identified in human serum, bile, and duodenal juices (Gawood et al., 1983; Iversen et al., 1985). CLA has been shown to exhibit a number of desirable physiological properties including anticarcinogenic (Ha et al., 1987, 1990; Shultz et al., 1992; Ip et al., 1991, 1994) and hypocholesterolemic and antiatherogenic effects (Lee et al., 1994; Nicolosi and Laitinen, 1996). The mechanisms responsible for these unique physiological effects are not yet known.

In the search for a possible anticarcinogenic mechanism, CLA was tested for its antioxidant effects using autoxidation models (Ha et al., 1990; Ip et al., 1991; van den Berg et al., 1995; Chen et al., 1997). Two studies have suggested CLA to possess antioxidative properties (Ha et al., 1990; Ip et al., 1991), whereas other studies showed that CLA was not an antioxidant and may even act as a prooxidant (van den Berg et al., 1995; Chen et al., 1997). The controversy in this issue warrants further investigations on the oxidation mechanisms of CLA. The conjugated structure of this fatty acid suggests possible interactions with active oxygen species, for example, singlet oxygen generated during photooxidation. Therefore, this investigation was initiated to compare the photooxidation of methyl-conjugated linoleate (MCL) and methyl linoleate (ML) using methylene blue (MB) as a sensitizer in a model system.

MATERIALS AND METHODS

Chemicals. The CLA standard was a gift from Dr. S. F. Chin (Food Research Institute, University of Wisconsin, Madison) and was a mixture of the following isomers: 9,11 (cis,trans and trans,cis) 48.5%; 10,12 (trans,cis) 48.2%; 9,11 and 10,12 (cis,cis) 1.8%; 9,11 and 10,12 (trans,trans) 1.5%. CLA was methylated according to the method of Werner et al. (1992) using 14% boron trifluoride in methanol as reagent (Sigma Chemical Co., St. Louis, MO). MCL produced were used in all experiments. ML (99% pure), MB, and butylated hydroxytoluene (BHT) were also purchased from Sigma. Methyl eicosanoate (20:0), used as internal standard for GC analysis of residual fatty acids, was purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Sodium borohydride powder ($NaBH_4$, 98%) was from Aldrich (Gillingham, JL), and the plates used for thin-layer chromatography (TLC, 0.25 mm silica gel 60) were from Merck (Darmstadt, Germany). All other reagents and organic solvents used were of analytical grade.

Photooxidation. Photosensitized oxidation was carried out in ethanol solution containing 0.11×10^{-3} M MB. The light source was a Philips HPI-T 400 W fluorescent lamp of wavelength 350−700 nm. Samples were put into clear glass vials (Glaswarenfabrik Karl Hecht, GmbH, Sondheim-rhön, Germany) of 14 mL volume (24 mm in diameter), which were tightly closed. The bottles were placed on a glass panel (0.6 cm in thickness), whereby the light of wavelengths <400 nm was filtered off. The glass panel was situated 1.5 m above the lamp. All experiments were performed at 25 °C, controlled by a fan working continuously. The light intensity at the samples was 190 ± 5 μmol s^{-1} m^{-2}, monitored by a quantum meter (Skye Instruments Ltd., Llandrindod Wells, Powys, U.K.). The distribution of the light wavelength was mainly between 500 and 700 nm.

ML and MCL, dissolved in ethanol, were mixed in five different ratios (w/w): 10:0, 7.5:2.5, 5.0:5.0, 2.5:7.5, and 0:10, respectively. Samples containing a total of 10 mg of the mixtures were pipetted into glass vials with 2 mg of the internal standard (20:0) and MB. After the final volume was adjusted to 2 mL, samples were sealed and oxidized in the light for 5 days. Representative duplicate samples were removed each day and analyzed for peroxide value, residual fatty acids, and residual MB.

Peroxide Value (PV) Analysis. The PV was determined for each sample at the chosen time points by using the ferric thiocyanate method (Ueda et al., 1986). Sample (15−200 μL) was added to 10 mL of 99% ethanol containing 200 μL of an aqueous solution of ammonium thiocyanate (30%). After 200 μL of ferrous chloride solution (20 mM in 3.5% HCl) was added, the mixtures were stirred for 3 min and the absorbance of the pink ferric thiocyanate complex was measured at 500 nm. The

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PV, expressed as milliequivalents of peroxide per kilogram of sample, was calculated by using the formula (Shantha et al., 1994)

$$\mathrm{PV} = \frac{(A_s - A_b)m}{55.84 m_0 \times 2}$$

where A_s is the absorbance of the sample; A_b is the absorbance of the blank, m is the slope, obtained from the standard curve (38.63 in this experiment); 55.84 is the atomic weight of iron; m_0 is the sample mass in grams; and 2 expresses the PV as milliequivalents of peroxide instead of milliequivalents of oxygen.

Analysis of Residual Fatty Acids. To extract the lipid, 1 mL of sample was mixed with 2 mL of diethyl ether and 3 mL of distilled water. After vigorous shaking, the ether layer was collected while MB was left in the water phase. A 20 μL aliquot of BHT (10 mg/mL ethanol) was then added to the lipid extracts to prevent further oxidation during sample workup, and the solvents were evaporated under a stream of nitrogen. The hydroperoxides in the sample were chemically reduced to alcohols by incubation with 2 mL of $NaBH_4$ solution (10 mg/mL ethanol) at 4 °C for 1 h. After incubation, 2 mL of water was added to decompose the excess reagent and the lipids were extracted in 2 mL of dichloromethane and dried using anhydrous sodium sulfate. The dichloromethane was then evaporated under a stream of nitrogen, and lipids were dissolved in dichloromethane/hexane (1:2 v/v).

To purify the fatty acid methyl esters (FAME) for GC analysis, 100 μL of this lipid solution was applied as 1.5 cm bands on single-dimension TLC plates which were developed with a mixture of hexane/diethyl ether/acetic acid (85:15:1 v/v). The reference spots were located using iodine vapor while the other portion of the plate was covered. Areas corresponding to methyl esters were removed from the plate and eluted with 3 mL of diethyl ether. The ether was evaporated, and the FAME were dissolved in 50 μL of hexane for GC analysis.

Residual FAME were analyzed essentially as described before (Jiang et al., 1996) using an HP5890 series II gas chromatography (Hewlett-Packard Co., Rolling Meadows, IL), fitted with a flame ionization detector. Samples were injected through the split injection port (split ratio = 40:1) onto a CP Sil 88 fused silica capillary column (50 m × 0.22 mm, 0.2 mm film thickness; Chrompack, Middelburg, The Netherlands). The injector and detector temperatures were 250 °C. The temperature program used was as follows: 180 °C held for 3 min, increased from 180 to 200 °C at 5 °C/min, and then held for 10 min. The identification of individual FAME was performed by comparing their relative retention times with those of individual FAME standards.

Measurements of Residual Methylene Blue. After the lipid had been extracted from each sample, the water phase containing MB was transferred to a 1 cm quartz cell. Residual MB in the samples was monitored spectrophotometrically from its absorbance at λ_{max} = 664 nm.

RESULTS AND DISCUSSION

The rates of the peroxide formation during the photooxidation of pure ML and MCL differed substantially (Figure 1a). MCL consistently yielded a lower amount of peroxides than ML. Similar results were obtained by Ha et al. (1987, 1990), who found that peroxides are not readily formed from CLA as opposed to LA during autoxidation. The percentages of residual ML and MCL are shown in Table 1 (samples A and E, respectively). MCL was lost at a much lower rate compared to ML during the course of oxidation. By day 5, the amount of residual MCL (~38%) was much higher than that of ML (~10%). These results are different from those published by van den Berg et al. (1995), who studied the autoxidation of CLA and LA exposed to air by measuring residual FAME and found that CLA is more susceptible to oxidation than LA.

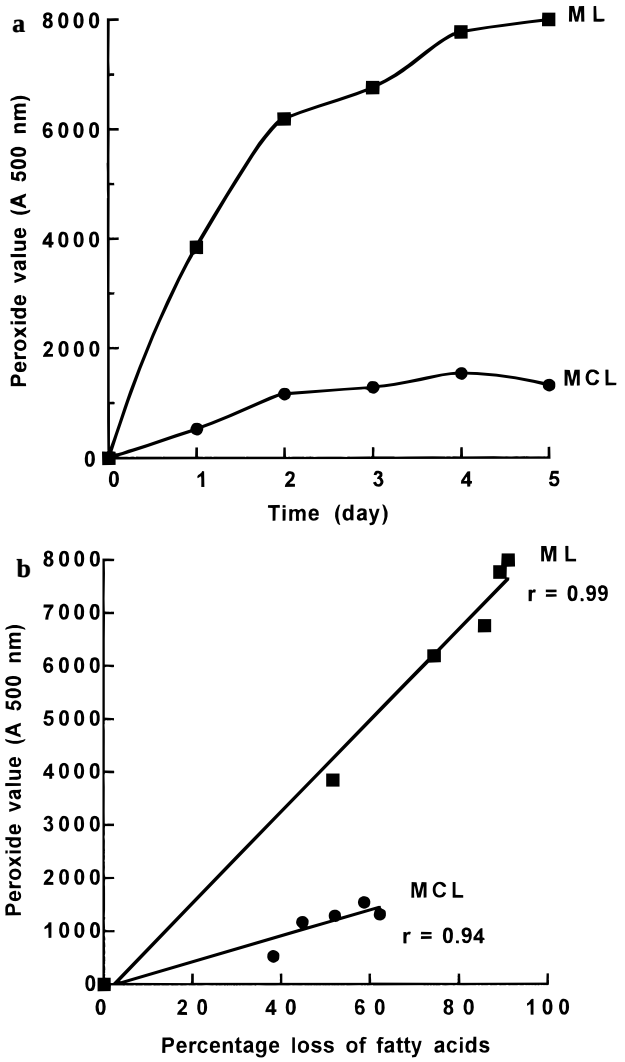


Figure 1. MB-sensitized photooxidation of pure ML and pure MCL: (a) PV as a function of time; (b) relationship between the loss of fatty acids and the development of PV.

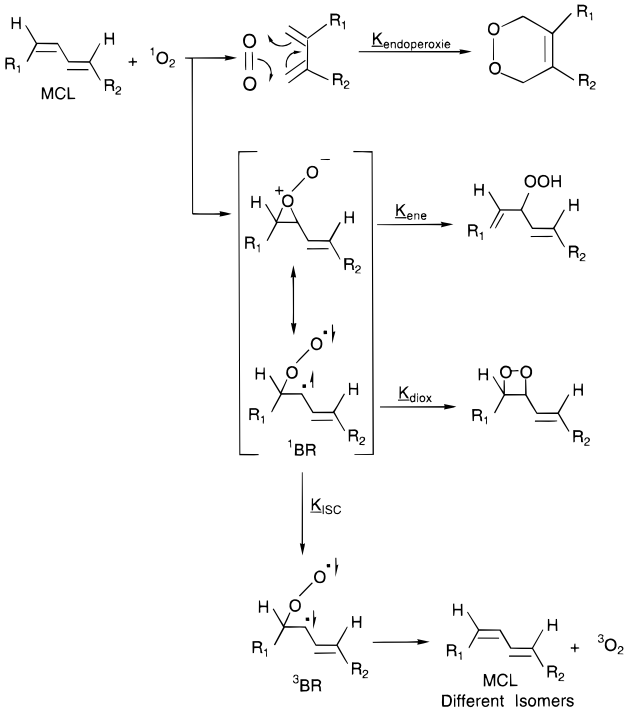
Table 1. Mean Residual Fatty Acids (n = 2) of Different Mixtures[a] of ML and MCL during 5 Days of Photooxidation

time (day)	A	B	C	D	E
		ML (Weight Percent)			
1	48.4	46.3	47.0	47.6	
2	25.5	23.5	24.4	26.8	
3	14.2	12.2	13.8	17.0	
4	10.8	8.3	7.7	9.4	
5	9.9	7.1	6.0	7.8	
		MCL (Weight Percent)			
1		55.8	58.0	61.2	61.8
2		50.0	54.1	50.5	55.2
3		37.2	40.8	45.3	48.0
4		33.2	37.2	39.1	41.3
5		28.4	30.8	35.1	37.8

[a] All samples contained a total of 10 mg of ML + MCL; the different mixtures had the following compositions: A (10 + 0); B (7.5 + 2.5); C (5.0 + 5.0); D (2.5 + 7.5); and E (0 +10), respectively.

The relationship between the loss of FAME and the development of peroxides is shown in Figure 1b. For the same percentage loss of FAME, much higher PVs were obtained from ML than MCL, suggesting that

Scheme 1. Schematic Representation of the Reaction of MCL with Singlet Oxygen ($^1O_2$)[a]



[a] After Manring and Foote (1983), Manring et al. (1983), Clennan and L'Esperane (1985a,b), and O'Shea and Foote (1988). ^{1}BR and ^{3}BR, singlet and triplet biradicals, respectively.

hydroperoxides were not the only primary oxidation products of MCL and/or that they were decomposed, at a rate similar to that of their formation, to secondary products. In the previous papers (Ha et al., 1987, 1990), CLA has been shown to be more resistant to oxidation than LA and has been suggested to act as an antioxidant. However, in those studies PV was the only measurement performed to follow the course of oxidation. According to our results, PV measurements may not be enough to understand the entire fatty acid oxidation/degradation process. Residual fatty acids and other oxidation parameters should also be determined.

Photosensitized oxidations are categorized into free radical-derived (type I) and singlet oxygen-derived reactions (type II) (Foote, 1976, 1982). There is evidence that MB-photosensitized oxidations of electron-rich compounds (including olefins and dienes) favor singlet oxygen reactions (Foote, 1976; Porter et al., 1979; Frankel et al., 1979; Terao et al., 1981). The reaction of ML with $^1O_2$ generated during photosensitization is known to proceed by the ene mechanism, via a six-membered ring intermediate, to yield the 9-, 10-, 12-, and 13- hydroperoxides of octadecadienoate as the main photooxidation products (Bradley and Min, 1992). The reaction of MCL with $^1O_2$ has not been studied before but may be compared to that published for other conjugated dienes. Conjugated dienes were shown to react with $^1O_2$ to form ene-hydroperoxides, dioxetanes, and endoperoxides, depending on the solvent and steric restrictions of the substrate (Manring and Foote, 1983; Manring et al., 1983; Clennan and L'Esperane, 1985a,b). The dioxetanes were often found to be the major products, and their formation was reported to proceed through the intermediary of zwitterions and 1,4-biradicals (Manring and Foote, 1983; Clennan and L'Esperane, 1985b) (Scheme 1).

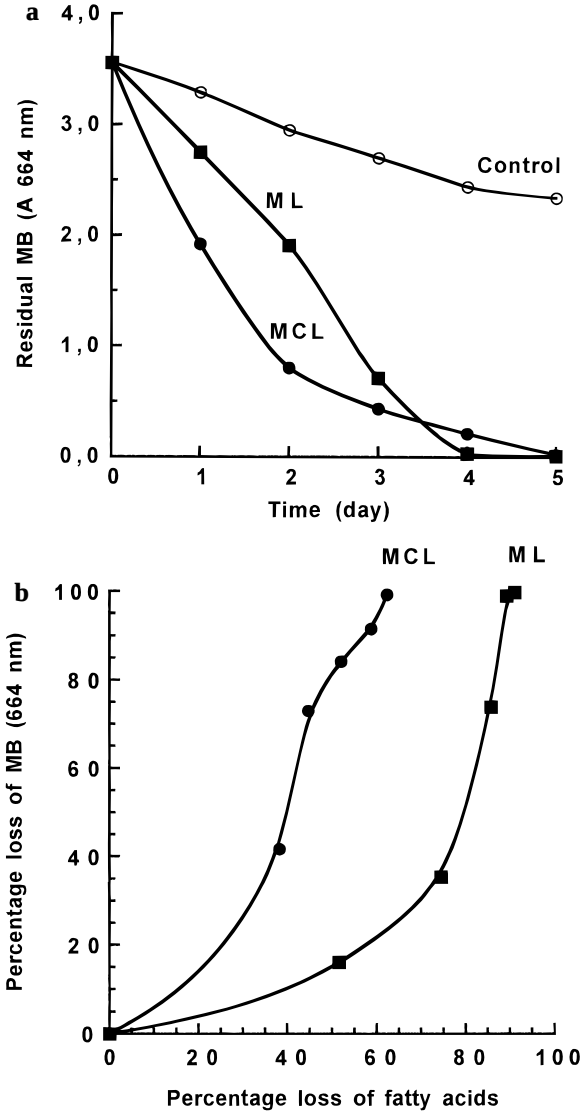


Figure 2. Bleaching of MB in samples containing pure ML and pure MCL during 5 days of photooxidation: (a) comparison of residual MB in ML and MCL samples with that in control samples containing only MB; (b) relationship between the loss of MB and the loss of fatty acids.

In this experiment, it is important to note that although total amounts of MCL decreased significantly upon photosensitization, its different isomers were not equally lost (Figure 3). The main CLA isomers (9,11-cis,trans + 9,11-trans,cis, and 10,12-trans,cis) were found to be lost at similar rates, and they were the only isomers that were lost. The level of the cis,cis isomers of 9,11- and 10,12-MCL was constant but the amount of total trans,trans isomers (9,11 + 10,12) increased ($P < 0.01$) during the course of photooxidation. This observation is in agreement with the finding that rapid isomerization (cis-to-trans) of conjugated dienes occurs during photooxidation, where $^1O_2$ was reported to be directly involved in conversion of the cis,trans isomers of hexadiene to trans,trans isomers (O'Shea and Foote, 1988). The cis,cis isomer, which is roughly 5 kcal higher in energy than the others (Jensen and Foote, 1987), is not formed from the other isomers. The isomerization is caused by the rotation of the single bonds of the

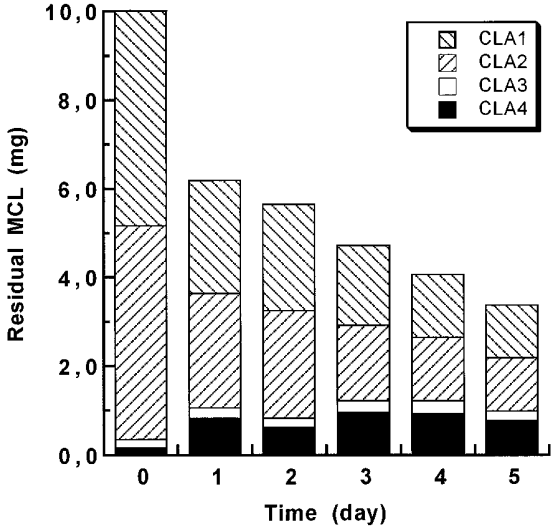


Figure 3. Decrease in total MCL and changes in its isomer composition during 5 days of MB-sensitized photooxidation: CLA1, 9,11-cis,trans + 9,11-trans,cis isomers; CLA2, 10,12-trans,cis isomer; CLA3, 9,11- and 10,12-cis,cis isomers; CLA4, 9,11- and 10,12-trans,trans isomers.

biradicals followed by relaxation to the ground state by intersystem crossing (Scheme 1) (O'Shea and Foote, 1988). Interisomerization of MCL supports our assumption and confirms the literature findings (Manring and Foote, 1983; Clennan and L'Esperane, 1985b) that hydroperoxides are not the only primary photooxidation products of conjugated dienes (Scheme 1).

In addition to the differences in PV and residual fatty acids, MB was found to be bleached differently by MCL or ML (Figure 2a). In samples of ML, MB was bleached at a nearly constant rate until it was all consumed. Compared to ML, MCL bleached MB at a higher rate during the first 2 days and at a lower rate afterward. Different correlations between the bleaching of MB and the loss of the FAME were obtained for MCL and ML (Figure 2b). For the same percentage loss of fatty acids, much more MB was bleached by MCL than by ML. It is known that MB specifically picks a hydrogen atom from the hydroperoxides of ML and becomes reduced to a colorless form (Steward et al., 1983; Tanielian et al., 1992). On the other hand, because the formation of hydroperoxides by MCL was found to be minor compared to ML, the bleaching of MB by MCL should be due to a different mechanism. Thus, the loss of MCL may be partly attributed to unknown interactions with MB, which lead to a fast bleaching of MB.

Therefore, the lower PV obtained for MCL than for ML in this study can be explained by two reasons: (i) MCL was oxidized to a lower extent compared to ML; (ii) the formation of ene-hydroperoxides was not the only factor responsible for the loss of MCL as supported by the interisomerization and the rapid bleaching of MB by MCL.

Mixtures containing different ratios of ML and MCL [(A) 10:0, (B) 7.5:2.5, (C) 5.0:5.0, (D) 2.5: 7.5, and (E) 0: 10 ratios (w/w), respectively] were photooxidized for 5 days. Figure 4a shows the development of peroxides in the different samples (A−E) during the photooxidation. In all mixtures, most of the peroxides were formed during the first 2 days of oxidation. The PV in the mixtures was linearly correlated with the relative amounts of ML and MCL and the rate of peroxide development in their pure samples. Another major

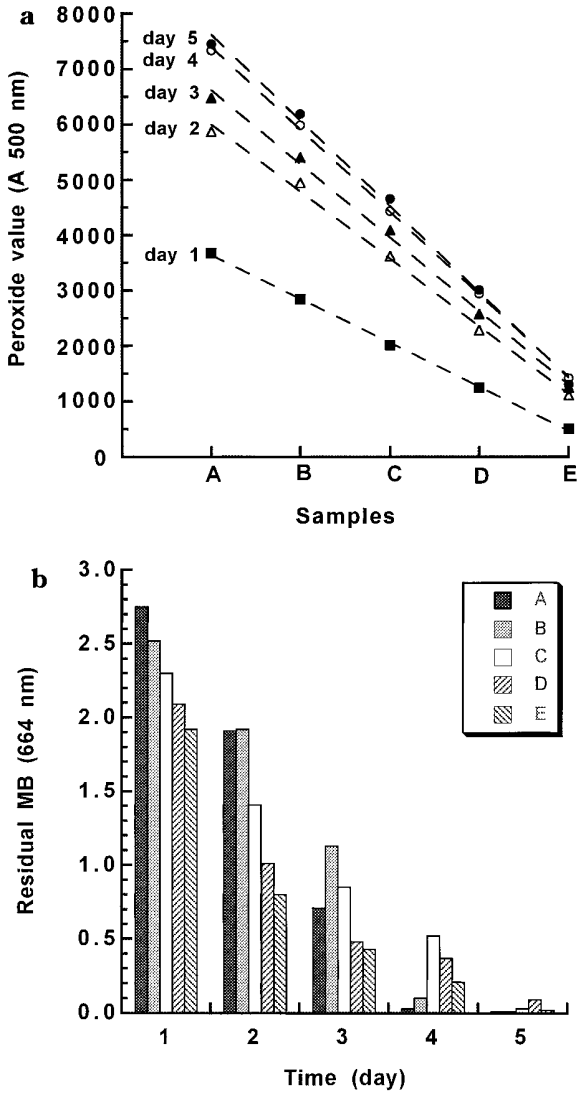


Figure 4. Photooxidation of mixtures of ML and MCL: (a) PV in the mixtures; (b) changes of residual MB in mixtures during 5 days of photooxidation. Samples contained a total of 10 mg of ML + MCL; the different mixtures had the following compositions: A (10 + 0); B (7.5 + 2.5); C (5.0 + 5.0); D (2.5 + 7.5); and E (0 + 10), respectively.

effect of mixing ML and MCL was observed on the different degrees of MB bleaching in the mixtures (Figure 4b). The order of samples with maximum residual MB was found to change during the 5 days. At day 1, sample A (pure ML) had the highest residual MB, whereas sample E (pure MCL) had the lowest value. After day 2, more bleaching occurred in the samples containing more ML. The highest residual MB was found in samples B (day 3), C (day 4), and D (day 5). This shift in the order of samples with highest residual MB was a result of the relative amounts of MCL and ML in the mixtures and their different patterns of bleaching MB as described before (Figure 2a).

The results obtained from this work showed that MCL and ML reacted differently during MB-photosensitized oxidation. The results of the mixing experiment suggested that there were no nonlinearities in the development of PV due to the mixing of these two fatty acids during the course of photooxidation. Further studies of full factorial design on the interaction of ML, MCL,

and MB are warranted to understand the effect of MCL on ML during photooxidation.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; LA, linoleic acid; MCL, methyl conjugated linoleate; ML, methyl linoleate; MB, methylene blue; BHT, butylated hydroxytoluene; PV, peroxide value; FAME, fatty acid methyl esters; TLC, thin-layer chromatography.

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