Determination of Hydroperoxides in Edible Oils by Electron Spin Resonance, Thiobarbituric Acid Assay, and Liquid Chromatography–Chemiluminescence Techniques

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The increasing concern over the possible relationship between lipid peroxidation and certain ailments, and correspondingly with food processing and storage, has prompted our examination of various techniques for the determination of hydroperoxides formed during lipid oxidation. A reversed-phase liquid chromatography method was used to separate the hydroperoxides, followed by postcolumn chemiluminescence detection. In addition, we compared these results with those obtained by the conventional thiobarbituric acid assay and a new procedure involving the oxidation of 2,2,6,6-tetramethyl-4-piperidone to nitroxides in the presence of hydroperoxides, followed by electron spin resonance spectroscopy. The formation and decay of hydroperoxides of methyl linoleate and corn oil under constant heating and oxygen purging are described.

INTRODUCTION

Lipid oxidation, one of the major causes of food spoilage, leads to rancidity in fat-containing food. Furthermore, some of the oxidative products are potentially toxic (Frankel, 1987). Peroxidation of lipids has been implicated in several diseases and in aging (Ames, 1983; Bors et al., 1984; McBrien et al., 1982). Extensive research has been carried out on the mechanism of lipid oxidation, identification of various oxidative products, and their possible interaction with biological molecules (Horton et al., 1987; Kanner et al., 1987). Since lipid hydroperoxides are the primary products of lipid oxidation, a sensitive, specific, and accurate method for measuring these hydroperoxides is needed to provide the necessary data to understand the oxidative mechanisms for lipids and to evaluate the efficacy of the many natural and artifical antioxidants present in foods.

In the oxidation of fats and oils, the initial rate of formation of hydroperoxides exceeds their rate of decomposition. The relative rates of these reactions reverse at later stages. Therefore, monitoring the amount of hydroperoxides as a function of time will indicate whether a lipid is in the growth or decay portion of the hydroperoxide concentration curve. This information can be used as a guide to the acceptability of a food product with respect to the extent of product deterioration. Similarly, by measuring the amount of hydroperoxides before and after certain food-processing procedures, the effects of the processing procedures on the rate of oxidation can be determined. By monitoring the incubation period before the appearance of hydroperoxides, one can assess the effectiveness of added antioxidants on the stability of a commodity. Thus, if the lipid hydroperoxide concentration is measured initially and then during and after the time the food has interacted with an added constituent or with its packaging and storage environment, or if the concentration has changed because of a processing technique such as heating and cooking, our understanding of lipid oxidation in foods will be enhanced.

Several techniques for measuring the extent of lipid oxidation have been published. These include the popular 2-thiobarbituric acid (TBA) test (Pokorny et al., 1985) and the peroxide value test [for a review see Gray (1978)]. These two techniques suffer from limited sensitivity and specificity and require large test portions. In the work reported here, we have applied a recently developed postcolumn chemiluminescence detection method to the measurement of hydroperoxides (Yamamoto et al., 1987) after separation by liquid chromatography (LC). In addition, we have previously demonstrated that hydroxyl radicals oxidized 2,2,6,6-tetramethyl-4-piperidone to stable free radicals (Rosenthal et al., 1987). In the present work, we found that lipid hydroperoxides are also capable of oxidizing 2,2,6,6-tetramethyl-4-piperidone to stable nitroxide radicals, which can be determined by electron spin resonance (ESR) spectroscopy. We have compared the results obtained with LC–chemiluminescence and the ESR technique for measuring oxidized fatty acids with those from the standard TBA test on simple fatty acid esters and edible oils.

EXPERIMENTAL PROCEDURES

Materials and Reagents. Figure 1 is a schematic diagram of the LC–chemiluminescence instrumentation. The LC system consists of a Shimadzu Model C-R2A solvent delivery system and Shimadzu C-R2XA data system with a Shimadzu SPD-6AV UV-VIS spectrophotometric detector and a Kratos FS 950 fluorescence detector. A luminol solution containing 1.24 g of Na_2CO_3, 124 mg of luminol (Sigma), and 2.5 mg of bovine hemin (Aldrich) per liter, adjusted to pH 11, was delivered by an Applied Biosystems Model 400 solvent delivery system to an Applied Biosystems Model PCRS 510/520 heater system for LC postcolumn reaction. Two Rainin 5-μm reversed-phase Microsorb columns, C-8 and C-18, were connected in series and heated to

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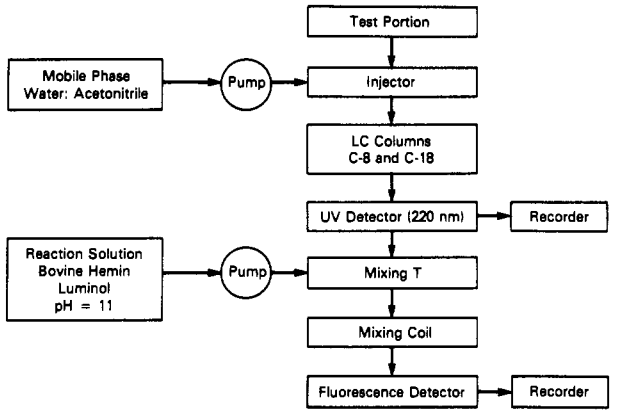

Figure 1. Schematic diagram of LC–chemiluminescence detection system.

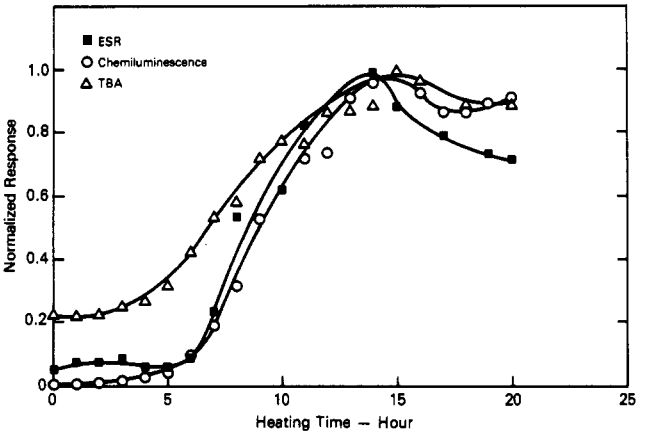

Figure 2. Detector responses for thermally oxidized corn oil, at 60 °C under constant oxygen purging, by chemiluminescence, ESR, and TBA assay techniques.

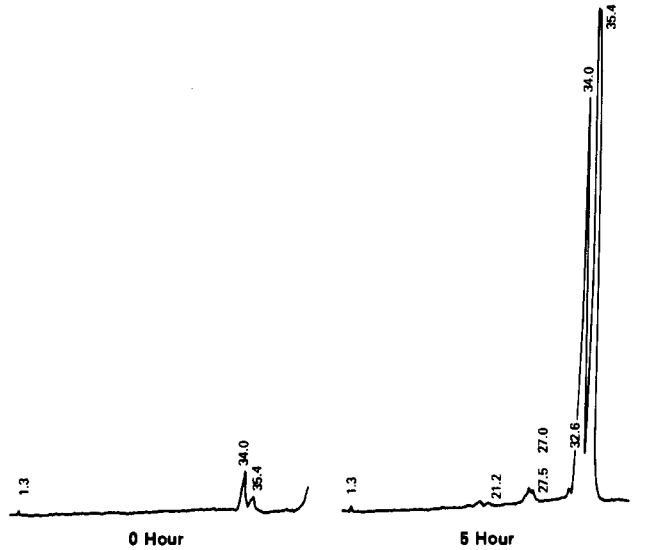

Figure 3. LC chromatograms of thermally oxidized methyl linoleate, at 60 °C and under constant oxygen purging, for 0 and 5 h.

60 °C. From the absence of any chemiluminescence response for an unoxidized lipid after LC separation at 60 °C, it can be safely concluded that no hydroperoxide is formed during column transit. Conversely, incorporating an oxidized lipid in the LC solvent without oxygen for up to 0.5 h at 60 °C shows no loss of chemiluminescence response. Water–acetonitrile, initially 60:40, with a linear gradient to 0:100 water–acetonitrile in 40 min, was used as the mobile phase at a flow rate of 1.3 mL/min and mixed with the luminol solution at a flow rate of 1.0 mL/min.

Commercially obtained corn oil and diluted methyl linoleate (Nu Check) were heated separately in a constant-temperature

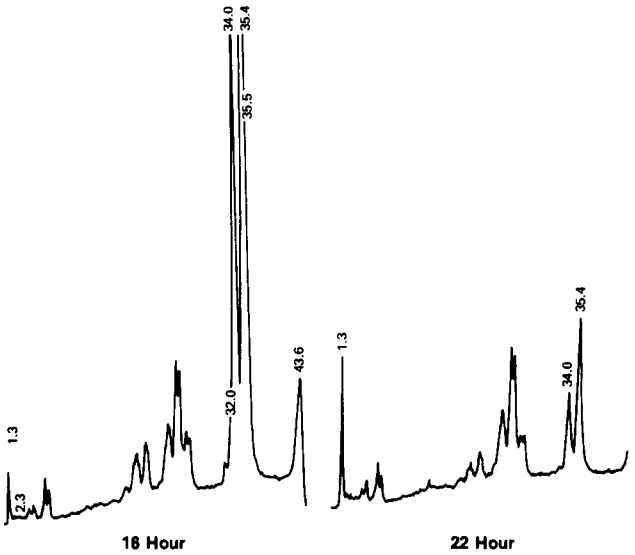

Figure 4. LC chromatograms of thermally oxidized methyl linoleate, at 60 °C and under constant oxygen purging, for 16 and 22 h.

bath at 60 °C under constant oxygen purging. Aliquots (100 μL) were withdrawn periodically and dissolved in 10 mL of acetonitrile prior to LC injection.

A Varian E-109 X-band ESR spectrometer was operated at a microwave power of 10 mW with a modulation of 0.5 G. Oxidized corn oil or methyl linoleate was added to a solution of 2,2,6,6-tetramethyl-4-piperidone monohydrate (Aldrich) in acetonitrile (10 mg/mL) and incubated for 6 h before measurement.

TBA values were determined according to the procedures of Pokorny et al. (1985) for fats and oils.

RESULTS AND DISCUSSION

The response of the chemiluminescence detector with peroxide concentration was investigated with benzoyl peroxide as a test compound. A linear relationship for benzoyl peroxide was observed when the combined LC postcolumn chemiluminescence detection scheme was used. With prior separation by LC, impurities such as antioxidants do not interfere with the oxidized lipids during measurement, thus offering additional specifity and sensitivity. With our instrumentation, each individual lipid hydroperoxide compound can easily be measured at a level of 1 ng with a signal-to-noise ratio of 2:1. For the ESR determination of hydroperoxides, 2,2,6,6-tetramethyl-4-piperidone was oxidized to a nitroxide radical in the presence of hydroperoxides (Rosenthal et al., 1987). We have also observed that the nitroxide concentration, as reflected by the ESR amplitude, is directly proportional to the concentration of benzoyl peroxide.

Hydroperoxide formation in heated corn oil, as measured by chemiluminescence, nitroxide concentration, and TBA assay for up to 20 h, is illustrated in Figure 2, which provides a relative comparison of the time dependence of the concentration of hydroperoxides for the three techniques. Since each technique involved a different principle of measurement with vastly disparate responses toward various hydroperoxide moieties, it was necessary to normalize the quantitative responses to the maximum response for each technique used. In addition, the corn oil chemiluminescence response was obtained by adding the various peaks after LC separation. The initial incubation period was followed by a rapid rise. A maximum value was reached, and upon further oxidation, a decrease was observed. At this time the corn oil became extremely viscous. Figure 2 shows that the measurements by the three techniques were in generally good agreement.

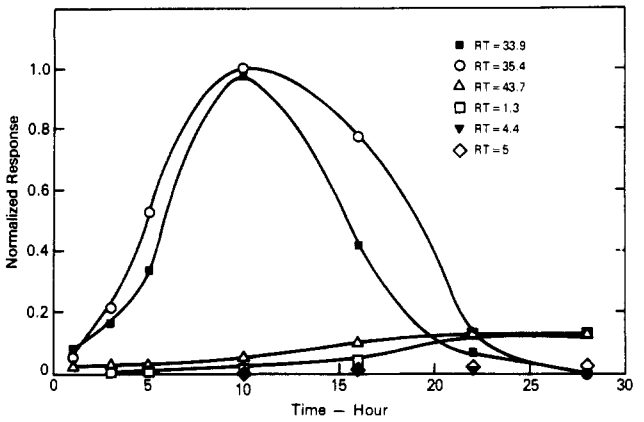

Figure 5. Time-dependent LC peak responses as detected by the chemiluminescence of thermally oxidized methyl linoleate, at 60 °C and under constant oxygen purging.

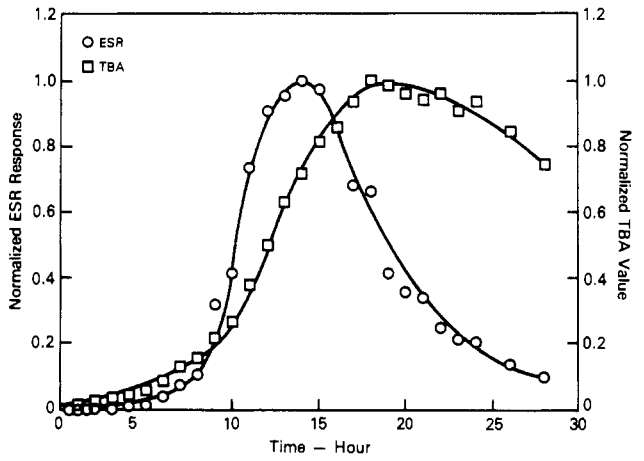

Figure 6. ESR and TBA determination of thermally oxidized methyl linoleate, at 60 °C and under constant oxygen purging.

To utilize the separation capability of LC, we re-examined the oxidation of methyl linoleate, using post-column chemiluminescence detection. Hydrogen abstraction from carbon 11 of methyl linoleate forms a pentadiene radical, which reacts with oxygen to form an equal concentration of conjugated 9- and 13-hydroperoxide isomers with the trans,cis conformation (Frankel, 1984). Figures 3 and 4 show the chromatographic response with chemiluminescence detection for methyl linoleate oxidized by constant purging with oxygen and heating at 60 °C for 0, 5, 16, and 22 h. Initially two small peaks appear at retention times of 33.9 and 35.3 min. The result is similar to the reversed-phase chromatographic separation of oxidized methyl linoleate reported by Chan and Levett (1977). The ratio of detector response between UV (205 nm) and chemiluminescence is at least 10:1.

With increasing time, these two peaks increased, as did others, which were unidentified but assumed to be due to other hydroperoxides. It should be noted that these peaks represent hydroperoxides as judged by the characteristic chemiluminescence response. Additional spectroscopic studies to identify these products are in progress. After 22 h, the original two peaks decreased, while the other "hydroperoxides" continued to increase. Eventually all peaks associated with the hydroperoxides decayed and disappeared. These chromatograms clearly demonstrate the utility of the LC–chemiluminescence technique, which permits the study of the detailed kinetic reactions of each hydroperoxide.

Figure 5 shows the time dependence of the chemiluminescence responses for the two major peaks occurring at 33.9 and 35.4 min, as well as for the other minor peaks at 1.3, 4.4, 5, and 43.7 min. The minor peaks produced by the more polar compounds (RT = 1.3, 4.4, and 5 min) are probably attributable to the secondary hydroperoxides and continue to grow after 25 h; their identities are currently under study. TBA and ESR measurements made as a function of time (up to 28 h) for the same oxidized methyl linoleate are shown in Figure 6. Compared with the ESR and chemiluminescence measurements, the TBA assay value reached a maximum at a later time and did not decay as rapidly as the values obtained by the other two techniques.

For the analysis of the overall oxidation of corn oil, the three techniques exhibit a good correlation, as demonstrated in Figure 2. TBA and ESR determinations essentially gave an overall total oxidation without individual hydroperoxide profile and suffered from possible interfering compounds. To resolve this situation, prior separation by LC is essential. A combination of LC with chemiluminescence not only provides the specificity rendered by the unique interaction among luminol, hemin, and hydroperoxides enhanced by the resolving separation power of LC but also enables a lower level of determination by virtue of the excellent sensitivity offered by the chemiluminescence detector. Application of this technique is especially useful in the biological area, where measurement is often hampered by the limited amounts of material available.

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