Quantitative HPLC Determination of the Antioxidant Activity of Capsaicin on the Formation of Lipid Hydroperoxides of Linoleic Acid: A Comparative Study against BHT and Melatonin

David E. Henderson* and Adam M. Slickman

Chemistry Department, Trinity College, Hartford, Connecticut 06106

Susan K. Henderson

Chemistry Department, Quinnipiac College, Hamden, Connecticut 06518

The antioxidant activity of capsaicin, as compared to BHT and melatonin, was determined by the direct measurement of lipid hydroperoxides formed upon linoleic acid autoxidation initiated by AIBN. The formation of four isomeric lipid hydroperoxides was detected after reverse-phase HPLC separation. Data from three detectors, UV absorption, glassy carbon electrode electrochemical detection, and postcolumn chemiluminescence using luminol, were compared. Capsaicin was more effective than melatonin in suppressing the formation of lipid hydroperoxides but not as effective as BHT. The formation of capsaicin and BHT dimers was observed during oxidation, and the dimers were characterized using APCI MS^n.

Keywords: *Lipid hydroperoxide; capsaicin; butylated hydroxytoluene; melatonin; electrochemical detection; HPLC; APCI-MS; antioxidant; linoleic acid autoxidation*

INTRODUCTION

The search for plant-derived antioxidants is a topic currently receiving a great deal of attention, partly due to the suspected connection between lipid oxidation and various pathologies such as cancer, heart disease, and aging (Dix and Aikens, 1993). This study reports the development of a simple, quantitative LC−EC procedure for the comparison of antioxidant effectiveness by the direct measurement of the first formed stable products of lipid oxidation, namely, lipid hydroperoxides. This procedure was used to compare the antioxidant (AO) activity of capsaicin (CAP) to butylated hydroxytoluene (BHT), a commonly used synthetic AO, and melatonin, a hormone believed to have AO activity.

Capsaicin is the principle ingredient responsible for the irritating and pungent effects of hot chile peppers and has become more widely used as the popularity of cuisines featuring hot and spicy foods has increased. In a previous study (Henderson and Henderson, 1992) it was observed that the oxidation of oleic acid at cooking temperatures was inhibited by the presence of CAP.

CAP, shown in Figure 1, is an intriguing molecule for use as an AO because it incorporates the phenolic structure found in the widely used synthetic antioxidant BHT. In addition, CAP contains an amide group which has been associated with AO activity and is the most probable site for free-radical attack in molecular modeling studies using Personal CAChe (Oxford Molecular Group, Oxford, U.K.). Further, the amide group of CAP is involved in thermal oxidation reactions of CAP (Henderson, 1992). MEL (Figure 1) has been shown to exhibit AO activity (Pieri, 1994) and was included in the study to evaluate possible effects of the amide group.

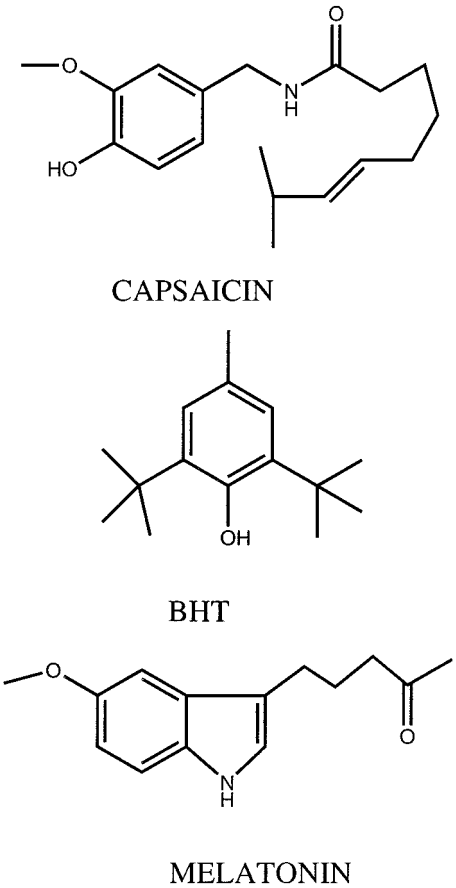

Figure 1. Structures of antioxidants.

* To whom correspondence should be addressed (e-mail david.henderson@trincoll.edu).

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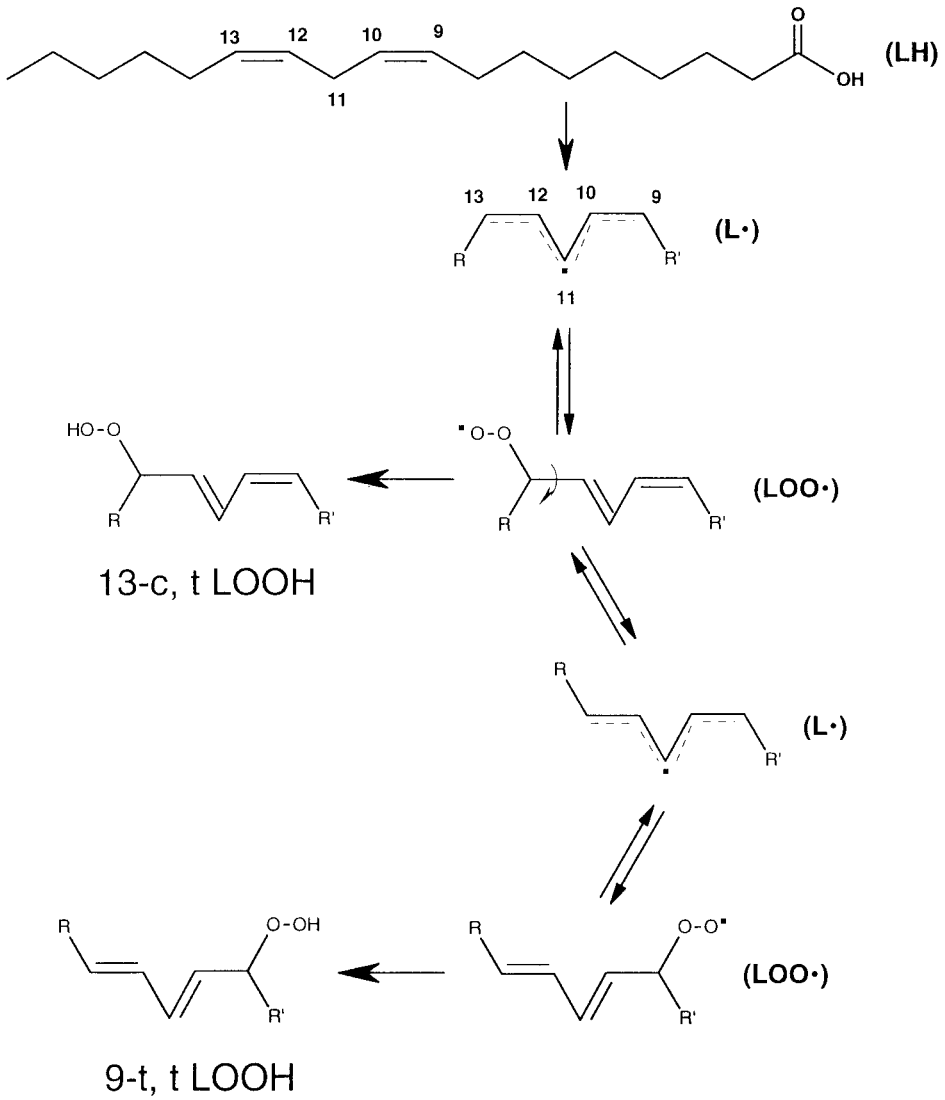


Figure 2. Partial mechanism of oxidation of linoleic acid (Adapted from Porter, 1984).

It is noted that recent reports indicate that MEL's AO activity is not due to radical scavenging (Antunes, 1999).

It is widely accepted that the general pathway for lipid autoxidation is initiated by the formation of a lipid radical (L•) which reacts with molecular oxygen at a rate limited only by the diffusion of the gas in the system. The resulting lipid peroxyl radical (LOO•) is believed to propagate a free-radical chain mechanism by abstracting allylic hydrogen from a lipid molecule to generate a new L• and the first stable molecular product of this process, a lipid hydroperoxide (LOOH). This process is shown schematically in Figure 2. Free-radical oxidation can be interrupted by antioxidants which can react with either alkyl or peroxyl radicals (Frankel, 1991).

The fatty acid chosen for this study, *cis*-9-*cis*-12-octadecadienoic acid (linoleic acid), was selected for two reasons. First, linoleic acid is rapidly oxidized in the presence of the radical initiator 2,2′-azobisisobutyronitrile (AIBN) (Yamamoto et al., 1982). This is in contrast to our initial studies which had indicated that the time required to obtain a measurable level of oleic acid oxidation at noncooking temperatures was too great to be useful. Second, the LOOHs produced (Figure 2) contain a conjugated diene group which is UV-active with a molar absorptivity of 26 000 (Chan and Levett, 1977). This provided a detection method during the development of the parameters for the other detectors used in this study.

A number of recent studies have reported HPLC methods that provide very sensitive and selective detection of LOOHs. The use of chemiluminescence detection was first reported by Yamamoto et al. (1982), and the use of reagents other than luminol has been reported (Akaska et al., 1987; Ohshima et al., 1996). The measurement of various LOOHs has been accomplished by normal-phase (Hopia et al., 1996) and reverse-phase HPLC separation (Korytowski et al., 1999) with various modes of detection. The identification of the specific LOOH isomers from linoleic acid in this report was based upon the work of Chan et al. (1975) and the more recent work of Schneider et al. (1997). Figure 2 shows the formation of two isomers; however, the hydroperoxide can form at either the 9 or 13 position for both cis−trans and trans−trans forms. The specific mechanisms for the formation of each isomer have been reported in detail by Porter et al. (1984, 1995).

Electrochemical detection has also been used to measure LOOHs. Photochemically generated cholesterol hydroperoxides were analyzed by reverse-phase HPLC with electrochemical detection performed in the reduction mode under anaerobic conditions (Korytowski et al.,

1991, 1999). Terao et al. (1988) reported the measurement of arachidonic acid hydroperoxides and their hydroxy derivatives by reverse-phase HPLC using combined UV absorption and reductive electrochemical detection.

One goal of this study was to select the optimal detector for ongoing studies of antioxidant effectiveness of natural products. The UV detector is limited to polyunsaturated fats, and the wavelength used is absorbed by many other substances, leading to potential interferences. Postcolumn chemiluminescence, like all postcolumn methods, suffers from degraded chromatography and instrumental complexity. Electrochemical detection should offer a true measure of the hydroperoxide functional group and allow efficient and simple detection of a wide range of conjugated and nonconjugated lipid hydroperoxides with minimal interferences.

This study reports the first use of direct reverse-phase HPLC analysis of LOOHs for the evaluation of AO effectiveness. Hopia, Huang, and Frankel have previously used normal-phase HPLC for studies of the antioxidants α-tocopherol and Trolox (Hopia et al., 1996). Since LOOHs are the first nonradical products formed in the lipid oxidation process and hydroperoxides are known to be responsible for tissue damage and toxicity in both oxidized food products and in vivo, their direct measurement offers the best single measure of the effectiveness of an AO. The analysis of small-chain volatile acid decomposition products in the Rancimat method or malonaldehyde in the TBA method (Pokorny et al., 1985) would not be able to differentiate between antioxidants which prevented LOOH formation and those which allowed peroxides to accumulate by blocking their subsequent degradation to secondary products. Thus, direct hydroperoxide measurement should provide more specific information on the mechanisms and true effectiveness of antioxidants.

MATERIALS AND METHODS

Reagents. CAP, linoleic acid, luminol (3-aminophthalhydrazide), hemin chloride (bovine), BHT, MEL, AIBN, and sodium carbonate were purchased from Sigma Chemical Co. (St. Louis, MO). HPLC solvents were Fisher HPLC grade. Tetraethylammonium perchlorate was prepared by mixing equimolar quantities of tetraethylammonium hydroxide (Aldrich Chemical Co., Milwauke, WI) and concentrated perchloric acid (Fisher Scientific, Springfield, NJ) in 0 °C water. The resulting precipitate was vacuum filtered, rinsed with water, and air-dried.

Instrumentation. Chemiluminescence detection was performed using a Hitachi (Tokyo, Japan) model 6300 dual-pump solvent delivery system equipped with a Hitachi AS 4000 intelligent auto sampler, Hitachi model D6100 data system, Hitachi model L3000 photo diode array detector at 233 nm, and Hitachi F1050 fluorescence detector with the lamp turned off and the emission wavelength set at 430 nm. A column oven, Hitachi L 5020, was used to maintain the column temperature at 50 °C. A luminol solution containing 15 g of sodium carbonate, 0.0025 g of bovine hemin, and 0.1234 g of luminol was prepared. This solution was found to have a pH of 11. The HPLC column mobile phase used was 70:30 methanol−water. The addition of 2% acetic acid to the water improved the separation of the hydroperoxide peaks and was used throughout the study. Flow rates used were 1.3 mL/min for the column mobile phase and 3.0 mL/min for the postcolumn luminol solution. The HPLC column used was a Microsorb Short One, C-18, 3 μm, 20 cm in length, equipped with a 2 cm guard column (Ranin, Emeryville, CA). Postcolumn detection was accomplished by passing the sample−luminol mixture through a reaction coil consisting of 6 ft of 0.010 in. i.d. stainless steel tubing and subsequent fluorescence detection at 430 nm.

Electrochemical detection was performed using a BAS-200 HPLC (West Lafayette, IN) with dual-channel glassy carbon amperometric detection. The system was operated by a PC running BAS Control and BAS Report software in Windows 95. Solvents were preheated to 50 °C and degassed with helium. The column oven was operated at 50 °C throughout the study. Injection was by means of a Hitachi AS4000 autosampler for initial studies and kinetics studies. Quantitative comparison of antioxidants were performed using a CMA 200 refrigerated autosampler kept at 4 °C (Acton, MA). The outlet of the electrochemical detector was connected to a Gilson (Middleton, WI) 115 UV detector for initial studies and to a Hewlett-Packard1040 diode array detector (Palo Alto, CA) for later studies. The HP detector was operated by a HP 300 Chemstation for data acquisition and signal processing. Detection was done at 233 and 280 nm.

The mobile phase for electrochemical detection was identical to that for chemiluminescence except for the addition of 0.030 M tetraethylammonium perchlorate as supporting electrolyte. Oxidative mode detection was optimized at +1300 mV vs a AgCl reference electrode. The second channel of the detector was operated in reductive mode at +600 mV vs a AgCl reference.

Mass spectra were obtained using a VG-Trio-2 quadrapole MS (Micromass, Danvers, MA) with LabBase software and using the atmospheric pressure chemical ionization (APCI) LC−MS interface of the LCQ (Finnigan, Piscataway, NJ). The LCQ included a TSP HPLC system with a TSP 6000 diode array detector (Thermo Separations Products, Piscataway, NJ), which was used to determine the wavelengths of hydroperoxide peaks for this report. The APCI source was operated at 1.3 mL/min flow of 70:29.5:0.5 methanol:water:acetic acid. Sheath gas flow 70, auxiliary gas flow 10, discharge current 5 μA, capillary temperature 150 °C, vaporizer temperature 350 °C for LOOH and 400 °C for dicapsaicin. Lipid hydroperoxides were analyzed in negative-ion mode, while the capsaicin dimer analysis was done in positive-ion mode.

Sample Preparation for Oxidation Study. A 0.30 M solution of linoleic acid (180.4 g/mol) was prepared by dissolving 0.54 g of linoleic acid in 10.00 mL of methanol. Linoleic acid was stored at −70 °C under nitrogen prior to use. A 0.20 M solution of AIBN (164.2 g/mol) was prepared by dissolving 32.8 mg of AIBN in 1.00 mL of methanol. Antioxidant solutions of 0.20 M were prepared by dissolving 61 mg of CAP in 1.00 mL of methanol, 44 mg of BHT in 1.00 mL of methanol, and 46 mg of MEL in 1.00 mL of methanol. A series of duplicate samples were prepared by placing 1.00 mL of the linoleic acid solution in each of 10 amber autosampler vials. Two vials were used as controls to monitor the lipid without the addition of AIBN or AO. To an additional two vials, 0.10 mL of AIBN solution was added in order to measure the concentration of LOOH in the presence of a free-radical initiator without the addition of AO. To the remaining five vials, 0.10 mL of AIBN solution was added and 0.10 mL of either CAP, BHT, or MEL was added in duplicate. All of the vials were sealed with PTFE caps. Each of the vials was immediately analyzed by HPLC and stored at room temperature in the autosampler for the time series analysis. This procedure produced a 1:1 molar ratio of AIBN to AO and a 12:1 molar ratio of linoleic acid to AIBN.

Samples for quantitative comparison of antioxidants and for concentration dependence studies were prepared as for the time series analysis; however, four different volumes, 0.10, 0.050, 0.010, and 0.0050 mL, of each of the AO solutions were added to separate vials. Appropriate volumes of methanol were added to each vial in order to bring the final volume to 1.20 mL. Vials were filled in air and sealed. They were then transferred to a constant-temperature bath operating at 30.0 °C for 1 week. They were then stored at −70 °C until they were analyzed and then kept at 4 °C during analysis.

The oxidation products of capsaicin were purified for further analysis by solid-phase extraction using 1000 mg C_{18} SPE

cartridges (Alltech Assoc., Deerfield, IL). The cartridge was prepared for use according to instructions. A 1.0 mL amount of oxidized CAP was absorbed. Elution of free CAP was done with 7 mL of 70% methanol−water. Oxidized CAP was then eluted with 3 mL of 100% methanol. The product was dried with anhydrous Na_2SO_4 and evaporated to dryness under nitrogen. Trimethylsilyl derivatives of capsaicin oxidation products were prepared using Tri-Sil (Pierce, Rockford, IL).

Synthesis of 13-Hydroperoxy Linoleic Acid. A standard solution of 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid was prepared using the procedure of Hamberg and Samuelsson, using type I−B soybean lipoxidase (Sigma). Linoleic acid was reacted for 30 min under oxygen at room temperature. Samples were extracted with dichloromethane and stored in amber vials at −70 °C. Before use, the dichloromethane was evaporated using a helium stream at reduced pressure. Samples were then redissolved in methanol for spectroscopic and HPLC analysis. Calibration curves for the UV and EC detectors were run using this solution to produce seven solutions in successive 1:4 dilutions. UV absorption at 233 nm was used with an absorptivity of 26 000 (Chan and Levett, 1997) to determine the concentration of LOOH. Calibration curves covered the range from 5400 ng of LOOH injected (in 10 μL) to 5.4 ng of LOOH. Peak areas of duplicate injections were averaged.

RESULTS AND DISCUSSION

Direct HPLC Analysis of Lipid Hydroperoxides. Chromatograms for the separation of LOOHs using (a) electrochemical detection, (b) UV absorption detection, and (c) chemiluminescence detection are shown in Figure 3a, b, and c, respectively. The identification of the peaks, which represent four geometrical isomers, was based upon the work of Schneider et al. (1997). Early in this study, three major peaks were obtained for the LOOHs. The first major peak, labeled peak 2 (λ_{max} = 237 nm) in Figure 3, was identified as an unresolved mixture of the 9- and 13-hydroperoxy cis−trans isomers. The retention time of the enzymatically synthesized 13-hydroperoxy-*cis*-9-*trans*-11-octadecadieneoic acid matched this peak, confirming this part of the peak assignment. Peak 3 was identified as 13-hydroperoxy-*trans*-9-*trans*-11-octadecadienoic acid (λ_{max} = 238 nm), and peak 4 was identified as 9-hydroperoxy-*trans*-10-*trans*-12-octadecadienoic acid (λ_{max} = 238 nm). All previous reverse-phase HPLC separations indicate that the trans−trans isomers elute after the cis−trans isomers (Schneider et al., 1997).

During the course of the study, two additional peaks appeared. Peak 1, a small peak preceding the 13-hydroperoxy-*cis*-9-*trans*-11-octadecadieneoic acid peak, was observed on all three detectors (λ_{max} = 234 nm) and is thus believed to be a LOOH. However, the expectation that the 9- and 13-hydroperoxides should be formed in approximately equal concentrations argues against assigning this peak to one of these isomers. Schneider et al. (1997) reported the presence of other, unidentified hydroperoxides in their mass spectral study, and this peak may correspond to one of those LOOHs found in their study. Negative-ion APCI spectra of all four peaks show two major ions, *m*/*z* 311 ($M-H^-$) and 371 (M + acetate$^-$). MS−MS spectra yielded a peak at *m*/*z* 293 ($M - H_2O^-$). MS^3 spectra were below detection limits and did not yield useful information on the identity of the isomers. The values of λ_{max} observed for the lipid hydroperoxide peaks were measured at 1 nm resolution using the TSP detector. All were shifted to longer wavelengths than those reported by Hopia et al. (1996). This shift is attributed to the higher polarity of the aqueous methanol mobile phase when compared with the nonpolar hexane−diethyl ether mobile phase used for normal-phase HPLC.

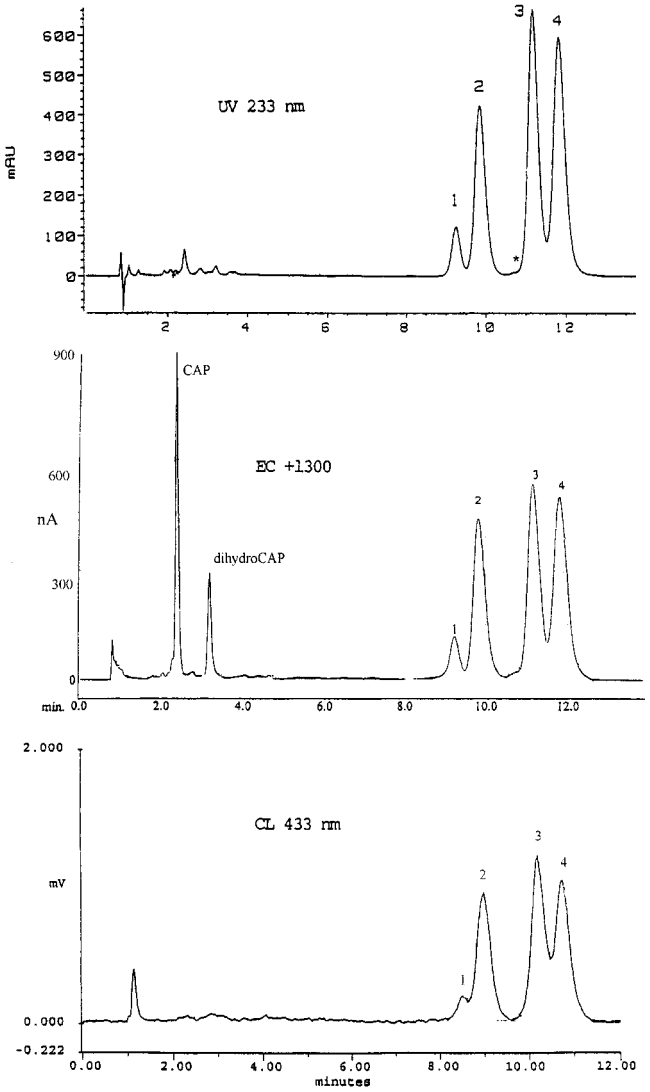


Figure 3. Chromatograms of 0.20 M linoleic acid, 0.001 M capsaicin, 0.02 M AIBN mixture after extensive lipid oxidation. Flow rate 1.3 mL/min; column temperature 50 °C; mobile phase 70% methanol, 30% water, with 2% acetic acid. For EC and UV detection, mobile phase was also 0.030 M in tetraethylammonium perchlorate. Chemiluminescence luminol solution added postcolumn at 3.0 mL/min. Peaks between 2 and 4 min in EC chromatogram are capsaicin and dihydrocapsaicin. Hydroperoxide peaks identified in text. Asterisk (*) indicates the position of peak 5.

A fifth small peak was observed between the cis−trans peak and the two trans−trans peaks. This peak has not been identified and is not included in the quantization since it is below the detection limits on most samples. It was not definitively detected in either positive or negative APCI LC-MS chromatograms.

The retention time of each peak slowly increased as the column aged, though the data were stable within the time scale of several days. This was due to a buildup of free linoleic acid on the column. Since linoleic acid is not detected by EC, A_{233}, or CL detectors, it had no effects other than those on retention time. Periodic flushing of the column with methanol restored the retention times to their initial values. LC−MS studies which did detect the linoleic acid showed that with a short methanol flush after every three analyses, linoleic

Table 1. Linear Regression for EC and UV Detector Calibration Curves for 13-c-t-LOOH

detector	slope	intercept	correlation coeficient
UV	1.035	−2.148	0.99999
EC	1.033	8.88	0.99995

acid accumulation was completely eliminated and never coeluted with the analytes.

Comparison of Detectors for Lipid Hydroperoxides. One objective of this study was the comparison of the EC detector with UV and CL detectors noted above. Hydroperoxides are amenable to both oxidation and reduction in electrochemical systems. Both modes were explored using glassy carbon electrodes. The transition from oxidation to reduction for the LOOH peaks occurs at approximately +600 mV vs AgCl in the mobile phase used. The highest sensitivity was observed with the highest positive potential which could be achieved without excessive background, +1300 mV. At this potential, the possibility of interferences by other electrochemically active species is a concern. The second cell of the detector was operated at +520 mV at which the LOOH's were reduced very selectively.

The calibration curves prepared using UV and EC detection at 1300 mV showed excellent linearity over the 3 orders of magnitude studied. Linear regression results for both detectors for the enzymatically produced 13-*cis*-9-*trans*-11-LOOH are given in Table 1. The data for the EC detector were divided by 266 to normalize the differences in the numerical values of peak area produced by the two data systems. The correlation coefficients of 0.9999 for both detectors are indicative of the excellent quantitative capability of both detectors. At quantities of 100 ng and greater, the reproducibility between injections of the same sample was excellent with precision better than 2% relative standard deviation. Below 95 ng, the precision of both detectors decreased in a similar fashion. More significantly, while the peaks at 5.9 ng were clearly distinguishable in chromatograms for both the EC and UV detectors, the peak areas were 50% smaller than those predicted by the linear regression line. This is interpreted as evidence of LOOH instability at these low concentrations. Similar instability has been observed for other peroxides studied in this laboratory and may be due to adsorption on surfaces or decomposition on contact with metal components in the injection system. The EC detector calibration curves at 520 mV were comparable to those at 1300 mV but an order of magnitude less sensitive. No peak was detectable below 95 ng of injected LOOH.

Calibration curves for 13-*cis*-9-*trans*-11-LOOH on the CL detector were not run. The data for the time series studies was used to estimate the relative sensitivity and reproducibility as compared with the UV detector. In general, the CL detector suffered from greater base line noise and lower overall sensitivity than the UV detector. The CL detector also suffers from increased instrumental complication, and it was the most difficult to operate in a reliable and reproducible manner. The postcolumn volume required for mixing reagents and delay time for reaction significantly degrades the chromatographic separation. This is shown clearly in Figure 3. The only advantage of the CL system is the ability to determine unequivocally the presence of hydroperoxide in a specific peak.

Time Series Study of Antioxidants. The formation of LOOHs as a function of time was studied by placing the samples in an autosampler at room temperature and sampling them repetitively over a period of more than 2 weeks. The study was conducted separately on both the LC/EC−UV instrument and on the UV/chemiluminescence system. The results of these studies are summarized in Figure 4 and showed that the addition of AIBN to linoleic acid caused rapid production of LOOHs. The rate of increase in LOOH concentration slows after 2000 min (about 33 h). This behavior may be due to depletion of AIBN after 2000 min or to the system approaching a steady state in which the LOOH is decomposing. Throughout the entire time period studied, the amount of LOOH produced in the presence of AIBN was substantially higher than that observed for linoleic acid alone. Both BHT and CAP consistently showed a lower concentration of LOOH produced in the presence of AIBN than that of the linoleic acid without AIBN. This data is consistent with the expected behavior of antioxidants.

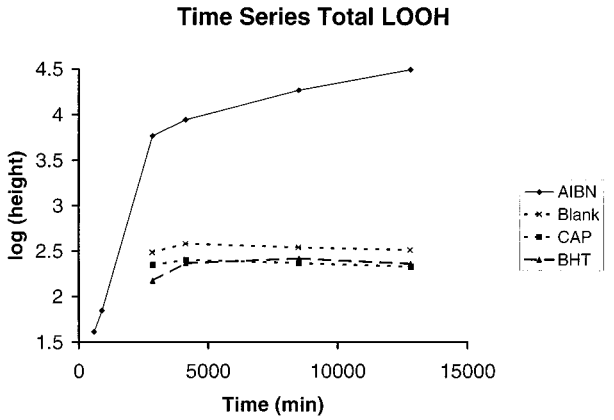


Figure 4. Time series plot of the log of total lipid hydroperoxide peak height (peaks 2−4) using chemiluminescence detector. Solutions 0.20 M lipid, 0.020 M AIBN, and 0.020 M antioxidant in air.

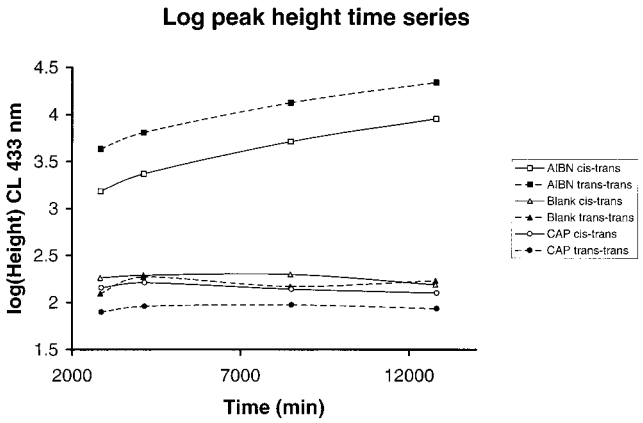


Figure 5. Comparison of the log of total cis−trans hydroperoxide peak height and the log of total trans−trans hydroperoxide peak height as a function of time. Lipid solutions as in Figure 4.

Figure 5 compares the formation of the cis−trans isomers to the formation of the trans−trans isomers as a function of time. In the blank, the peak height of the cis−trans isomer peak was approximately equal to the sum of the height of the two trans−trans isomer peaks, indicating that the air oxidation of linoleic acid produced equal amounts of the four isomers. When linoleic acid is oxidized in the presence of AIBN-generated free radicals, the trans−trans isomers clearly predominate

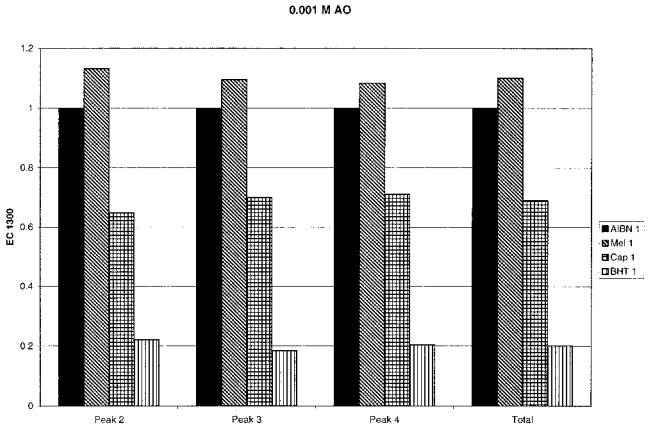


Figure 6. Comparison of antioxidant effectiveness of four antioxidants at 0.001 M concentration. Data from electrochemical detector at 1300 mV. All peak heights normalized to 0.020 M AIBN solution and 0.20 M linoleic acid in air without antioxidant. Oxidation at 30 °C for 8 days.

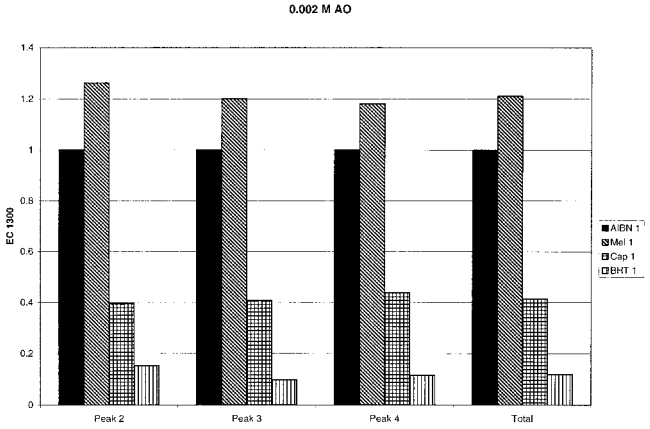


Figure 7. Comparison of antioxidant effectiveness of four antioxidants at 0.002 M concentration. Conditions as in Figure 6.

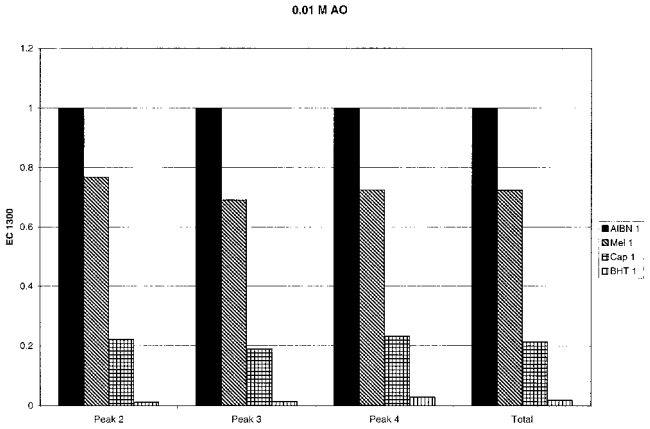


Figure 8. Comparison of antioxidant effectiveness of four antioxidants at 0.010 M concentration. Conditions as in Figure 6.

over the cis−trans isomers. In the presence of the antioxidants CAP and BHT (not shown), the cis−trans isomers appear first during oxidation and predominate over the trans−trans isomers. Linoleic acid without the radical initiator produces approximately equal quantities of the cis−trans and trans−trans isomers. The formation of the cis−trans isomers are kinetically favored. Their formation does not require bond rotation or the removal of oxygen from the intermediate hydroperoxy radical (Figure 2) (Porter, 1984). The presence of CAP and BHT favors cis−trans product formation by a small extent while reducing the total LOOH formation substantially.

Quantitative Studies of Antioxidant Efficacy. Quantitative determination of the production of each of the isomers specified above was based on peak heights using UV and electrochemical detection at 1300 mV. Electrochemical detection was used for all quantization because it was more selective than UV absorption detection and proved to be more sensitive and much more reproducible than chemiluminescence detection. On the basis of the results of the time series study, it was determined that a time of 10 000 min (7 days) was adequate to achieve significant lipid oxidation, even in the presence of the antioxidants, to allow reliable quantization of the relative effects of the various antioxidants with all three detectors.

Quantitative determination of the four hydroperoxide isomers was performed on blank samples containing only linoleic acid, samples containing linoleic acid and AIBN, and samples containing linoleic acid, AIBN, and each of the individual antioxidants. The concentration of each of the four isomers obtained at varying molar concentrations of each of the antioxidants used is shown in Figures 6−9. Figure 6 shows the normalized results for each isomer peak and the total LOOH formation at 0.001 M of each of the antioxidants. The results were normalized against samples containing only linoleic acid and AIBN. BHT was, by far, the most effective AO, in that the total of the lipid peroxides formed decreased by about 80% of those formed without AO. CAP was found to decrease the production of LOOHs by about 30%. Melatonin, under these conditions, increased the formation of LOOHs by about 10%, suggesting it acts as a pro-oxidant. At this low concentration of AO, the ratio of isomers formed in the presence of each of the antioxidants is similar.

Figure 7 demonstrates the effect of doubling the AO concentration to 0.002 M. This increases the effectiveness of all of the antioxidants. The production of LOOHs was decreased by 90% and 60% for BHT and CAP, respectively. The increased amount of MEL increased the formation of LOOHs by 20%, again indicating the pro-oxidant behavior under these conditions.

The concentration of each of the antioxidants was increased to 0.01 M, and Figure 8 shows that the level of LOOHs produced decreased substantially. A comparison of BHT, CAP, and MEL indicated that the reduction in overall LOOH formation was about 97%, 78%, and 28%, respectively. It is noteworthy that at this concentration, MEL switches from pro-oxidant to AO behavior. Figure 9 shows the effect of increasing the concentration of AO to 0.02 M. The formation of LOOHs overall was reduced by about 98% using BHT, by about 88% using CAP, and by about 10% using MEL.

The relationship between the concentration of linoleic hydroperoxides produced and the inverse of the concentration of AO is shown in Figure 10. The data shown for both BHT and CAP show a simple linear relationship with correlation coefficients greater than 0.99. The steepness of the slope of the line should be inversely related to the effectiveness of the AO, again indicating the effectiveness of CAP and the greater effectiveness of BHT.

The data shown for MEL indicate that a more complex relationship exists between the concentration of AO and the concentration of hydroperoxides produced.

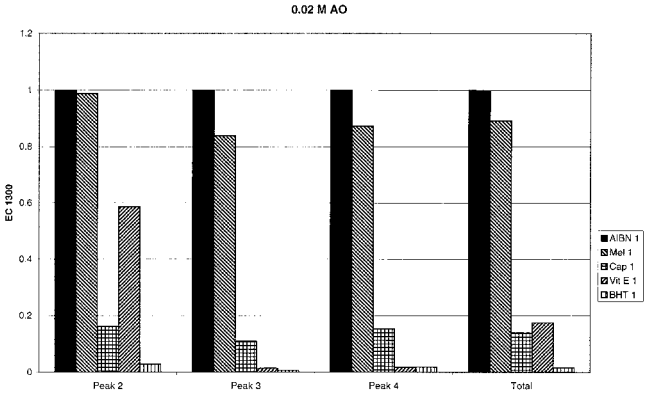


Figure 9. Comparison of antioxidant effectiveness of four antioxidants at 0.020 M concentration. Conditions as in Figure 6.

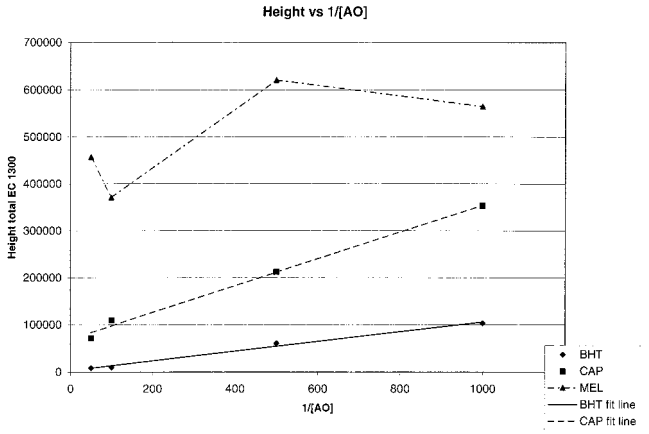
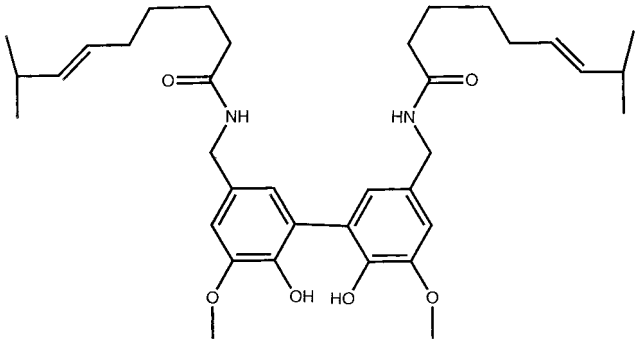


Figure 10. Plot of total lipid hydroperoxide peak height as a function of the reciprocal of antioxidant concentration. Least-squares lines for BHT and capsaicin with correlation better than 0.99. Line for melatonin shows nonlinear relationship.

The negative slope for melatonin at low concentrations is consistent with its action as a pro-oxidant under these conditions. The nonlinearity of these data is indicative of changes in the mechanism of action as a function of concentration.

Mass Spectral Analysis of Capsaicin Oxidation Products. During the course of oxidation reactions involving both BHT and CAP with AIBN, a peak at retention times much longer than that of the lipid hydroperoxides was observed which increased regularly as a function of time of oxidation. For the oxidation of samples containing pure capsaicin, a single oxidation peak was determined to be due to a coupling reaction to produce a capsaicin dimer at *m*/*z* 608. When capsaicin samples containing dihydrocapsaicin and other homologes of capsaicin were oxidized, the multiple HPLC peaks contained a mixture of dimers representing all possible combinations the various homologues. The MS−MS spectra of the CAP dimer showed major peaks at 273, 420, and 440. The peak at 440 was identified as the loss of one amide side chain from the dicapsaicin and shifted to 442 for the dihydrocapsaicin dimer (*m*/*z* 612). The peak at 273 is the methoxyphenol core of the dimer after loss of both side amide chains. The peak at 420 is the same for both capsaicin and dihydrocapsaicin and has not yet been assigned. APCI spectra of the silylated dicapsaicin shows the presence of two phenolic groups indicative of the structure shown in Figure 11. The exact position of the dimerization on the ring has not been determined. The oxidation peak for BHT was collected and subjected to GC−MS. It was identified as the dimer of BHT.



DICAPSAICIN

Figure 11. Structure of dicapsaicin from oxidation of capsaicin.

The presence of both BHT and CAP dimers in this reaction is consistent with the explanation that both of these act as antioxidants by the same process, the donation of a hydrogen from the aromatic ring to a more reactive radical.

CONCLUSIONS

There are significant differences in the effectiveness of BHT, CAP, and MEL as antioxidants. BHT proved to be the most effective AO in this study. Both BHT and CAP appear to operate by a simple mechanism which does not change over the range of concentrations studied, with effectiveness being linearly related to the reciprocal of AO concentration.

Under the conditions of this study, melatonin showed very limited AO activity at higher concentrations and pro-oxidant activity at low concentrations. This finding is consistent with recent reports that melatonin is not a free-radical scavenger antioxidant (Antunes, 1999). This leads the authors to conclude that the amide group present in the CAP molecule probably does not play a major role in its AO activity under free-radical oxidation conditions. Thus, the AO behavior observed for CAP under these conditions is due primarily to the phenolic moiety in the molecule. Previous work on the antioxidant effects of CAP under high-temperature conditions showed considerable involvement of the amide group in formation of a range of amides and imides (Henderson, 1992). Further studies of the formation of lipid hydroperoxides under thermal conditions and the effects of CAP and BHT on this process will be conducted to clarify the mechanism of CAP during thermal oxidation.

This study demonstrates the ability to analyze LOOHs directly and to use this analysis to evaluate the effectiveness and mechanism of action of antioxidants. Ongoing studies in our laboratories are directed at evaluating the effectiveness of other natural product compounds as potential antioxidants in lipid systems. In addition, studies are underway to evaluate the effect of oxygen pressure on the effectiveness and mechanisms of AO action. The HPLC methodology developed here is also being employed to study thermal oxidation of lipids.

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