Retardation of 4-Hydroxy-2-Transnonenal (HNE), a Toxic Aldehyde Formation by Antioxidants in Heat-Treated Corn Oil at Frying Temperature

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Abstract

The antioxidative properties of four antioxidants such as rosemary extracts (RE), tert-butylhydroquinone (TBHQ), ascorbyl palmitate (AP), citric acid (CA) and their mixtures were investigated on the formation of 4-hydroxy-2-transnonenal (HNE) in commercial corn oil heated at 185˚C for up to 6 hours. Among the antioxidants 100 ppm RE and a mixture of 200 ppm tertiary butylated hydroquinone (TBHQ) + 100 ppm ascorbyl palmitate (AP) + 50 ppm citric acid (CA) exhibited excellent antioxidative activity, as determined by the thiobarbituric acid reaction (TBARS) assay, measuring the formations of the secondary lipid oxidation products and by high-performance liquid chromatography (HPLC), measuring the formation of the toxic α, β-unsaturated hydroxyaldehyde HNE after heat treatment of corn oil at 185˚C up to 6 hours. TBHQ, AP and CA alone did not show much protective properties. The synergistic effects of TBHQ + AP + CA mixture shown to reduce the formation of HNE after 6 hours heat-treated corn oil by 27%. RE 100 ppm was also found to be a very effective antioxidant, reducing the formation of HNE after 6 hours heat-treated corn oil in the same condition by 29%.

Keywords

Antioxidants, Ascorbyl Palmitate, Citric Acid, Corn Oil, HNE, Rosemary Extracts, TBHQ

1. Introduction

Lipid peroxidation of polyunsaturated fatty acids (PUFA) leads to a large variety

of secondary lipid peroxidation products, including the α, β-unsaturated hydroxyaldehydes such as the toxic aldehyde 4-hydroxy-2-transnonenal (HNE) [1] [2] [3]. This polar very reactive aldehyde containing unsaturation between the α and β carbons and has a hydroxyl group on the carbon 4 position. The chemical structure makes this aldehyde very reactive to amino, sulfhydryl and thiol groups and therefore reacts aggressively with biological compounds. Among the four α, β-unsaturated hydroxyaldehydes (HHE, HOE, HNE, HDE), the 9 carbon containing HNE was found to be the most reactive and toxic compound [4]-[12]. Its toxicity has been demonstrated in the literature by its reaction to DNA and RNA in low concentrations and related to a number of pathological conditions including inflammatory and degenerative processes such as atherosclerosis, liver damage, Parkinson's, Alzheimer's and other diseases [13]-[19].

It has been shown that the precursor of HNE is linoleic acid [20] and corn oil together with soybean oil contain high levels of this fatty acid. Corn oil contains about 60% linoleic acid and is commonly used for frying in the food industry, frying fast foods commercially and used also in households for frying. A number of previous experiments have shown the formation of HNE due to heat treatments in the above oils by this laboratory [20]-[25]. It is known that certain antioxidants can delay the reactions of lipid peroxidation and therefore could cause some protection to heat-treated PUFA oils, and lower the formation of secondary lipid oxidation products. HNE, a toxic secondary lipid oxidation product, was shown to form linoleic acid due to heat treatment at frying temperatures and it has also been shown to incorporate into fried food [26] [27]. Since HNE has been shown to be absorbed from the diet and metabolized [28], it is important to investigate how its formation can be reduced during heat treatments at frying temperature. The objective of the present study was to measure the antioxidant effects of several synthetic and a naturally occurring antioxidant, rosemary [29] and measure the lowering effects of the formation of the most toxic and abundant α, β-unsaturated hydroxyaldehyde isomer, HNE in heat-treated corn oil which is high in linoleic acid, a precursor for HNE.

2. Materials and Methods

2.1. Chemicals and Instruments

Mazola corn oil was purchased from a local store (Roseville, MN). The 2,4-dinitrophenylhydazine was obtained from Eastman Kodak Company. (Rochester, NY). The HPLC-grade methanol, HPLC-grade water, HPLC-grade dichloromethane, trichloroacetic acid, boron trifluoride-methanol solution and TBHQ were all from Sigma Chemical Company (St. Louis, MO). Rosemary extract (RE), Fortium R30 containing 4% of carnosoic acid and carnosol mixture in sunflower oil. It was obtained from Kemin Industries Inc. (Des Moines, IA). Sodium thiosulfate, and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Hydrochloric acid and potassium iodide were obtained from Mallinckrodt Baker Inc. (Paris, KY). HPLC-grade hexane was obtained from EMD Chemicals,

Inc. (Gibbstown, NJ). Citric acid and L-ascorbyl palmitate were purchased from MC/B Manufacturing Chemists (Norwood, OH) and MP Biomedicals, LLC (Solon, OH) respectively. No. 1 filter paper and 0.45 μm syringe filters were obtained from Whatman Ltd. (Kent, England). Thin layer chromatographic (TLC) plates were purchased from EMD Millipore, Inc. (Billerica, MA). HNE was purchased from Cayman Chemical Company (Ann Arbor, MI).

The fatty acid distribution was measured on a gas chromatograph containing an 18835B capillary inlet system (5830A Gas Chromatograph, Hewlett-Packard, Saginaw, MI).

Thiobarbituric acid (TBA) reactivity was measured using a UV/vis Bausch and Lomb Spectronic 20 spectrophotometer at 535 nm.

The high-performance liquid chromatograph (HPLC) system contained a sample injector (712 WISP, Waters, Milford MA, USA), a solvent delivery system (9050, Varian, Walnut Creek, CA) and a UV-Vis detector (9010, Varian). The HPLC column was an Ultrasphere ODS 5 × 4.6 mm, 25 cm column (Hichrom, Berkshire, UK) with a guard column (Deerfield, IL).

2.2. Methods

The detailed methods used in the present experiments was described by Seppanen and Csallany [21] [22] and recently by Juan, Shoeman and Csallany [25].

Stock Solutions in 10 mL Ethanol:

1) 5 mg Rosemary extract (RE);
2) 10 mg Rosemary extract (RE);
3) 20 mg Tertiary Butylhydroquinone (TBHQ);
4) 20 mgTBHQ + 5 mg Citric acid (CA);
5) 20 mg TBHQ + 10 mg Ascorbyl palmitate (AP);
6) 20 mg TBHQ + 5 mg CA + 10 mg AP;
7) Control; 10 mL Ethanol.

Sample Preparations

The 0.5 mL stock solutions of antioxidants were added to 5 mL of corn oil samples to produce the following mg/L (ppm) concentrations: a) RE 50 and 100 ppm, b) TBHQ 200 ppm, c) TBHQ 200 ppm + CA 50 ppm, d) TBHQ 200 ppm + AP 100 ppm, e) TBHQ 200 ppm + CA 50 ppm + AP 100 ppm, f) Control only ethanol, no antioxidants.

Heating Conditions of Commercial Corn Oil Samples

Duplicate samples of 5 g commercial corn oil and 0.5 mL of the various stock solutions in ethanol were mixed using a shaker for 5 min before heating. All samples were heated in the center of the preheated 185˚C sand bath in a 5 cm × 15 cm open glass tube. The test tubes were inserted 10 cm down into the sand bath. The temperature of the oil was monitored and in about 20 min the oil reached to 185˚C in the test tube and then held for the entire heating time of 1 or 2 or 4 or 6 hours. The duplicate samples were heated together for each time period and removed from the sand bath at the same time. The samples, after heat

treatments, were cooled and used for analysis immediately. From each duplicate samples, two 1 mL samples (a total of 4 samples) used for the TBARS assays. For the HPLC analysis of HNE, the oil samples were heated again as before in duplicates, for 1 or 2 or 4 or 6 hours at 185˚C separately and used for the analyses. The unheated samples were (0 heating time) used as references for measuring the start of the thermally induced oxidation process of the commercial corn oil. The stock solution No. 7, contained no antioxidant only ethanol.

Thiobarbituric Acid Reactive Substances (TBARS)

The TBARS method of Buege and Aust [30] was used to monitor the oxidation and accumulation of the secondary lipid peroxidation products, such as aldehydes and related carbonyl compounds, in the commercial corn oil after heated for 0, 1, 2, 4, 6 h. The TBAR reagent was prepared with equal volumes of 15% w/v TCA, 0.375% w/v 2-thiobarbituric acid (TBA), and 0.25N hydrochloric acid. Samples of 200 μL of oil were combined with 4 mL of the reagent, and the mixture was heated for 15 min in boiling water. The absorbance of the sample was measured in triplicates at 535 nm with a UV/Vis spectrophotometer. The calibration curve was prepared with a pure malondialdehyde (MDA) standard, and the results were expressed as nmol MDA equivalents per g oil.

HPLC Analysis of HNE

The method used as described in detail [21] [22] [25].

Brief description of the method:

1) The preparation of 2,4-dinitrophenylhydrazine (DNPH) reagent was made from 10 mg DNPH, three times recrystallized from methanol. The recrystallized DNPH was mixed with 20 mL 1N HCl and heated at 50˚C for 1 h. After cooling it was extracted four times with HPLC-grade hexane to remove impurities. The DNPH reagent was used immediately.

2) Transesterification of aldehydes and related carbonyl compounds was carried out in duplicates by using 1 gr of unheated or previously heated oil samples, and 5 mL of freshly prepared DNPH reagents, the samples were shaken overnight at room temperature in the dark.

3) The DNPH derivatives were extracted from the oil with 10 mL of methanol:water (75:25, v/v) repeatedly three times.

4) From the combined methanol:water samples, the DNPH derivatives were extracted three times with 10 mL of dichloromethane. The combined dichloromethane extracts were concentrated under N_2 gas to 0.5 mL.

5) The DNPH derivatives, from the 0.5 mL dichloromethane extracts, were separated to polar and nonpolar compounds and osozones by thin-layer chromatography (TLC) using 2 silica gel plates per sample and developed in dichloromethane.

6) The polar compounds were individually eluted from the thin plates 3 times with 10 mL methanol. The combined methanol extracts, after centrifugation, to remove residual silica gel, were concentrated under N_2 gas to 1 mL. The concentrated polar DNPH derivatives were filtered by a syringe filter nylon membrane

0.45 μm pore. Samples were stored in −20˚C until HPL analysis.

7) Aliquots of 100 μL samples of the polar DNPH derivatives in duplicates were injected into the HPLC system. The integration of peaks was completed with a Varian Star Chromatography Workstation installed on a computer and connected to the detector. The DNPH derivatives were separated using isocratic elution with methanol-water (50:50, v/v) for 10 min, then a linear gradient to 100% methanol for 30 min, after than maintaining 100% methanol for an additional 10 min. The flow rate was 0.8 mL min^{-1} and the absorbance was monitored at 378 nm. Since the retention time can change slightly, pure standard of HNE used as an external standard before each injection period. Identification of samples was by comparing the retention times of samples to the retention times of pure standard. For additional identification, co-chromatography was used. Known amount of sample was mixed with known amount of the pure standard. The recovery was calculated from the increased peak area due to the added standard. Recovery rates were between 95% and 105%. The detection limit of samples was 1 ng. Since every duplicate sample was analyzed separately and injected in duplicates for HPLC analysis, the minimum numbers of the HPLC chromatograms were 4 for each sample analyzed.

Fatty Acid Distribution

The fatty acid distribution of the unheated corn oil was measured by gas chromatography using the method of Metcalf and Schmitz [31]. The fatty acid distribution was measured comparing the fatty acid methyl esters retention times of the samples to the standards [32]. The results are expressed as % of total fatty acids.

Peroxide Value Determination

The peroxide value (PV) in unheated commercial corn oil was measured in duplicates by the Official Methods of the American Oil Chemists' Society. Four identical samples were used with triplicate titrations to determine the PV.

Statistical Analysis

Analysis of variance was used to determine the significant differences between groups. All measurements were replicated three times. The results obtained were statistically analyzed with two-way ANOVA. The Tukey test was conducted to calculate P values. Significant differences were determined at $P \leq 0.05$.

3. Results and Discussion

The main objective of the present experiments was to measure the heat-induced secondary lipid peroxidation, with special reference to the formations of the toxic 4-hydroxynonenal (HNE) in the presence and absence of certain antioxidants.

Before the major objective, to measure the formation of HNE, preliminary experiments were conducted including the TBARS assay to measure the formation of all aldehydes and related carbonyl compounds at 185˚C frying temperature, up to 6 hours, in the presence and absence of certain antioxidants [33].

Results of the TBARS assay were also used to determine the conditions to be used for the much longer HPLC based measurements of HNE retardation by antioxidants in the heat-treated corn oil samples at 185˚C, up to 6 hours duration.

Peroxide Value (PV)

Four identical commercial corn oil samples were used to measure the PV before the oil was heat-treated. The results showed that PV was 1.70 - 1.76 milliequivalents/1000g corn oil. This indicates that the corn oil used was minimally oxidized before the beginning of thermal treatments.

Fatty Acid Distribution

The commercial corn oil used in the following experiments contained 43.7% linoleic acid, 34.2% oleic acid, 19.3% palmitic acid and 2.8% stearic acid. It did not contain linolenic acid and arachidonic acid. Linoleic acid, which contains 2 double bonds, is a precursor for HNE, and oleic acid which contains only one double bond was shown not to be a precursor for HNE [20].

Thiobarbituric Acid Reactive Substances (TBARS)

Duplicate corn oil samples were heated individually for each time period of 0, 1, 2, 3, 4 and 6 hours at 185˚C. Results of the TBARS assay showed the highest level of secondary lipid oxidation product formations in corn oil without added antioxidants after 6 hours of thermal treatment at 185˚C. The formation of TBARS in the absence and the presence of various antioxidants are presented in detail in figures between **Figures 1(a)-3(b)**. The TBARS concentrations were expressed as malondialdehyde (MDA) equivalents per μg/mL of oil.

Figure 1(a) and **Figure 1(B)** show no measurable antioxidant activity in the presence of 100, 200 and 300 ppm TBHQ or 100 and 200 ppm AP alone. The middle concentration TBHQ 200 ppm was selected in the following measurements and mixed with other antioxidants or synergists. **Figure 2(a)** also shows no antioxidant effects for the mixture of TBHQ 200 ppm + AP 100 ppm or 200 ppm. **Figure 2(b)** also shows no antioxidants effects for the mixture of TBHQ 200 ppm + CA 50 ppm or 100 ppm.

Figure 3(a) shows that the mixture of TBHQ 200 ppm + AP 100 ppm + Ca 50 ppm, can significantly lower the oxidation, at frying temperature, the 6 hour heat treated corn oil. A previous study reported that AP has antioxidant activity [34]. It was also reported that AP retarded the oxidation in oil at frying temperature [35]. However, in the present study, AP alone had no lowering effects of TBARS formation of heat-treated corn oil.

Figure 3(b) shows the antioxidant activity of 50, 100 and 200 ppm of RE. Results show that as low of 50 ppm RE in the oil reduced statistically the TBARS formation at frying temperature already after 2 hours of heating time.

Present results indicate that AP and CA, secondary antioxidants, act as synergists with the primary antioxidant TBHQ and retard the oxidation of corn oil at frying temperature measured by the TBARS assay.

In summary, it was found that the maximum suppression of lipid oxidation

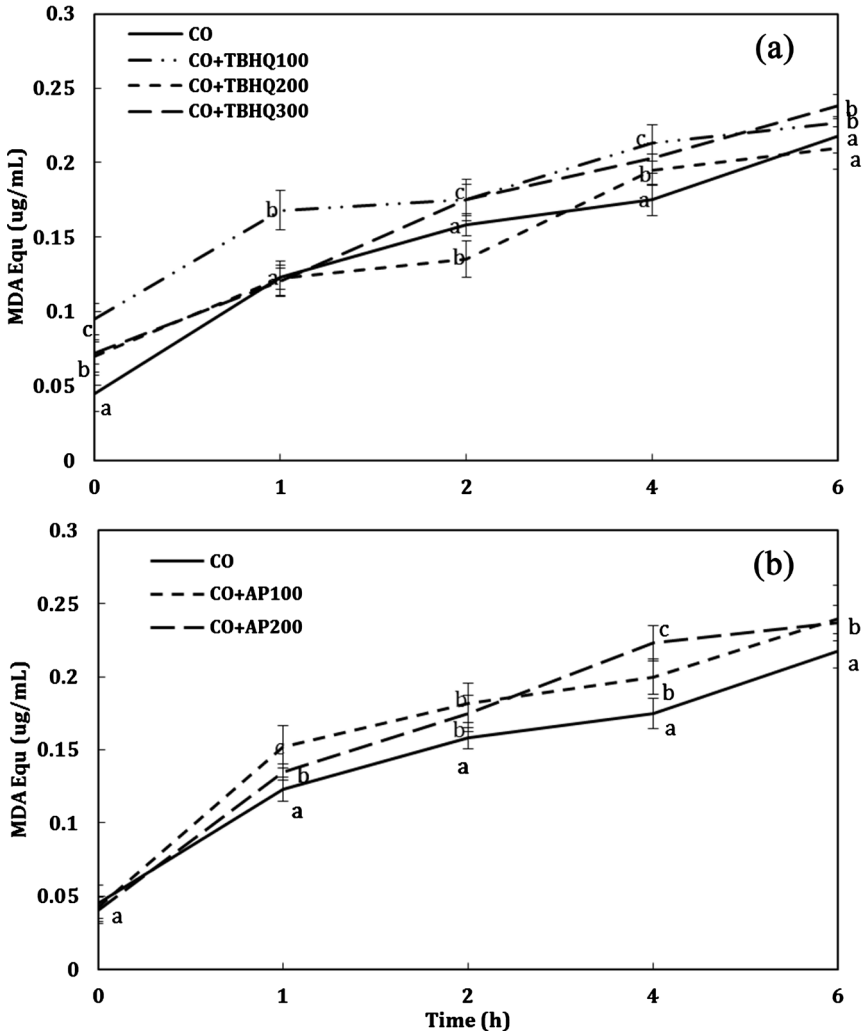
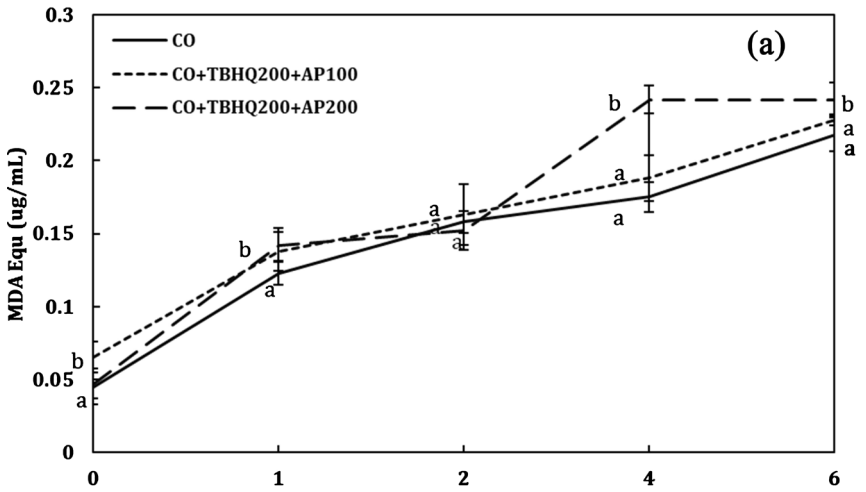


Figure 1. (a) Comparison of TBARS expressed as MDA equivalents of heat-treated corn oil for 0, 1, 2, 4 and 6 hours at 185˚C in the presence and absence of 100 ppm, 200 ppm and 300 ppm TBHQ. (b) Comparison of TBARS expressed as MDA equivalents of heat-treated corn oil for 0, 1, 2, 4 and 6 hours at 185˚C in the presence and absence of 100 ppm and 200 ppm ascorbyl palmitate (AP).



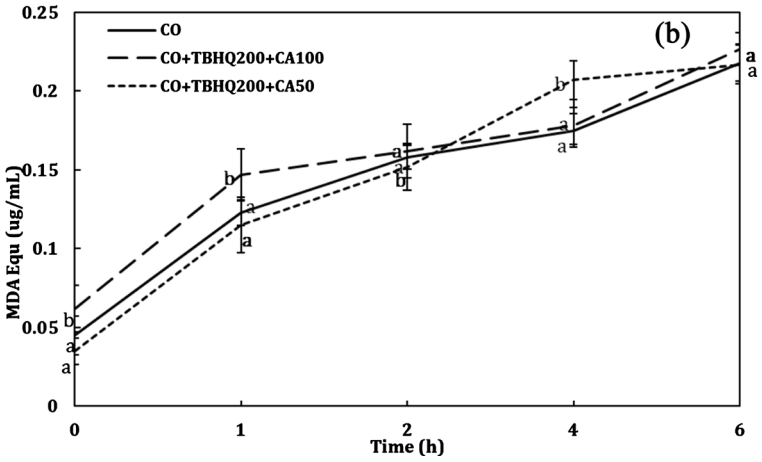


Figure 2. (a) Comparison of TBARS expressed as MDA equivalents of heat-treated corn oil for 0, 1, 2, 4 and 6 hours at 185˚C in the presence and absence of 200 ppm TBHQ and 100 ppm or 200 ppm ascorbyl palmitate (AP). (b) Comparison of TBARS expressed as MDA equivalents of heat-treated corn oil for 0, 1, 2, 4 and 6 hours at 185˚C in the presence and absence of 200 ppm TBHQ and 50 ppm or 100 ppm citric acid (CA).

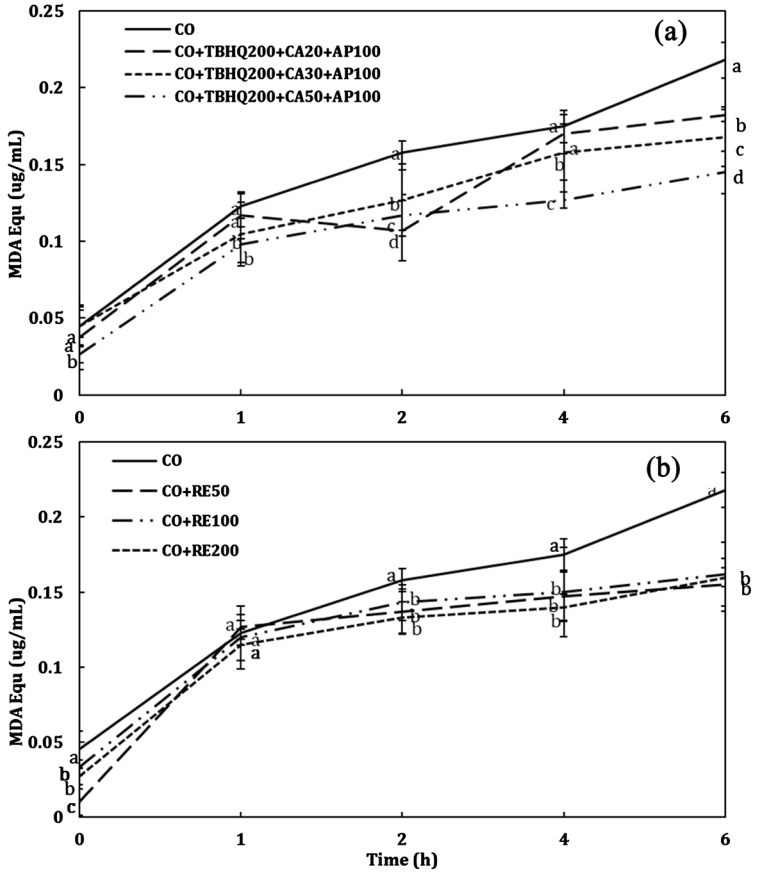


Figure 3. (a) Comparison of TBARS expressed as MDA equivalents of heat-treated corn oil for 0, 1, 2, 4 and 6 hours at 185˚C in the presence and absence of a mixture of 200 ppm TBHQ, 100 ppm ascorbyl palmitate (AP) and 20 ppm or 30 ppm or 50 ppm citric acid (CA). (b) Comparison of TBARS expressed as MDA equivalents of heat-treated corn oil for 0, 1, 2, 4 and 6 hours at 185˚C in the presence and absence of 50 ppm, 100 ppm or 200 ppm rosemary extracts (RE).

measured by the TBARS assay, in corn oil heated for 6 hours at 185˚C, was 33% by the mixture of 200 ppm TBHQ + 50 ppm citric acid (CA) + 100 ppm ascorbyl palmitate (AP). The suppression of oxidation by rosemary extracts (RE) 50 or 100 ppm was 29% for heat-treated corn oil. Several studies have recommended the use of rosemary extracts as an alternative antioxidant to synthetic antioxidants [36] [37]. The compounds responsible for rosemary extracts' antioxidant properties are mainly phenolic diterpenes, such as carnosol, carnosic acid, rosmanol, epirosmanol and isorosmanol [28].

The deterioration of frying fats and oils at high temperatures is complicated, because oxidative and thermolytic reactions are occurring simultaneously, and both the saturated and unsaturated fatty acids undergo chemical degradations when exposed to high temperatures and oxygen [38] resulting in the formation of aldehydes, ketones and related carbonyl compounds.

After the suppression of oxidation of heat-treated corn oil was established by the mixture of synthetic and one natural antioxidant by the measurements of TBARS, the main objective was to measure the suppressing effects of these antioxidants on the formation of the toxic α, β-unsaturated-4-hydroxyaldehyde HNE, in heat-treated corn oil at frying temperature (185˚C) up to 6 hours.

Figure 4(a) illustrates the formation of HNE in corn oil heated at 185˚C for 0, 1, 2, 4 and 6 hours in the absence and the presence of synthetic antioxidants and their mixtures. These are 200 ppm TBHQ alone, 200 ppm TBHQ + 50 ppm CA, 200 ppm TBHQ + 100 ppm AP and 200 ppm TBHQ + 50 ppm CA + 100 ppm AP. Results show that after 2, 4 and 6 hours of heat treatment at 185˚C, the HNE concentration was significantly suppressed by 27% in the corn oil by both mixtures of 200 ppm TBHQ + 50 ppm CA and 200 ppm TBHQ + 50 ppm CA + 100 ppm AP. While the synergistic effect of AP and CA was evident in measuring the TBARS reaction in heated corn oil, for the HNE formation of AP was not significant. While the TBARS are measuring all the various secondary oxidation products including the polar and nonpolar aldehydes and related carbonyl compounds, HNE is only a 9 carbon α, β-unsaturated-4-hydroxyaldehyde. It seems that the synergistic effect of AP with TBHQ, a primary antioxidant, is not affecting the formation of HNE.

Figure 4(b) shows the retardation of HNE, in corn oil heated at 185˚C up to 6 hours in the absence and presence of 50 ppm or 100 ppm RE and it was retarded by 29%. Significant suppression of HNE formation due to both 50 ppm or 100 ppm RE can be seen after 4 and 6 hours of heat treatment of the corn oil.

In summary, the results of **Figure 4(a)** and **Figure 4(b)** show the best combination of synthetic antioxidants used in this study, to suppress the formation of HNE after 6 hours of thermal treatment at 185˚C. This was 200 ppm TBHQ + 50 ppm CA + 100 ppm AP. Results of RE of 50 or 100 ppm under the same conditions, similarly reduced the HNE formation.

Previously, investigators have reported the protective effect of polymethylsiloxane (PDMS) in the formation of HNE in soybean oil at frying temperature.

However, the protective effect was not due to antioxidants, but the protection was due to the prevention of oxygen penetration into the monolayer of the oil surface, therefore lowering the oxidation of the oil and lowering the decomposition of linoleate and the formation of HNE [39].

Figure 5(a) illustrates the sum total of polar aldehydes (PA) and related carbonyl compounds (RCC) formations in corn oil heat-treated at 185˚C for 0, 1, 2, 4 and 6 hours in the absence and presence of synthetic antioxidants and their mixtures measured by HPLC. Results show that PA and RCC concentrations were very low at 0 time. After 6 hours of thermal treatment, the combination of 200 ppm TBHA + 50 ppm CA + 100 ppm AP suppressed the formation of the sum total of polar aldehydes by 72% as expressed as hexanal equivalents.

Figure 5(b) shows the effect of RE on the sum total of polar aldehydes (PA) and

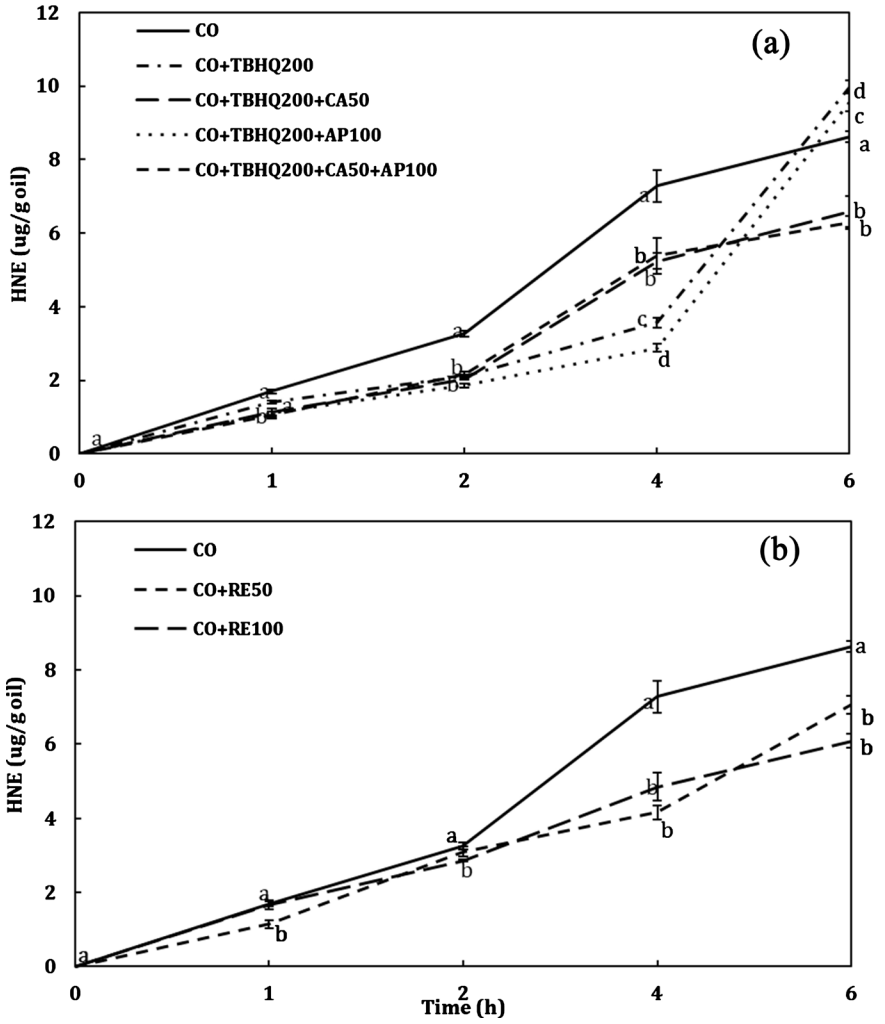


Figure 4. (a) Formation of HNE in corn oil heat-treated at 185˚C for 0, 1, 2, 4 and 6 hours in the presence and absence of synthetic antioxidants and their mixtures. 200 ppm TBHQ, 200 ppm TBHQ and 50 ppm CA, 200 ppm TBHQ and 100 ppm AP, 200 ppm TBHQ and 50 ppm CA and 100 ppm AP. (b) Formation of HNE in corn oil heat-treated at 185˚C for 0, 1, 2, 4 and 6 hours in the presence and absence of 50 ppm and 100 ppm rosemary extracts (RE).

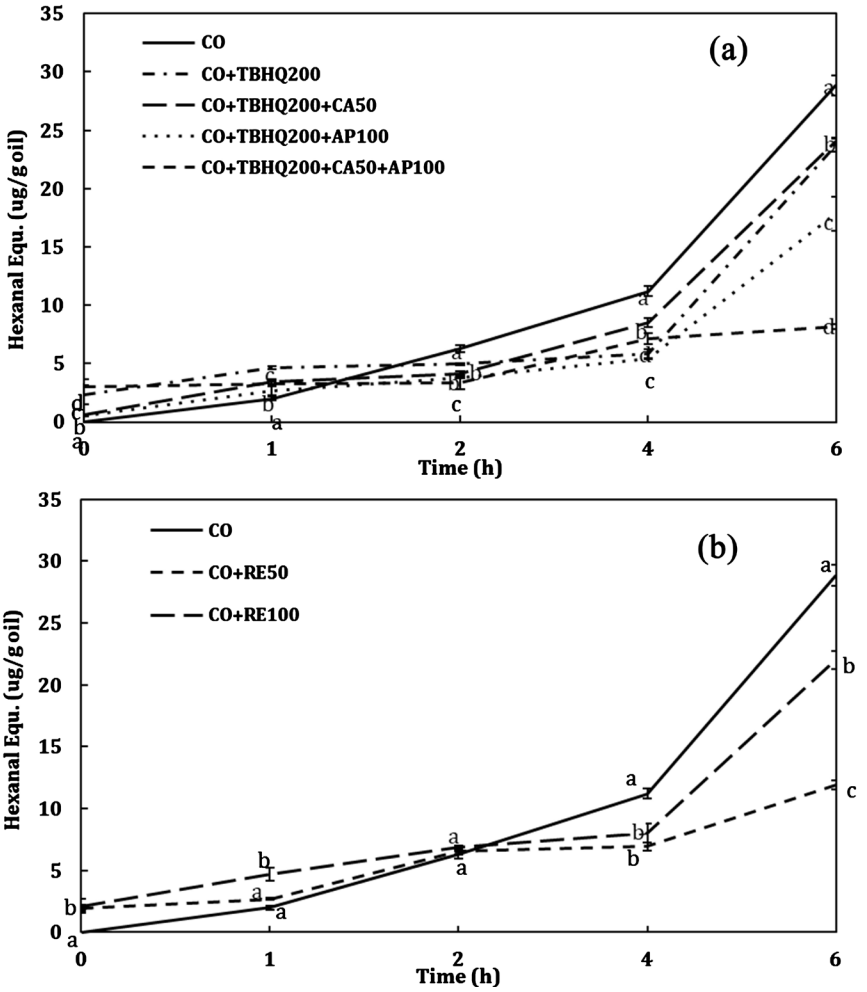


Figure 5. (a) The sum total of polar aldehydes and related carbonyl compounds in corn oil heat-treated at 185˚C for 0, 1, 2, 4 and 6 hours in the presence and absence of synthetic antioxidants and their mixtures. 200 ppm TBHQ, 200 ppm TBHQ and 50 ppm CA, 200 ppm TBHQ and 100 ppm AP, 200 ppm TBHQ and 50 ppm CA and 100 ppm AP. Expressed as hexanal equivalents/g oil. (b) The sum total of polar aldehydes and related carbonyl compounds in corn oil heat-treated at 185˚C for 0, 1, 2, 4 and 6 hours in the presence and absence of 50 ppm and 100 ppm rosemary extracts (RE). Expressed as hexanal equivalents/g oil.

related carbonyl compounds (RCC) formation measured by HPLC in corn oil in the presence of up to 6 hours of heat treatments in 185˚C. After 4 hours of heat treatment, both 50 and 100 ppm REs are significantly lowered by the formation of PA and RCC. However, after 6 hours of heat treatment, 50 ppm RE was found to be the most effective. It suppressed 59% of the sum total formations of PA and RCC expressed as hexanal equivalents.

When the antioxidant effects of the natural and synthetic antioxidants were measured by the TBARS assay, the mixture of TBHQ 200 ppm + CA 50 ppm + AP 100 ppm demonstrated 33% lower total lipid oxidation and as low as 50 ppm RE demonstrated 29% lower lipid oxidation compared to the untreated corn oil due to thermal treatment for 6 hours at 185˚C. It should be mentioned that corn

oil contains high concentration of linoleic acid, a precursor for HNE. The formation of HNE, a toxic aldehyde, form from ω-6 fatty acids which have been reported and confirmed in the literature [20] [40] [41].

In summary, the greatest reduction of HNE formation (29%) after 6 hours of heat treatment at 185˚C was produced by the addition of 100 ppm RE. This was followed by the suppression of 27% HNE by the combination of 200 ppm TBHQ + 100 ppm AP + 50 ppm CA.

In conclusion, the present experiments demonstrated that certain synthetic antioxidant mixtures and a naturally occurring antioxidant at certain low concentrations will retard the overall lipid peroxidation process of heat-treated commercial corn oil at of 185˚C up to 6 hours as measured by the TBARS assay and also reduce the formation of the most toxic α, β-unsaturated hydroxyaldehyde HNE, measured by HPLC.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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