The Effect of Intermittent and Continuous Heating of Soybean Oil at Frying Temperature on the Formation of 4-Hydroxy-2-*trans*-nonenal and Other α-, β-Unsaturated Hydroxyaldehydes

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ABSTRACT: 4-Hydroxy-2-*trans*-nonenal (HNE) is a cytotoxic secondary lipid peroxidation product of linoleic acid. Previous investigations in this laboratory showed that HNE is formed in thermally oxidized soybean oil, which is high in linoleic acid. Continuous exposure of the oil to frying temperature (185°C) for up to 6 h graduallyincreased the formation of HNE and other polar lipophilic aldehydes. Additional investigations in this laboratory showed that HNE is absorbed into food fried in thermally oxidized oil in the same concentration as was found in the oil. In the present experiment, the effect of intermittent heating on the formation of HNE in soybean oil was compared with continuous heating. Soybean oil samples were heated either for 1 h each day for five sequential days or for 5 h continuously at 185 ± 5°C. The thermally oxidized soybean oil samples were analyzed by HPLC for the presence of HNE and three other polar lipophilic α-,β-unsaturated hydroxyaldehydes: 4-hydroxy-2-*trans*-hexenal, 4-hydroxy-2-*trans*-octenal, and 4-hydroxy-2-*trans*-decenal. Under intermittent and continuous heating over a total of 5 h, the concentration of these compounds increased similarly. These results indicate that the formation of HNE and other hydroxyaldehydes at frying temperature is a cumulative result of oxidation of PUFA over time.

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KEY WORDS: HHE, HNE, 4-hydroxy-2-*trans*-decenal, 4-hydroxy-2-*trans*-hexenal, 4-hydroxy-2-*trans*-nonenal, 4-hydroxy-2-*trans*-octenal, secondary oxidation products, soybean oil, thermal oxidation, α-, β-unsaturated hydroxyaldehydes.

When subjected to heating at elevated temperatures, oils and fats undergo oxidation that results in changes in physical and chemical characteristics (1–3). Some of the reactions that occur in the heated oil include lipid peroxidation, hydrolysis, polymerization, cyclization, and pyrolysis. The rate at which these reactions occur is dependent on time and temperature. At typical frying temperatures in the presence of air, lipid peroxidation reactions proceed very quickly to form hydroperoxides that undergo further decomposition to yield a wide variety of secondary lipid peroxidation products. Highly unsaturated oils are frequently used for frying, especially in home applications, because of availability and cost; however, these oils are especially susceptible to oxidation at high temperatures because of their FA composition. It has been reported that both frying and thermal oxidation of oils result in degradation of linoleic acid (4–8).

We reported (9,10) that the thermal oxidation of soybean oil, which contains about 58% linoleic acid and about 6% linolenic acid, results in the formation of 4-hydroxy-2-*trans*-nonenal (HNE), an α-, β-unsaturated aldehyde and a secondary oxidation product of linoleic acid that has been shown to have cytotoxic and mutagenic effects (11). Experiments have related HNE toxicity to the incidence of atherosclerosis (12,13); LDL oxidation; stroke; Parkinson's, Alzheimer's, and Huntington's diseases (14–19); and liver disease (20) among others. HNE forms conjugates with proteins containing cysteine, histidine, and lysine residues and can damage DNA by inducing gene mutations and can alter the structure and function of cancer-related proteins (21).

In addition to HNE, two other α-, β-unsaturated hydroxyalkenals, 4-hydroxy-2-*trans*-hexenal (HHE), a degradation product of linolenic acid, and 4-hydroxy-2-*trans*-octenal (HOE), a degradation product of linoleic acid, were identified in heated soybean oil (9). Subsequent investigations have shown that HNE, when present in thermally oxidized oil, is incorporated into food fried in that oil *via* absorption during frying (22).

The focus of the present investigation was to measure the formation of HNE, HHE, and HOE, and a fourth α-, β-unsaturated hydroxyalkenal, 4-hydroxy-2-*trans*-decenal (HDE), in thermally oxidized soybean oil under two different conditions of heating, either continuously for 6 h or intermittently, 1 h per day for 5 d for a total of 5 h. The continuous heating of soybean oil for several hours may not be typical use of the oil for frying purposes in all circumstances. Heating periods are generally shorter, especially in home use where the oil used for frying may be saved for further use. The objective of the present experiment was to measure the similarities between the effect of intermittent heating and the effect of continuous heating on the formation of HNE in soybean oil heated at frying temperature (185°C). A second objective was to measure the effect of heating on the formation of other hydroxyalkenals, namely, HHE, HOE, and HDE.

EXPERIMENTAL PROCEDURES

Chemicals and materials. 2,4-Dinitrophenylhydrazine (DNPH) and hexanal were obtained from Sigma (St. Louis, MO);

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HPLC-grade acetone, dichloromethane, and methanol, from Mallinckrodt (Paris, KY); hydrochloric acid, from Fisher Scientific (Fair Lawn, NJ); and HPLC-grade water from EM Science (Gibbstown, NJ). Silica gel plates for TLC (Al Sil G, aluminum-backed, 20 × 20 cm, 250 μm thickness) were obtained from Whatman Ltd. (Maidstone, Kent, England). All solvents used were HPLC grade. Pure HNE, HHE, HOE, and HDE standards were synthesized and purified at the University of Graz (Graz, Austria) and were gifts from Dr. Hermann Esterbauer; these standards have been stored at −70°C under nitrogen since their receipt.

Preparation of thermally oxidized soybean oil. (i) Intermittent heating. A 2600-g portion of commercial soybean oil purchased at a local store was heated at 185 ± 5°C in a 5-L stainless steel deep fryer (Model #106770; General Electric) for 1 h each day for 5 d. The 1-h heating time included ~10 min for the oil to reach temperature, after which the oil was held at a constant temperature without stirring or agitation for the remaining time. At the end of the heating period, the oil was allowed to cool to room temperature and was kept at room temperature, in the dark, until the next heating session (23 h later). The oil in the fryer was sampled daily by removing 10 g of oil immediately after the heating period and before the next heating period started on the following day. The oil samples were immediately analyzed in triplicate for lipophilic aldehydes and related carbonyl compounds.

Preparation of thermally oxidized soybean oil. (ii) Continuous heating. Individual aliquots (5 g) of commercial soybean oil were heated in open test tubes (12 × 150 mm) without stirring at 185 ± 5°C in a sand bath for 6 h. Two tubes of heated oil were removed after each hour of heating and were immediately analyzed in duplicate for lipophilic aldehydes and related carbonyl compounds.

Measurement of polar lipophilic aldehydes and related carbonyl compounds in oil. The method described by Seppanen and Csallany (9) was used to analyze the thermally oxidized soybean oil. Duplicate aliquots of the oxidized oil (2 g) were reacted with 5 mL of DNPH reagent (prepared by combining 10 mg recrystallized DNPH with 20 mL 1 N HCl) overnight at room temperature to form hydrazone derivatives with the aldehydic secondary oxidation products. The DNPH derivatives were extracted from the oil first with methanol/water (75:25 vol/vol) (3 × 10 mL) and then dichloromethane (3 × 10 mL). The lipophilic DNPH derivatives were then separated into three groups (polar carbonyl compound derivatives, nonpolar carbonyl compound derivatives, and osazones) by TLC using dichloromethane as the solvent. The polar carbonyl compounds were eluted from the TLC plates with methanol (3 × 10 mL), and the combined solvent extract was evaporated under N_2 gas to 1 mL. Aliquots (100 μL) of the concentrated methanolic solution of polar carbonyl compound DNPH derivatives were analyzed by HPLC on a reversed-phase C18 column (Ultrasphere ODS, 25 cm × 4.6 mm i.d., 5 μm particle size; Altex, Berkeley, CA) with a guard column (2 cm × 2 mm i.d.; ChromTech, Apple Valley, MN). The lipophilic polar carbonyl hydrazones, including the hydroxyaldehydes HHE, HDE, HNE, and HOE, were eluted isocratically for 10 min with methanol/water (50:50 vol/vol) followed by a linear gradient to 100% methanol for a total elution time of 40 min at a flow rate of 0.8 mL/min. Absorbance was monitored at 378 nm. Disposable syringes used for sample injection were equipped with a 0.2 mm polyvinylidene difluoride filter (ChromTech, Apple Valley, MN). A mixture of hexanal-, 2-heptenal-, and decanal-DNPH standards was used daily to measure the reproducibility of the HPLC system before the application of samples.

Quantification of HHE, HOE, HNE, and HDE DNPH derivatives. The HPLC peak areas of each identified peak were converted to mass using a peak area of 13,000 mV equivalent to 1 ng pure HNE-DNPH standard. This value was determined by repeated injections of various concentrations of pure HNE-DNPH standard and comparison of the peak area response to the concentration. The masses of HHE, HOE, and HDE were calculated using a ratio of each compound's M.W. to the M.W. of HNE.

Statistical analysis. Where appropriate, Student's *t*-test was used to determine whether differences between treatments were statistically significant. A P-value < 0.01 was considered indicative of a statistically significant difference.

RESULTS AND DISCUSSION

A typical HPLC chromatogram showing the separation of the polar lipophilic aldehydes and related carbonyl compounds, including HHE, HOE, HNE, and HDE, in thermally oxidized soybean oil (heated at 185°C for 5 h) is illustrated in Figure 1. In this chromatogram HNE is the major polar lipophilic aldehyde, as we have reported previously (10). HHE, HOE, and HDE are also present but in much lesser amounts than HNE. The precursor for HNE is linoleic acid, which is present in soybean oil at about 58%; this FA is also the precursor to HOE. The precursor for HHE is linolenic acid, which is present at a much lower amount in soybean oil, about 6%. The precursor to HDE has not been reported in the literature.

The identification of HHE, HNE, and HOE in thermally oxidized soybean oil has been previously reported (9,10). The confirmation of the identity of HDE by co-chromatography is illustrated by the chromatograms shown in Figure 2. Three different polarity solvent systems were used for co-chromatography of HDE. The recoveries from the co-chromatography of pure HDE standard added to HDE derived from heated in soybean oil from the different polarity solvent systems were calculated as $[a/(b + c)] \times 100$, where a = area from the cochromatography of the compounds from the oil plus the standard, b = area of compound from oil, and c = area of standard and found to be: 50% methanol/50% water (vol/vol), 99.0%; 55% methanol/45% water, 91.9%; 60% methanol/40% water, 86.8%. In each case, the solvent system indicated was used during the initial 10 min of the HPLC elution, then followed by a linear gradient to 100% methanol. This is the first known report of the existence of HDE in thermally oxidized soybean oil. The toxicity of this compound is not known, but it is expected to have reactions similar to HNE because it also is an α-, β-unsaturated hydroxyaldehyde.

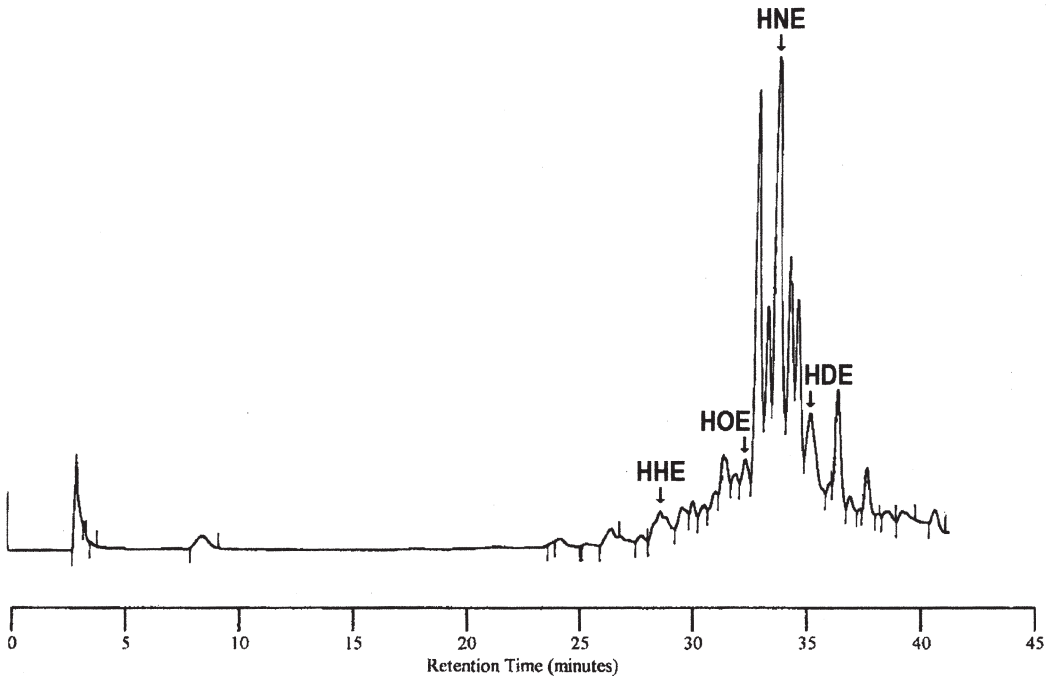

FIG. 1. HPLC separation of 2,4-dinitrophenylhydrazine (DNPH) derivatives of polar lipophilic aldehydes and related carbonyl compounds from thermally oxidized soybean oil (heated 5 h at 185°C). The identified compounds are HHE, 4-hydroxy-2-*trans*-hexenal; HOE, 4-hydroxy-2-*trans*-octenal; HNE, 4-hydroxy-2-*trans*-nonenal; and HDE, 4-hydroxy-2-*trans*-decenal. Other peaks are unidentified. Separation conditions: Ultrasphere ODS column (4.6 mm × 25 cm, 5 μm), isocratic elution with methanol/water (50:50 vol/vol) for 10 min, followed by a linear gradient to 100% methanol for 15 min; flow rate 0.8 mL/min; detector wavelength, 378 nm; injected volume, 100 μL.

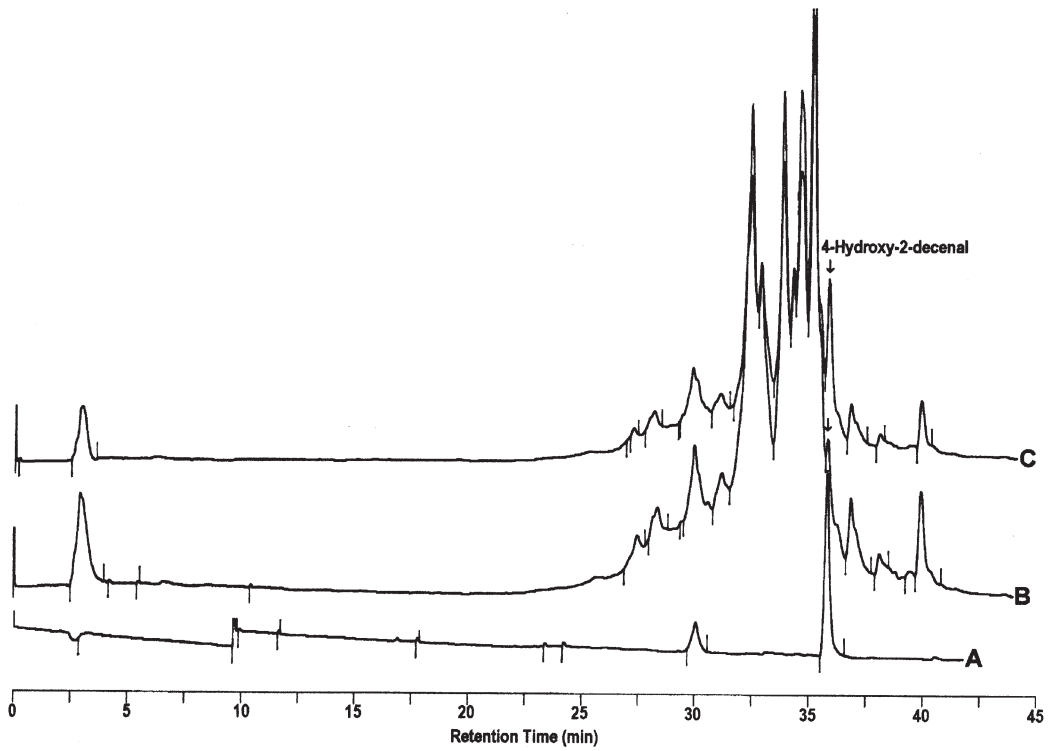

FIG. 2. Co-chromatography of DNPH derivatives of HDE by HPLC. (A) Pure HDE standard; (B) HDE from thermally oxidized soybean oil (heated 5 h at 185°C); (C) mixture of HDE standard and HDE from thermally oxidized soybean oil. Separation conditions and abbreviations are given in Figure 1.

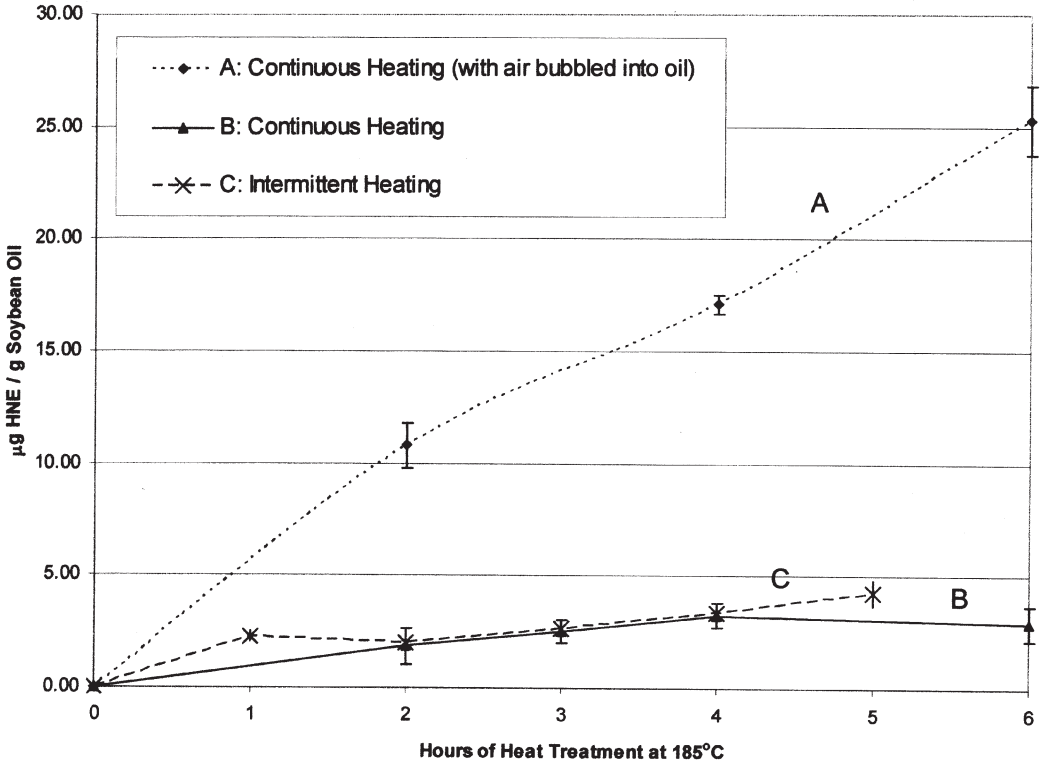

FIG. 3. HNE in thermally oxidized soybean oil heated at 185°C under three different conditions. (A) Oil heated continuously for 6 h with air bubbled into the oil during heating (97 mL/min); (B) oil heated continuously for 6 h; (C) oil heated intermittently for 5 h (1 h/d for 5 d). HNE measured as DNPH derivative by HPLC. Points represent mean ± SEM; n = 5–8 for each time point. Abbreviations given in Figure 1.

The main objective of this investigation was to determine whether continuous heating and repeated short-term intermittent heating of soybean oil had similar effects on the formation of HNE. The two treatments were found to be similar with respect to the total heating time and the temperature. However, the treatments differed in the volume of oil used. The different oil volumes were selected so as to allow for repeated sampling from the same volume of oil over the course of the entire heating time. In Figure 3 the formation of HNE under three different but related heating conditions is presented: heating continuously at 185°C while air was bubbled into the oil sample (10); heating continuously at 185°C without bubbling of air into the sample; and heating intermittently at 185°C for 1 h per day for 5 d without bubbling of air into the oil. Under all three conditions, heating at 185°C resulted in an increase in HNE concentration in relation to the length of heating time. When air was bubbled into the oil during heating, lipid peroxidation increased greatly and a much greater amount of HNE was formed. Under similar heating conditions without air bubbling into the oil, HNE formation was much less but essentially the same whether heating was continuous or intermittent.

The relationship between the formation and partial decomposition of HNE, HHE, and HDE in soybean oil during 6 h of continuous heating at 185°C is illustrated in Figure 4. HNE and HHE increased in concentration in a similar manner during the first 2 h of heating of the oil; however, HNE concentration increased and HHE concentration decreased with increased heating time. HHE concentration was at its maximum at 2 h; after that, HHE appeared to decompose or polymerize owing to heat. The concentration of HDE was much smaller than either HNE or HHE, and it remained fairly constant during the 6-h heating period. The increased stability of this lipophilic aldehyde may be due to its decreased volatility because of the length of the carbon chain. HOE was not measured in this heating experiment.

In Figure 5 the formation of HHE, HOE, HNE, and HDE in intermittently heated soybean oil is shown. Similarly to the continuously heated oil, HNE was present in the greatest concentration and showed an increase in concentration over the 5-h heating period. As already stated, the concentrations of HNE in the continuously and intermittently heated oil were essentially the same. The other three hydroxyalkenals, HHE, HOE, and HDE, were present in much lower concentrations than the HNE. A slight increase in the concentration of these three oxidation products occurred over the course of the five 1-h intermittent heating periods.

The intermittent heating conditions were designed to mimic frying conditions used in households where oil is heated and saved after use for future frying. Soybean oil used in the present experiments was heated for 1 h each day, cooled to room temperature in the dark, and reheated 23 h later for another 1 h. This procedure was repeated four additional times, and the concentrations of HHE, HOE, HNE, and HDE in the oil were analyzed be-

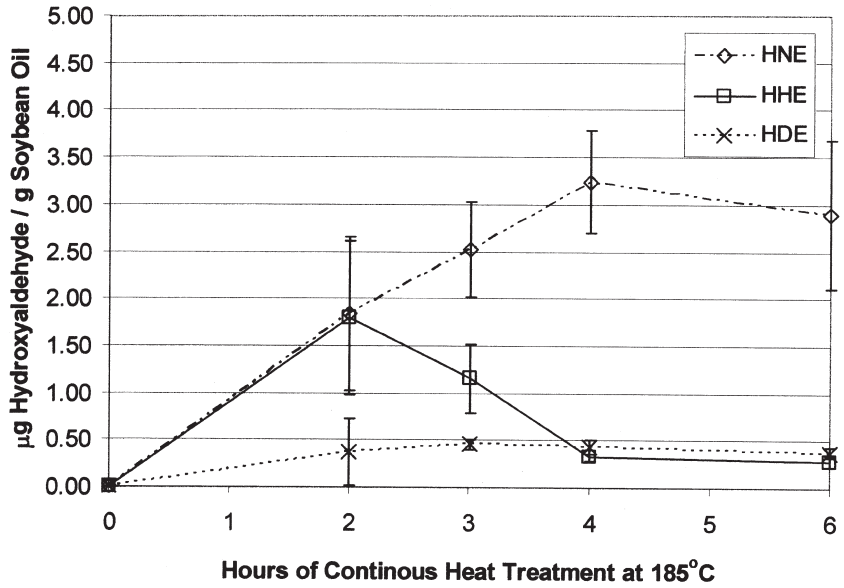

FIG. 4. Individual polar lipophilic α-, β-unsaturated hydroxyaldehydes, HHE, HNE, and HDE, in thermally oxidized soybean oil heated continuously at 185°C for 6 h. Points represent mean ± SEM; n = 4–8 for each time point. Abbreviations are given in Figure 1.

fore and after each heating period. Concentrations of each compound per gram of oil are listed in Table 1. In general, there was little change between the samples analyzed immediately after heating and before the next heating period, 23 h later. However, as demonstrated in Figure 5, HNE concentration increased with intermittent heating in a manner similar to that with continuous heat treatment (Fig. 4) under the same conditions. This indicates that the formation of these secondary oxidation products is cumulative in relation to heating time and that room temperature storage does not result in their decomposition or accumulation.

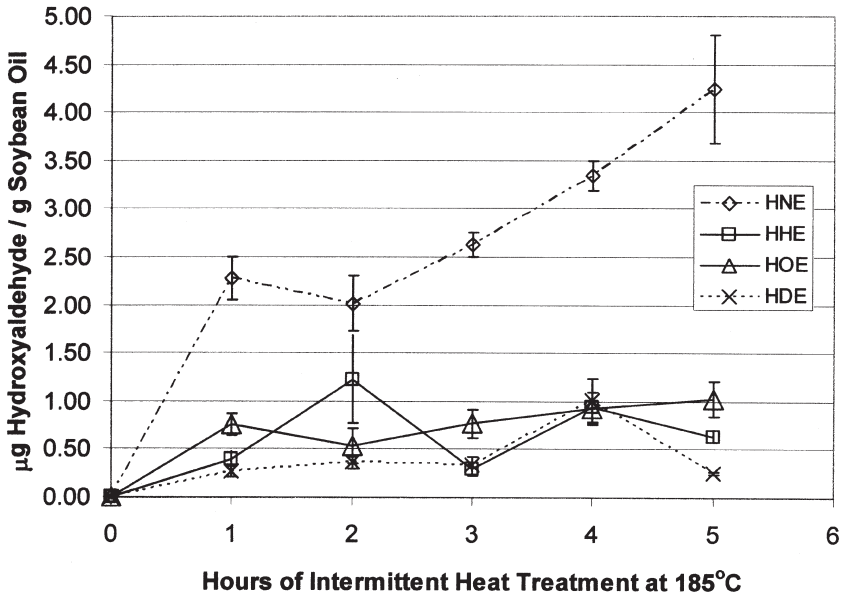

FIG. 5. Individual polar lipophilic α-, β-unsaturated hydroxyaldehydes, HHE, HOE, HNE, and HDE, in thermally oxidized soybean oil heated at 185°C for 1 h per day for 5 d with a 23-h hold at room temperature in the dark between heating sessions. Oil was sampled immediately after heating. Points represent mean ± SEM; n = 5–8 for each time point. Abbreviations are given in Figure 1.

TABLE 1
Change in Concentration[a] of the Four Polar Lipophilic α-, β-Unsaturated Hydroxyaldehydes[b] Measured in Thermally Oxidized Soybean Oil After Intermittent Heating at 185°C

Heating time (h)	HNE (μg/g oil)		HHE (μg/g oil)	
	A	B	A	B
1	2.27 ± 0.22	2.85 ± 0.65	0.40 ± 0.08	0.14 ± 0.01
2	2.01 ± 0.28	1.91 ± 0.36	1.23 ± 0.46	0.26 ± 0.03
3	2.62 ± 0.13	3.22 ± 0.17	0.30 ± 0.07	0.44 ± 0.07
4	3.33 ± 0.15	3.58 ± 0.56	0.94 ± 0.14	0.37 ± 0.09

Heating time (h)	HOE (μg/g oil)		HDE (μg/g oil)	
	A	B	A	B
1	0.75 ± 0.11	0.74 ± 0.16	0.27 ± 0.06	0.37 ± 0.05
2	0.53 ± 0.19	0.44 ± 0.11	0.36 ± 0.06	0.42 ± 0.09
3	0.77 ± 0.14	0.86 ± 0.18	0.33 ± 0.09*	0.72 ± 0.03*
4	0.93 ± 0.17	0.71 ± 0.18	1.00 ± 0.23	0.40 ± 0.11

[a]Values (μg α-, β-unsaturated hydroxyaldehyde/g heated soybean oil) represent mean ± SEM; $n = 5–8$. * indicates a statistically significant difference between samples at that time point ($P < 0.01$).
[b]Oil analyzed immediately after heating (A); oil analyzed after holding at room temperature for 23 h following heating (B). HHE, 4-hydroxy-2-*trans*-hexenal; HOE, 4-hydroxy-2-*trans*-octenal; HNE, 4-hydroxy-2-*trans*-nonenal; and HDE, 4-hydroxy-2-*trans*-decenal.

The concentration of HNE did not decrease between intermittent heating periods but did increase with increasing frying time whether the soybean oil was heated continuously or intermittently for several hours. This indicates that the practice of reusing soybean oil or other oils high in linoleic acid for repeated deep-frying will result in increased accumulation of HNE in the oil, which will be incorporated into the food as it has been previously reported by this laboratory (22).

These results confirm our previous findings that HNE is a major polar lipophilic secondary peroxidation product of soybean oil at frying temperature. Furthermore, intermittent heating of soybean oil results in a cumulative increase of HNE in relation to increased time at frying temperature in a manner similar to the continuous heating of soybean oil. Because soybean oil is high in linoleic acid, an ω-6 FA that is the precursor for HNE, we predict that other high-linoleic acid or ω-6 FA-containing oils will exhibit similar levels of HNE formation with similar heat treatment. In addition to HNE, this experiment has shown that three other α-, β-unsaturated hydroxyaldehydes, HHE, HOE, and HNE, are produced in heated soybean oil. Because of the known toxic effects of HNE and HHE and the probable toxicity of other α-, β-unsaturated hydroxyaldehydes and because these aldehydes are incorporated into fried food, it is important to understand the factors that lead to their formation and to seek methods to minimize their existence in edible oils.

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