# Formation of 4-Hydroxynonenal, a Toxic Aldehyde, in Soybean Oil at Frying Temperature

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**ABSTRACT:** The formation of 4-hydroxy-2-*trans*-nonenal (HNE), a mutagenic and cytotoxic product of the peroxidation of linoleic acid, was monitored in soybean oil that was heated at 185°C for 2, 4, 6, 8, and 10 h. Unheated soybean oil contained no HNE and a relatively low concentration of polar lipophilic secondary oxidation products (aldehydes and related carbonyl compounds), measured as 2,4-dinitrophenylhydrazine derivatives by HPLC. An increase in the concentration of both HNE and total lipophilic polar oxidation products was observed with increased exposure to frying temperature. A considerable concentration of HNE had already formed at 2 h and the concentration continued to increase at 4 and 6 h of heating. After 6 h the concentration of HNE decreased, possibly due to degradation of the aldehyde with further exposure to high temperature. The loss of endogenous tocopherols was also monitored in the heated oil, and the tocopherol concentration decreased as the secondary lipid oxidation products increased.

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**KEY WORDS:** Hydroxyaldehydes, 4-hydroxynonenal, lipid peroxidation, soybean oil, thermal oxidation, α,β-unsaturated aldehydes.

The oxidative degradation of PUFA and TG in vegetable oils such as soybean oil is enhanced at frying temperature (185°C). The degradation products produced by thermal oxidation of oils include hydroperoxides and secondary oxidation products. These secondary lipid peroxidation products include volatile and nonvolatile short-chain aldehydes, TG-bound aldehydes, other carbonyl compounds such as ketones, dimeric FA and TG, cyclic FA monomers, and polymerization products (1). Toxic effects due to the consumption of thermally oxidized oils, such as weight loss and high mortality in experimental animals, have been reported (2–7). The acute toxicity of hydroperoxides has been demonstrated with intravenous doses to rats and mice (8,9); however, equivalent or higher oral doses had no lethal effect (8–10). This evidence indicates that hydroperoxides are not absorbed; therefore, the toxicity of thermally oxidized oils appears to come from various low molecular weight secondary oxidation products such as hydroxy- and hydroperoxyalkenals rather than hydroperoxides (1). Billek (11) also concluded that nonvolatile secondary oxidation products are more likely to be detrimental to health than other oxidation products of thermally oxidized oils. This toxicity may be due in part to the formation of α,β-unsaturated aldehydes and α,β-unsaturated hydroxyaldehydes (12). These aldehydes are of particular interest because some of them are toxic and are readily absorbed from the diet (13–15). The toxicity arises because these aldehydes are highly reactive substances, which can modify proteins, nucleic acids, and other biomolecules *in vivo* (16–19). One of the α,β-unsaturated lipophilic aldehydes, 4-hydroxy-2-*trans*-nonenal (HNE), is formed from the oxidation of n-6 FA, including linoleic acid, and is cytotoxic and mutagenic (20,21). At cellular concentrations of 1–20 μM, hydroxyalkenals, including HNE, partially inhibit DNA and protein synthesis (22). At cellular concentrations >100 μM, acute effects were observed, including inhibition of catabolic (i.e., mitochondrial respiration) and anabolic (i.e., DNA, RNA, protein synthesis) functions that lead to cell death. Concentrations such as these may arise in the cells because of *in vivo* oxidative stress or possibly because of consumption of these oxidation products in foods.

Secondary lipid peroxidation products such as these aldehydes are formed as a consequence of FA deterioration in the presence of oxygen. When polyunsaturated vegetable oils are subjected to high temperatures, including frying temperatures, the formation of secondary lipid peroxidation products (e.g., HNE) is enhanced. Because fried foods are widely consumed, the extent of formation of toxic lipid degradation products in oils at frying temperature is important with regard to public health.

There are a few reports in the literature about the detection of HNE in edible oils, but none in oil at elevated temperature. Some investigators have shown the presence of HNE in unheated olive, sunflower, and pumpkin seed oils at levels ranging from 0.08 to 7.3 nmol/g oil (23) and in unheated soybean and sesame oils at 0.03–0.04 and 0.60–0.79 nmol/g oil, respectively (24). When cod liver oil was highly oxidized with Fenton's reagent, ~3 mmol HNE/g oil was found (25).

Recently we described a very sensitive HPLC method for the simultaneous measurement of nonpolar and polar lipophilic secondary lipid peroxidation products in thermally oxidized soybean oil (26). With this method, 13 nonpolar aldehydes and related carbonyl compounds and three polar hydroxyaldehydes, including HNE, were identified. Commercial soybean oil, bubbled with compressed air and heated at 185°C for 8 h in an open beaker, was determined to contain 21.49 ± 1.34 nmol HNE/g oil (26). The present paper reports the time course of HNE formation in commercial soybean oil heated at 185°C for up to 10 h.

## EXPERIMENTAL PROCEDURES

*Chemicals and materials.* 2,4-Dinitrophenylhydrazine (DNPH), hexanal, δ-tocopherol, and boron trifluoride/methanol were

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obtained from Sigma (St. Louis, MO); α-tocopherol (99%), β-tocopherol, and γ-tocopherol (97%), from Fluka Chemie (Milwaukee, WI); bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine, from Aldrich Chemical (Milwaukee, WI); HPLC-grade acetone, dichloromethane, and methanol, from Mallinckrodt (Paris, KY); hexane, hydrochloric acid, HPLC-grade isopropyl alcohol, thiobarbituric acid, and TCA, from Fisher Scientific (Fair Lawn, NJ); HPLC-grade water, from EM Science (Gibbstown, NJ); potassium iodide, from Spectrum Chemical Manufacturing (Redondo Beach, CA); and malonaldehyde tetramethyl acetal, from Eastman Kodak (Rochester, NY). The DNPH derivative of HNE was a gift from the Institute of Biochemistry, University of Graz (Graz, Austria). Silica gel TLC plates (Al Sil G, aluminum-backed, 20 × 20 cm, 250 µm layer) were obtained from Whatman Ltd. (Maidstone, Kent, England).

*Preparation of thermally oxidized soybean oil.* One hundred-gram portions of commercial soybean oil (Cargill, Wayzata, MN) were heated in 250-mL three-necked round-bottomed flasks at 185°C. Each oil sample was heated separately for 2, 4, 6, 8, or 10 h. During heating, compressed air, at 30 psi at room temperature (22–24°C), was bubbled into the sample continuously at a flow rate of 97 $cm^3$/min measured by a Matheson 621-PS flowmeter. The unheated and thermally oxidized oils were stored under $N_2$ in the dark at −20°C until analysis.

*Synthesis and isolation of DNPH-HNE.* The method described by Seppanen and Csallany (26) was used to synthesize the DNPH derivatives of the aldehydic secondary lipid peroxidation products in the soybean oil. The DNPH derivatives were first separated into two groups by TLC using dichloromethane as the solvent: Nonpolar carbonyl compounds were located between $R_f$ 0.55 and the solvent front, and polar carbonyl compounds were located between the origin and $R_f$ 0.23. The HNE-containing group of polar carbonyl compounds was eluted from the TLC plates with methanol, and aliquots (100 µL) of the concentrated (2 mL) DNPH derivatives were measured by HPLC with 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), using isocratic elution 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 µm polyvinylidene fluoride filter (ChromTech). A mixture of hexanal-, 2-heptenal-, and decanal-DNPH standards were used daily to measure the reproducibility of the HPLC system before the application of samples.

*Identification of DNPH-HNE.* Evidence of identity of HNE was obtained by co-chromatography using HPLC of the isolated DNPH derivatives from the oil with the DNPH derivative of pure HNE standard using three solvent systems of differing polarities to confirm the co-elution of the compounds (26).

Further evidence of identity of HNE was confirmed by the GC–MS analysis of silylated derivatives of DNPH-HNE by the method of Thomas *et al.* (27). The DNPH derivative of HNE, which was isolated from the oil by HPLC, was collected, and the trimethylsiloxy (TMS) derivative was prepared by combining about 40 nmol DNPH-HNE with 100 µL BSTFA in pyridine (35.62 mol/mL) and 2 mL pyridine. The mixture was incubated in a 50°C water bath for 30 min. The solvent was then evaporated, and 0.5 mL methanol was added. The TMS derivative of pure HNE-DNPH standard was identically prepared. Aliquots (5.0 µL) of the TMS-DNPH derivatives were injected onto a HP5-MS capillary column (30 m × 250 µm × 0.25 µm nominal; Hewlett-Packard, San Diego, CA) with splitless injection. The injection temperature was 245°C. Oven conditions were as follows: temperature gradient from 100 to 230°C at a rate of 35°C/min, followed by a 10-min hold at 230°C, then another gradient at a rate of 50°C/min to 300°C. Selective ion monitoring (SIM) of the 182 and 408 *m/z* ions, corresponding to the most abundant fragments of HNE, was used to detect the DNPH-TMS derivative (27).

*Quantification of DNPH-HNE.* HPLC peak areas were converted to mass using a peak area of 2000 equivalent to 1 ng pure hexanal standard. This value was determined by repeated injections of various concentrations of pure hexanal-DNPH standard and comparisons of the peak area response to the concentration (28). A similar molar extinction coefficient was assumed for all DNPH derivatives (29). The quantity of HNE was calculated from hexanal-equivalents using the molecular weight of DNPH-HNE and HNE.

*Tocopherol analysis.* The loss of endogenous tocopherols in soybean oil, owing to heat, air, and time, was monitored by a modified method of Carpenter (30). Aliquots of oil (0.5 g) were dissolved in 10 mL 1.5% isopropyl alcohol in hexane. Separation was achieved on a normal-phase HPLC column (4.6 mm × 25 cm Lichrobsorb Si-60 5µ; Alltech, Deerfield, IL, with guard column) with a mobile phase of 1.5% isopropyl alcohol in hexane at a flow rate of 1.0 mL/min. Fluorescence was monitored at 295 nm excitation and 330 nm emission for detection of α-, β-, γ-, and δ-tocopherols. Quantification of the tocopherols was made by preparing calibration curves using pure standards with the chromatographic conditions described above.

*TBARS.* The TBARS method of Buege and Aust (31) was used to monitor the accumulation of secondary lipid peroxidation products in the soybean oil after heating for 0, 2, 4, 6, 8, and 10 h. The TBARS reagent was prepared with equal volumes of 15% wt/vol TCA, 0.375% wt/vol 2-thiobarbituric acid (TBA), and 0.25 N hydrochloric acid. Oil (200 µL) was combined with 4 mL of reagent, and the mixture was heated in a boiling water bath for 15 min. Sample absorbance was measured at 535 nm. A calibration curve was prepared with pure malondialdehyde (MDA) standard, and results are expressed as nmol MDA equivalents per g oil.

*Peroxide value.* The PV in the unheated soybean oil was measured by the recommended practice of the American Oil Chemists' Society (32) to assess the initial state of oxidation. The PV was expressed as milliequivalents peroxide per 1,000 g oil.

*FA determination.* The FAME of the unheated soybean oil were prepared by the method of Metcalf and Schmitz (33). Aliquots (100 mg) of oil were combined with 3 mL $BF_3$/MeOH and placed in a boiling water bath for 1 h. Distilled water (3 mL) was added and then 9 mL hexane. The hexane layer was

removed, dried over $Na_2SO_4$, and evaporated under $N_2$ to 2 mL. The FAME in hexane were stored at −20°C until GC analysis. Soybean oil FAME were separated on a Supelco SP-2560 capillary column (100 m × 0.25 mm, 0.20 µm) with splitless injection and FID. The injection temperature was 230°C, and FID temperature 250°C. The oven condition was a temperature gradient from 150 to 220°C at 5°C/min.

*Statistical analysis.* All experiments were performed in duplicate or triplicate, and the average value of each measurement was calculated. Where appropriate, results are expressed as mean ± SEM.

## RESULTS AND DISCUSSION

Unheated commercial soybean oil, which was stored in the dark at −20°C under nitrogen, showed very little oxidation, as indicated by a PV of 0.07 mequiv peroxide/kg oil and an initial TBARS value of 2.59 MDA equiv/g oil. As the amount of time over which the oil was heated increased, the TBARS concentration increased (Fig. 1) up to 8 h. After that, there was a decrease in the concentration of secondary lipid peroxidation products, reflecting the decomposition or the polymerization of these products due to the continued exposure to heat. The TBARS assay measures all carbonyl-containing secondary lipid peroxidation products that are reactive to TBA including HNE, MDA, and numerous other aldehydes such as alkanals, alkenals, hydroxyalkenals, and various ketones. Thus, the TBARS reaction gives information only on the general level of oxidative deterioration.

A typical HPLC chromatogram (Fig. 2) shows the separation of the DNPH derivatives of the polar lipophilic aldehydes and related carbonyl compounds in unheated soybean oil (Fig. 2, line A) and in soybean oil after 8 h of heating at 185°C with air bubbled into the oil during heating (Fig. 2, line B). The chromatogram of the heated oil sample shows that the lipophilic polar secondary oxidation products are present at high concentrations in the heated oil. We previously identified three of these polar aldehydes—HNE, 4-hydroxy-2-*trans*-hexenal (HHE), and 4-hydroxy-2-*trans*-octenal (HOE)—by co-chromatography in thermally oxidized soybean oil (26). The toxic effects of HHE and HOE have been established (12), but they have not been studied as extensively as HNE. These oxidation products do not appear to be present in unheated soybean oil in our experiments. The identities of the other polar aldehydes appearing in heated and unheated soybean oil have not yet been determined. The total concentrations of polar lipophilic carbonyl compounds in unheated and heated soybean oil were determined as the sum of the individual HPLC-separated peak areas (Fig. 3). The total amount of polar lipophilic secondary peroxidation products showed an increase similar to the TBARS value as the oil was heated. The maximal concentration of polar aldehydes and related carbonyl compounds was reached at 6 h of heating, after which a decrease in concentration occurred. This was possibly due to decomposition or further oxidation of the secondary oxidation products. The amount of polar lipophilic secondary oxidation products in the unheated soybean oil was quite small, reflecting the minimal oxidation in the initial state; however, the concentration quickly began to increase with exposure to 185°C.

Co-chromatography of pure standard HNE-DNPH with oil-derived HNE-DNPH is illustrated in Figure 4. The retention time of the pure standard (Fig. 4, line A) is the same as the retention time of the corresponding DNPH derivative from the heated soybean oil (line B). The mixture of the oil-derived compound with a known amount of the corresponding pure standard resulted in co-elution shown in Figure 4, line C. Similar co-elutions were obtained using solvent systems of differing polarity, which confirmed the identity of the HNE in heated soybean oil.

The presence of HNE in heated soybean oil was further confirmed by GC–MS analysis of the silylated DNPH derivative

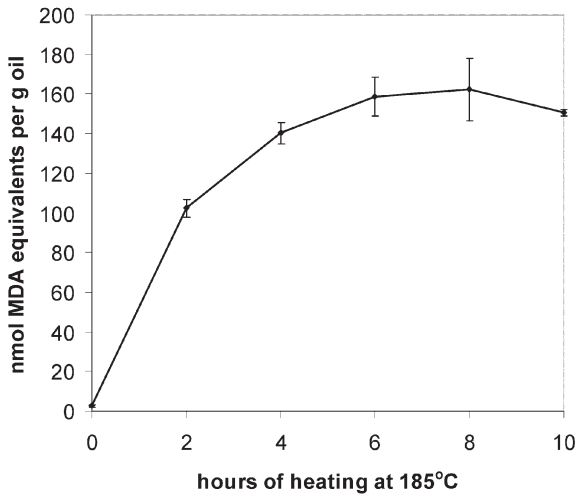


**FIG. 1.** TBARS in soybean oil heated at 185°C for 0–10 h. Error bars represent SEM. MDA, malondialdehyde.

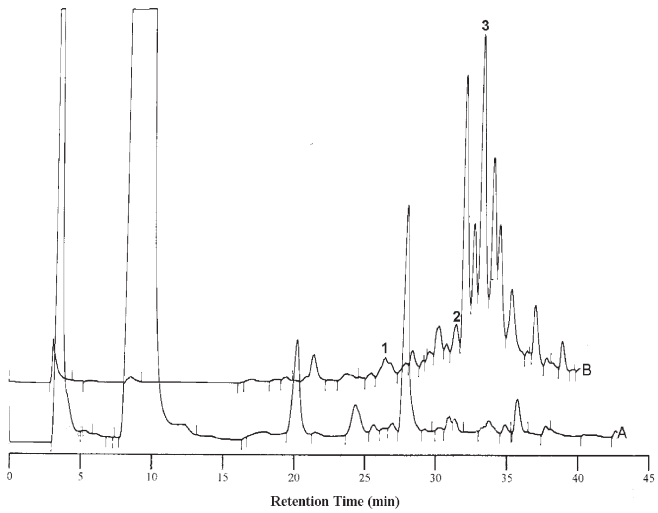


**FIG. 2.** HPLC separation of 2,4-dinitrophenylhydrazine (DNPH) derivatives of polar aldehydes and carbonyl compounds from soybean oil. (A) Unheated oil; (B) oil heated for 8 h at 185°C in the presence of air. Identified compounds are **1**: 4-hydroxy-2-hexenal; **2**: 4-hydroxy-2-octenal; **3**: 4-hydroxy-2-nonenal. 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.

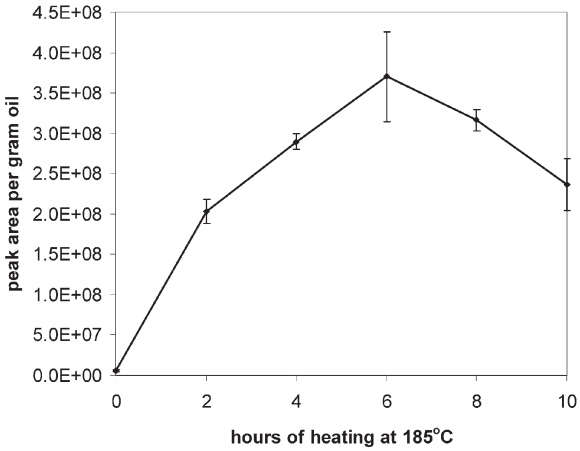


**FIG. 3.** Total polar lipophilic aldehydes and related carbonyl compounds, determined as the sum of the individual HPLC-separated DNPH derivatives of polar compounds, in soybean oil heated at 185°C for 0–10 h. See Figure 2 for abbreviation. Error bars represent SEM.

of HNE using SIM. Figure 5 is a SIM GC–MS trace showing the co-elution of the silylated HNE hydrazone separated from heated soybean oil and the silylated pure HNE hydrazone standard with negative ion detection at 182 *m/z*. Thomas *et al.* (27) have shown that the chemical ion–negative ion mass spectrum of DNPH derivatives gives two prominent ions: the characteristic negative ion for MS-HNE hydrazone at 408 *m/z* and a base peak at 182 *m/z*, most likely arising from cleavage of the nitrogen–nitrogen bond. Each compound, i.e., the TMS-HNE-DNPH separated from the oil and the pure standard, was chromatographed separately, and the peaks eluting at 7 min were found to contain both the 182 and 408 *m/z* ions. The use of this second method of identification of HNE serves to verify the presence of this toxic aldehyde in heated soybean oil.

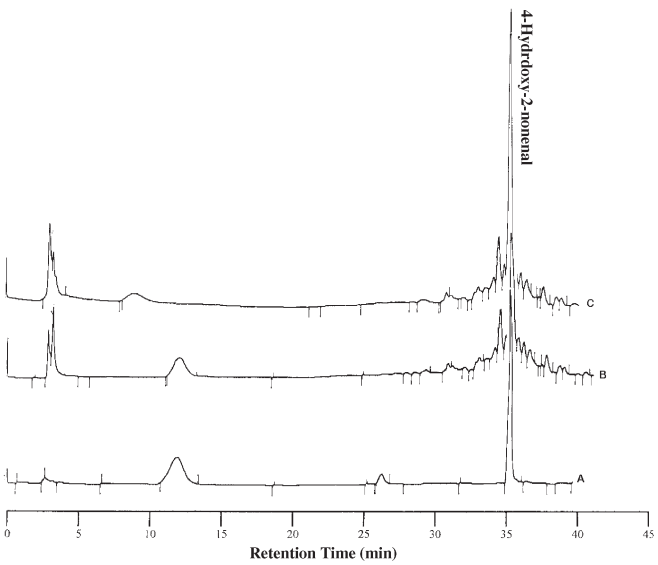


**FIG. 4.** Co-chromatography by HPLC of DNPH derivatives of 4-hydroxynonenal (HNE) derived from heated soybean oil with HNE standard. (A) HNE standard; (B) polar lipophilic carbonyl compounds from soybean oil heated 8 h at 185°C in the presence of air; (C) co-chromatogram of oil-derived polar lipophilic carbonyl compounds with HNE standard. Separation conditions and abbreviation are given in Figure 2.

The concentration of HNE was determined in each oil sample and is shown in Figure 6. No HNE was detected in the unheated soybean oil, but after 2 h of heating HNE was the major polar lipophilic lipid peroxidation product in the oil. The amount of HNE increased with continued exposure to the 185°C frying temperature, up to a maximal concentration at 6 h, followed by a decrease in concentration, presumably as the aldehyde was degraded or decomposed by further thermal oxidation. At its maximum, the concentration of HNE was 42.5 μg/g oil. Previously, we reported a concentration of 2.45 ± 0.15 μg/g in commercial soybean oil that had been heated at 185°C for 8 h (26). In the present experiments the oil was heated in a round-bottomed flask with a small vent through one of the necks of the flask; in earlier experiments the oil was heated in an open beaker. In both experiments compressed air was bubbled into the oil during the heating period. HNE is a somewhat volatile aldehyde, and greater loss would be expected from the open beaker than from the more nearly closed flask. A small amount of HNE also has been detected in the volatile phase arising from soybean oil heated in a closed system by direct condensation into DNPH reagent, but most of the HNE remained in the oil (Seppanen, C.M., and A.S. Csallany, unpublished data). Further experiments in this laboratory showed that when air was bubbled into the oil during the heating period, the concentration of the lipophilic aldehydes was lower than that in oil heated without air bubbling. This suggests that air bubbling strips out some of the volatile lipophilic secondary oxidation products from the oil.

HNE arises from the oxidation of n-6 FA including linoleic acid, the major FA in soybean oil. The FA distribution of unheated soybean oil is listed in Table 1 along with a range of values from the literature (34–36). The data indicate that the soybean oil used in this experiment was typical and that comparable results would be expected from other soybean oils with similar linoleic acid contents.

The concentrations of the endogenous tocopherols during the heating period are illustrated in Figure 7. γ-Tocopherol was the primary tocopherol homolog in soybean oil, and δ- and α-tocopherols were also present in relatively large amounts. The oil contained a trace amount of β-tocopherol which was already undetectable after 6 h of heating. A small amount of α-tocopherol remained in the oil at 8 h, but this disappeared at 10 h of heating. γ-Tocopherol showed the greatest loss over the 10 h of heat exposure. This loss is likely due to the high initial concentration and the antioxidant effect of this tocopherol in oils. The concentration of δ-tocopherol decreased the least during the 10 h of heating, from 400 ppm initially to a final concentration of 100 ppm. It has been reported that in oils the tocopherols with less antioxidant activity are destroyed more rapidly ($\alpha < \gamma < \delta$) (37) and that at high temperatures α-tocopherol is decomposed more readily than γ-tocopherol, which is less stable in turn than δ-tocopherol (38). Others have found similar results (39–41). At high temperatures, when autoxidation of FA occurs at a very fast rate, antioxidants can be quickly consumed but may also be destroyed by exposure to heat. Apparently the presence of tocopherols somewhat restricts the formation of HNE in the first few hours of heating until the antioxidant protection is

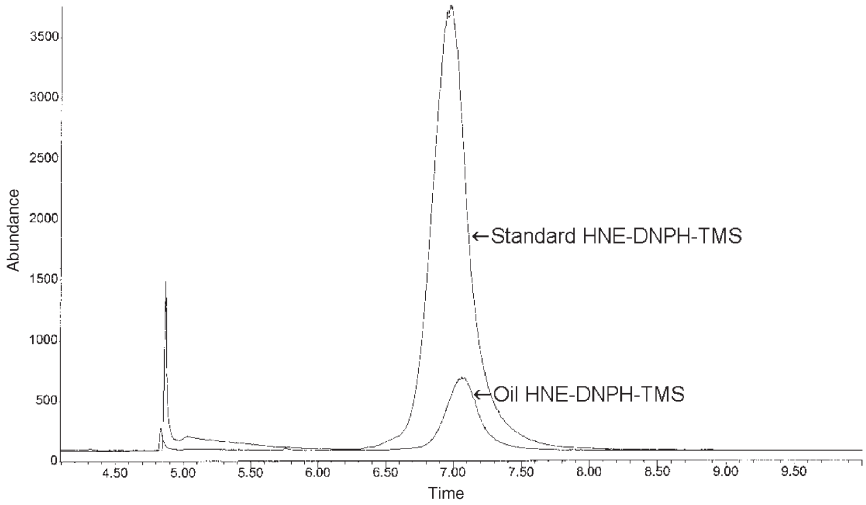


**FIG. 5.** Selective ion monitoring GC–MS trace for the trimethyl silyl (TMS) derivative of the DNPH derivative of HNE separated from heated soybean oil and the silylated pure HNE hydrazone standard with negative ion detection at 182 *m/z*. See Experimental Procedures section for analysis conditions. Abbreviations given in Figures 2 and 4.

diminished. At 4 h of heating about two-thirds of the total tocopherol concentration already was lost. Further investigation is needed to determine the protective effects of individual tocopherols and the concentrations that will minimize HNE formation in heated oils.

The formation of HNE in soybean oil at frying temperatures is of great importance because of the well-documented toxic effects of this aldehyde (20,21). Further investigation is warranted on the formation of HNE under conditions that more closely simulate deep-frying as practiced in the home or food service industry. Research is also needed to determine the protective effects of natural and synthetic antioxidants on the formation of this toxic aldehyde in various edible oils.

**TABLE 1**
**FA Distribution (relative % area) of Soybean Oil**

| FA | | Soybean oil used in present experiment | Reference values[a] |
|---|---|---|---|
| Palmitic | 16:0 | 11.96 | 7–11 |
| Stearic | 18:0 | 4.49 | 2–6 |
| Oleic | 18:1 *c*9 | 21.98 | 15–33 |
| *cis*-Vaccenic | 18:1 *c*11 | 1.35 | NA |
| | 18:2 *t*9,*t*12 | 0.79 | NA |
| Linoleic | 18:2 *c*9,*c*12 | 52.36 | 43–56 |
| Linolenic | 18:3n-6 + n-3 | 5.22 | 5–11 |
| Eicosenoic | 20:1n-9 | 0.75 | 0–0.22 |
| Arachidic | 20:0 | 0.09 | 0–0.3 |
| Behenic | 22:0 | 0.05 | 0–0.3 |

[a] From References 21–23. NA, not available.

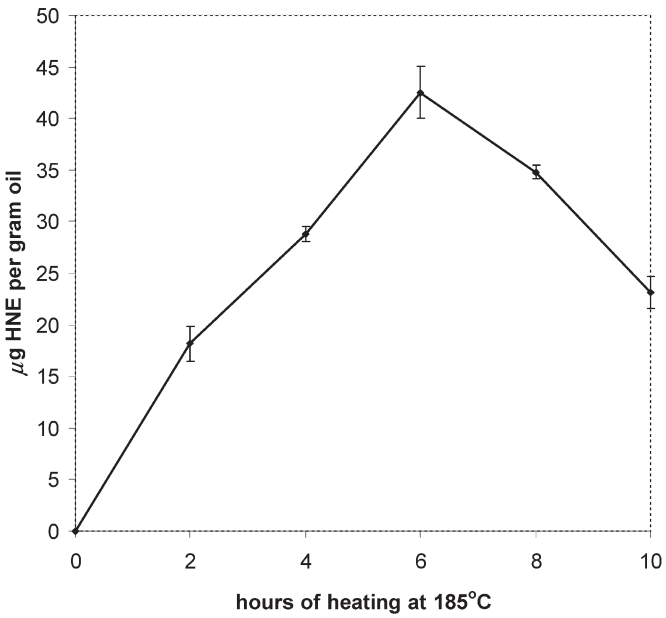


**FIG. 6.** HNE in soybean oil heated at 185°C for 0–10 h measured by HPLC as a DNPH-derivative. Abbreviations given in Figures 2 and 4. Error bars represent SEM.

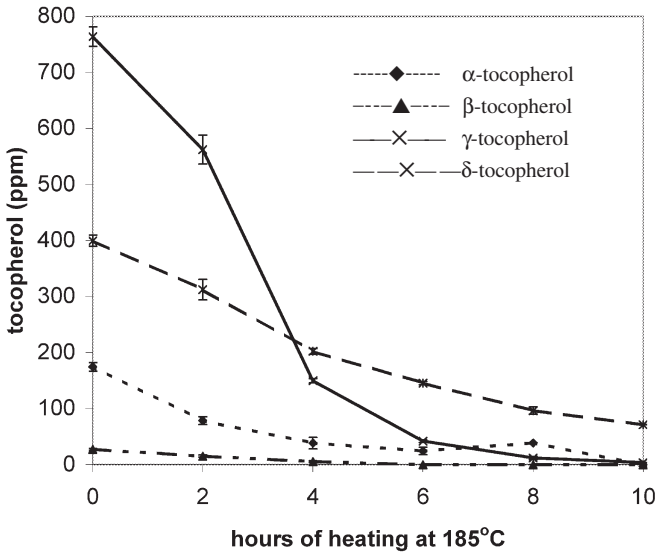


**FIG. 7.** Tocopherol contents of soybean oil heated at 185°C for 0–10 h measured by HPLC. Error bars represent SEM.

## ACKNOWLEDGMENTS

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