Simultaneous Determination of Lipophilic Aldehydes by High-Performance Liquid Chromatography in Vegetable Oil

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ABSTRACT: A very sensitive high-performance liquid chromatography (HPLC) method was developed for the simultaneous separation and measurement of nonpolar and polar lipophilic secondary lipid peroxidation products in vegetable oil. Seventeen nonpolar and 13 polar lipophilic aldehydes and related carbonyl compounds, derived from thermally oxidized soybean oil as 2,4-dinitrophenyl hydrazones, were separated simultaneously by reversed-phase HPLC. Detection limit for the individual compounds is 1 ng. Thirteen of the nonpolar carbonyl compounds were identified as: butanal, 2-butanone, pentanal, 2-pentanone, hexenal, hexanal, 2,4-heptadienal, 2-heptenal, octanal, 2-nonenal, 2,4-decadienal, decanal, and undecanal. Three of the polar carbonyl compounds were identified as: 4-hydroxy-2-hexenal, 4-hydroxy-2-octenal, and 4-hydroxy-2-nonenal. The detection of the toxic 4-hydroxy-2-nonenal, a major compound, and 4-hydroxy-2-hexenal, a minor compound, in heated soybean oil is of particular importance because these toxic compounds have been shown to be absorbed from the diet.

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KEY WORDS: 4-Hydroxyhexenal, 4-hydroxynonenal, 4-hydroxyoctenal, heated soybean oil, HPLC, lipid oxidation, lipophilic aldehydes, secondary oxidation products, toxic aldehydes, vegetable oil.

Lipid peroxidation, the oxidative deterioration of fatty acids through free radical mechanisms in the presence of oxygen, causes major chemical changes in oils and fats, especially at elevated temperatures. Lipid peroxidation of oils and fats leads to the generation of hydroperoxides, free radical intermediates, and a wide variety of secondary lipid peroxidation products. These products include various lipophilic aldehydes such as alkanals, alkenals, alkadienals, and hydroxyalkenals and are readily absorbed from the diet (1–3).

Among the lipophilic aldehydes, the α,β-unsaturated aldehydes and the α,β-unsaturated hydroxyaldehydes are of particular interest since they are highly reactive substances. Because of their high reactivity, they can modify proteins, nucleic acids, and other biomolecules. Several studies have presented evidence that the toxic lipophilic aldehydes are capable of inhibiting enzyme activities, causing cell lysis, and promoting loss of cellular reproductive integrity (4–7). They can also inhibit DNA and RNA synthesis, respiration, glycolysis (8) and can be involved in low-density lipoprotein modification and the consequent formation of atherosclerotic lesions (9–12). Two of the α,β-unsaturated lipophilic aldehydes, 4-hydroxy-2-*trans*-hexenal (HHE) and 4-hydroxy-2-*trans*-nonenal (HNE), have also been found to be cytotoxic and mutagenic (13,14).

In the presence of heat and atmospheric oxygen, the formation of secondary lipid peroxidation products is greatly enhanced; thus, the potential for the formation of these toxic compounds is greater in highly unsaturated vegetable oils that have been subjected to high temperatures encountered during frying. Determining the level of consumption of the toxic carbonyl compounds from oils is of paramount importance. Consequently, detecting the lipophilic aldehydes arising from the heating of vegetable oils in the presence of atmospheric oxygen is equally important.

Many methods are available for assessing the oxidative status of thermally oxidized oils such as peroxide, iodine, and carbonyl values, free fatty acid content, thiobarbituric acid test (15–20), and the measurement of malondialdehyde, a secondary oxidation product (20). Other oxidation products, such as volatile compounds produced during thermal oxidation of oils, have been measured by gas chromatography–mass spectroscopy (GC–MS) (21–23) or by high-performance liquid chromatography (HPLC) (24). Spectroscopic and chromatographic methods used for the same purpose include thin-layer chromatography (TLC) coupled with spectral analysis (25), gel permeation chromatography, (26,27), infrared analysis (28,29), and ^{1}H nuclear magnetic resonance (30–33). The latter method detects protons associated with alkanals, alkenals, alkadienals, and α,β-unsaturated aldehydes, but the separation and identification of the individual secondary peroxidation products are not achieved. One of the α-,β-unsaturated aldehydes, HNE, has been detected in edible oils (34–36), but no information is presently available on the presence of any other lipophilic hydroxyaldehydes or α,β-unsaturated aldehydes in oils. At present, no methods have been reported that achieve the separation and simultaneous measurement of the wide range of nonpolar and polar lipophilic secondary peroxidation products from vegetable oils and fats.

The method described here was based on a method previously developed in this laboratory that measures the secondary lipid peroxidation products formed *in vivo* and excreted in the urine and that has been used in several experiments (1,37–39).

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The present method measures the lipophilic nonpolar and polar aldehydes and related carbonyl compounds, in oils, as 2,4-dinitrophenylhydrazones. These secondary lipid peroxidation products include alkanals, alkenals, alkadienals, and hydroxyaldehydes, including the toxic α,β-unsaturated aldehydes such as HHE, HNE, and 4-hydroxy-2-*trans*-octenal (HOE). In this method, an HPLC profile is produced that allows for simultaneously assessing the occurrence and relative amounts of the lipophilic secondary lipid oxidation products present in oils and fats during the course of peroxidation.

EXPERIMENTAL PROCEDURES

Chemicals. 2,4-Dinitrophenylhydrazine (DNPH) and hexanal were obtained from Sigma (St. Louis, MO); 2-pentanone (97%), 2-heptenal (97%), 2,4-heptadienal (90%), decanal, 2,4-decadienal, and undecanal from Aldrich Chemical Co. (Milwaukee, WI); hydrochloric acid, acetone, methanol, dichloromethane, hexane, and water from EM Science (Gibbstown, NJ). All solvents used were HPLC grade. DNPH derivatives of butanal, butanone, hexanal, octanal, 2-nonenal, HHE, HOE, and HNE were received as a gift from the Department of Biochemistry, University of Graz (Graz, Austria). Silica gel TLC plates (Silica gel 60, aluminum-backed, 20 × 20 cm, 0.2 mm thickness) were purchased from Altex (Berkeley, CA).

Instrumentation. The HPLC system consisted of an Altex model 110A solvent metering pump and sample injector (Beckman Instruments, Berkeley, CA), SP8400 UV/Vis detector (Spectra-Physics, Arlington, IL), and HP3380A computing integrator (Hewlett-Packard, San Diego, CA). The HPLC separations were performed on an Ultrasphere ODS C18 reversed-phase column (25 cm × 4.6 mm i.d., 5 µm particle size) (Altex) with a guard column (2 cm × 2 mm i.d.) (ChromTech, Apple Valley, MN). Disposable syringes used for sample injection were equipped with a 0.2 µm PVDF filter (ChromTech).

Preparation of DNPH reagent. The reagent was prepared daily by combining 10 mg DNPH, recrystallized three times from methanol, with 20 mL 1 N HCl at 50°C for about 1 h. After cooling, the mixture was extracted four times with HPLC-grade hexane in a separatory funnel to remove impurities. The aqueous purified DNPH reagent was used immediately.

Synthesis of 2,4-dinitrophenylhydrazones of standards. DNPH derivatives of 2-pentanone, hexanal, 2-heptenal, 2,4-heptadienal, decanal, 2,4-decadienal, and undecanal were synthesized as follows: 80 mg of freshly prepared DNPH regent, 8 mL warm methanol, 0.2 mL 6 N HCl, and 100 µL of the aldehyde or ketone standard were mixed and heated in a water bath at 60°C for 10 min. After cooling overnight at 4°C, 10 drops of water were added to induce crystallization. The fine crystals were filtered using a Buchner funnel and recrystallized from 20 mL of methanol.

Synthesis of DNPH reagent standard and acetone–DNPH standard. The DNPH reagent and acetone–DNPH standards were prepared by combining equal volumes of water or acetone/water (1:99, vol/vol), respectively, with the freshly prepared DNPH reagent, incubating overnight at room temperature, and subsequently extracting three times with dichloromethane. The solvent was evaporated from the combined extracts of each standard using N_2 until the sample size reached 0.5 mL.

Preparation of thermally oxidized soybean oil. Commercial soybean oil (50 g) (Cargill, Inc., Wayzata, MN) was heated in an open vessel for 8 h at 185°C. 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 with a Matheson model 610 (East Rutherford, NJ) flow meter. The thermally oxidized oil was cooled and was stored under N_2 gas in the dark at –20°C until analysis.

Synthesis and isolation of DNPH derivatives of lipophilic aldehydes and related carbonyl compounds in thermally oxidized soybean oil. Thermally oxidized soybean oil (3 g) was reacted with 6 mL of freshly prepared DNPH reagent. The mixture was incubated overnight at room temperature in the dark with shaking at ~120 oscillations per minute.

The DNPH derivatives from the oil were extracted three times with 10 mL HPLC-grade methanol/water (75:25, vol/vol) and separated by centrifugation at 1360 × *g* for 10 min. The DNPH derivatives in the combined methanol extracts were further extracted three times with 10 mL dichloromethane and separated by centrifugation as before. Dichloromethane was evaporated away from the pooled extracts with N_2 until the sample size was ~1 mL.

Aliqots (500 µL) of the DNPH derivatives in the CH_2Cl_2 extract were applied to silica gel TLC plates and were developed with CH_2Cl_2. The nonpolar and polar carbonyl compounds were identified by comparison of R_f values to those for the acetone–DNPH standard (0.55) and the DNPH reagent standard (0.23). The nonpolar carbonyl compounds (NPC) such as alkanals, alkenals, ketones, and dienals were located between R_f 0.55 and the solvent front. Osazones were separated from the NPC and polar carbonyl compounds (PC) on TLC between the acetone–DNPH standard (R_f 0.55) and the DNPH reagent standard (R_f 0.23) reference bands. The PC, including HHE, HOE and HNE, were located between the origin and R_f

TABLE 1
Solvent Systems Used for Co-chromatography of Polar Lipophilic Aldehydes[a]

	45% MeOH/ 55% water	50% MeOH/ 50% water	55% MeOH/ 45% water	60% MeOH/ 30% water	68% MeOH/ 32% water
4-Hydroxy-2-hexenal		*		*	*
4-Hydroxy-2-octenal		*	*		
4-Hydroxy-2-nonenal	*	*	*		

[a]The solvent systems used for co-chromatography of the individual polar carbonyl compounds are indicated by an asterisk.

0.23 (DNPH reagent standard). The nonpolar and polar regions were individually removed from the TLC plates. Analytes were eluted from the TLC plate sections with 3 × 10 mL methanol. The combined methanol extracts were centrifuged at 1360 × *g* for 15 min to precipitate the residual silica. The clarified supernatant fractions were concentrated under N_2 to 2.0 mL.

Identification of DNPH derivatives by HPLC. DNPH derivatives of NPC (100-µL aliquots) were injected onto an HPLC reversed-phase C18 column, equipped with a guard column, using isocratic elution for 10 min with methanol/water (75:25 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. For the HPLC separation of the DNPH derivatives of PC, a more polar solvent, methanol/water (50:50, vol/vol), was used as the starting eluent. The gradient and other operating conditions were the same as for NPC. Absorbance was monitored at 378 nm. 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.

Identification of individual NPC and PC DNPH derivatives from the oil samples was accomplished (i) by comparing peak retention times of DNPH derivatives of nonpolar and polar standards to the retention times of peaks derived from the oil and (ii) by co-chromatography by HPLC in solvent systems of differing polarities. Evidence of identity was obtained by the co-chromatography of the isolated DNPH derivatives from the oil with pure DNPH derivatives of standards using multiple solvent systems of differing polarities to confirm the co-elution of the compounds. The solvent systems used for co-chromatography of the polar lipophilic aldehydes are listed in Table 1. The quantity of the added derivatized standard was selected so as not to increase the peak area of the DNPH derivative from the oil more than two times.

Statistical analysis. The average and standard error of the mean (SEM) were calculated for each identified compound and unidentified major peak in both the nonpolar and polar fractions.

RESULTS AND DISCUSSION

Typical HPLC chromatograms illustrate the simultaneous separation of nonpolar and polar 2,4-dinitrophenylhydrazones of lipophilic aldehydes and related carbonyl compounds

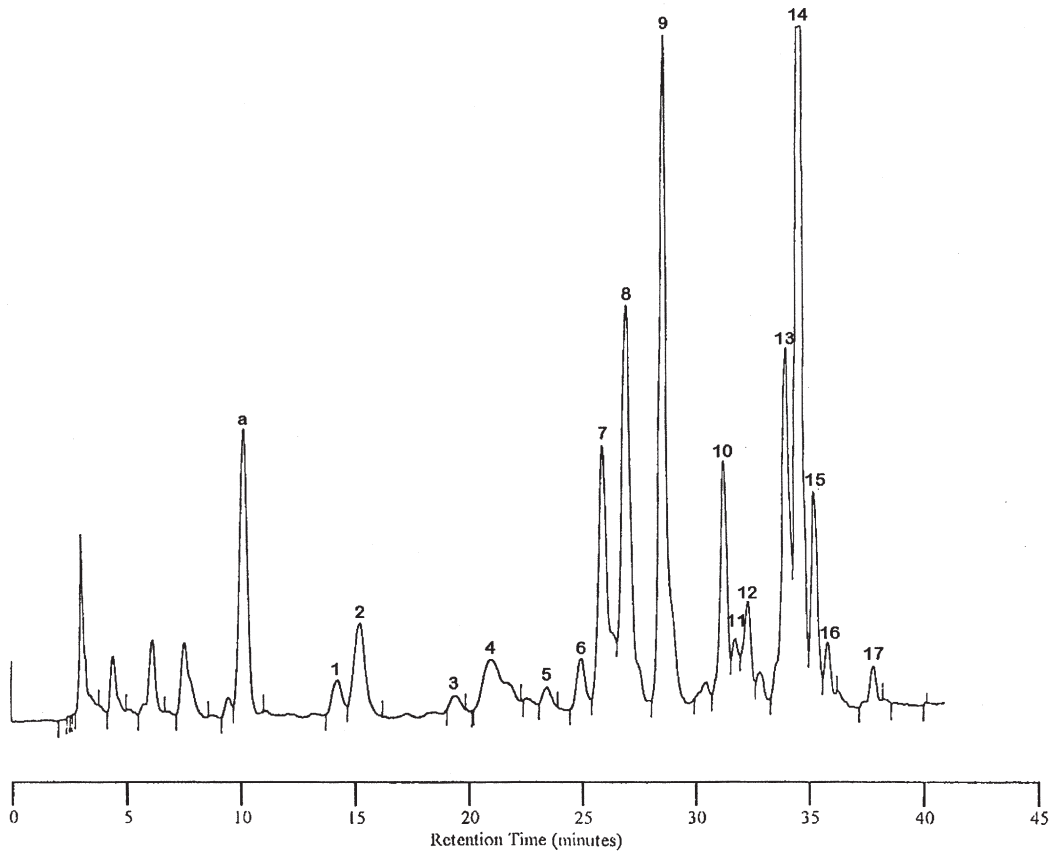

FIG. 1. High-performance liquid chromatography (HPLC) separation of 2,4-dinitrophenylhydrazine (DNPH) derivatives of nonpolar aldehydes and carbonyl compounds from soybean oil heated 8 h at 185°C in the presence of air. a: acetone; 1: butanal; 2: 2-butanone; 3: pentanal; 4: pentanone; 6: 2-hexenal; 7: hexanal; 8: 2,4-heptadienal; 9: 2-heptenal; 10: octanal; 13: 2-nonenal; 14: 2,4-decadienal; 15: decanal; 17: undecanal. 5, 11, 12, 16: unidentified. Separation conditions: Ultrasphere ODS column (4.6 mm × 25 cm, 5 µm; Altex, Berkeley, CA), isocratic elution with methanol/water (75:25, 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.

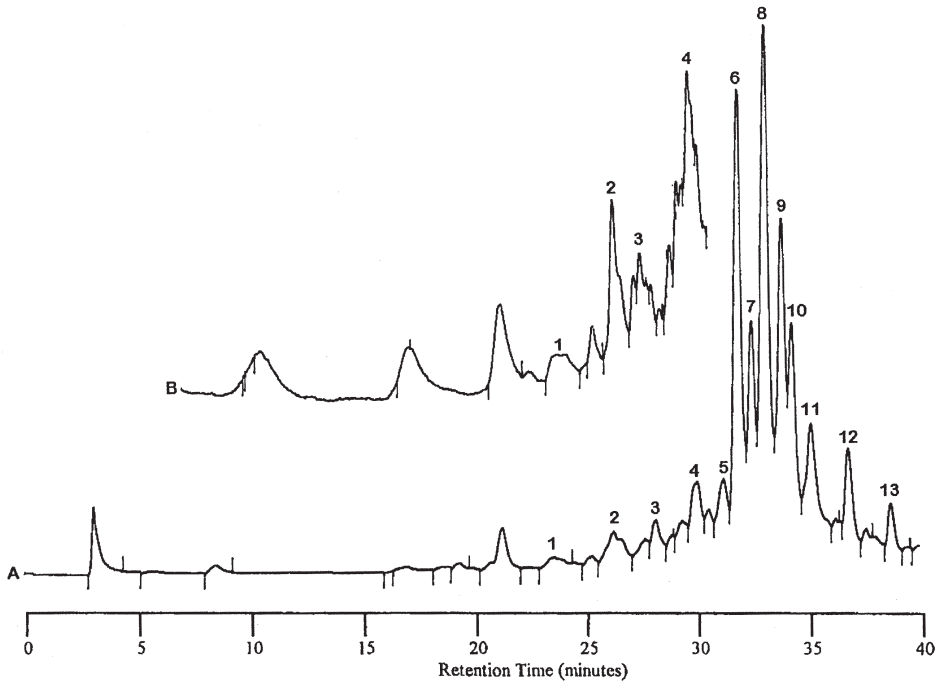

FIG. 2. HPLC separation of DNPH derivatives of polar aldehydes and carbonyl compounds from soybean oil heated 8 h at 185°C in the presence of air (A). 2: 4-hydroxy-2-hexenal; 5: 4-hydroxy-2-octenal; 8: 4-hydroxy-2-nonenal. 1, 3, 4, 6, 7, 9–13: unidentified. Chromatogram B shows enlargement of chromatogram region containing peaks 1, 2 (4-hydroxy-2-hexenal), and 3 at a different attentuation and concentration from chromatogram A. Separation conditions: Ultrasphere ODS column (4.6 mm × 25 cm, 5 µm; Altex), 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. For abbreviations see Figure 1.

extracted from the heated soybean oil (Figs. 1 and 2, respectively). The nonpolar and polar DNPH derivatives are well separated on the C18 reverse-phase column. Thirteen of the 17 major nonpolar lipophilic aldehydes and related carbonyl compounds in heated soybean oil have been identified by comparison of retention times to the retention times of pure standards. The identified compounds are butanal, 2-butanone, pentanal, 2-pentanone, hexenal, hexanal, 2,4-heptadienal, 2-heptenal, octanal, 2-nonenal, 2,4-decadienal, decanal, and undecanal. Further confirmation of the identity of the nonpolar carbonyl compounds was made by co-chromatography with pure standards, conducted as in previous experiments in this laboratory (1). The remaining nonpolar compounds separated from the oil were not identified at present because of the lack of appropriate standards.

Three of the major polar lipophilic aldehydes, HHE, HOE and HNE, have also been identified by comparison of retention times and by co-chromatography with pure standards. Co-chromatograms of pure standard HHE–DNPH, HOE–DNPH, and HNE–DNPH with their oil-derived counterparts are illustrated in Figures 3, 4, and 5. The elution of the pure standards (Figs. 3A, 4A, and 5A) is the same as the elution of the corresponding DNPH derivatives from the heated soybean oil (Figs. 3B, 4B, and 5B). The mixture of the oil-derived compounds with a known amount of the corresponding pure standard resulted in co-elution of the HHE, HOE, and HNE as shown in Figures 3C, 4C, and 5C. Similar co-elutions were obtained for HHE, HOE, and HNE using solvent systems of differing polarity, which confirmed the identity of the compounds derived from the oil. The percent recovery for each co-elution was calculated by comparing the peak area of the mixture of pure standard and oil-derived compounds to the sum of the peak area of the pure standard and the peak area of the compound derived from heated soybean oil (Table 2). The remaining polar lipophilic aldehydes and related carbonyl compounds from heated soybean oil were not identified at this time owing to lack of appropriate standards.

The average peak areas per gram of peroxidized oil (mean peak area ± SEM, $n = 4$ replicates) for the 17 major nonpolar carbonyl compounds, separated by HPLC, found in thermally oxidized soybean oil are shown in Figure 6. The concentrations (µg/g oil) for the 13 identified compounds are listed in Table 3. Quantitation was based on 1 ng pure hexanal standard equivalent to a peak area of 2000. This value was determined by repeated injections of various concentrations of pure hexanal–DNPH standard (38). A similar molar extinction coefficient was assumed for all DNPH deriatives (40). The quantity of each aldehyde was calculated from hexanal equivalents using the molecular weight for each individual compound. The detection limit for individual compounds is less than 1 ng per injection (1).

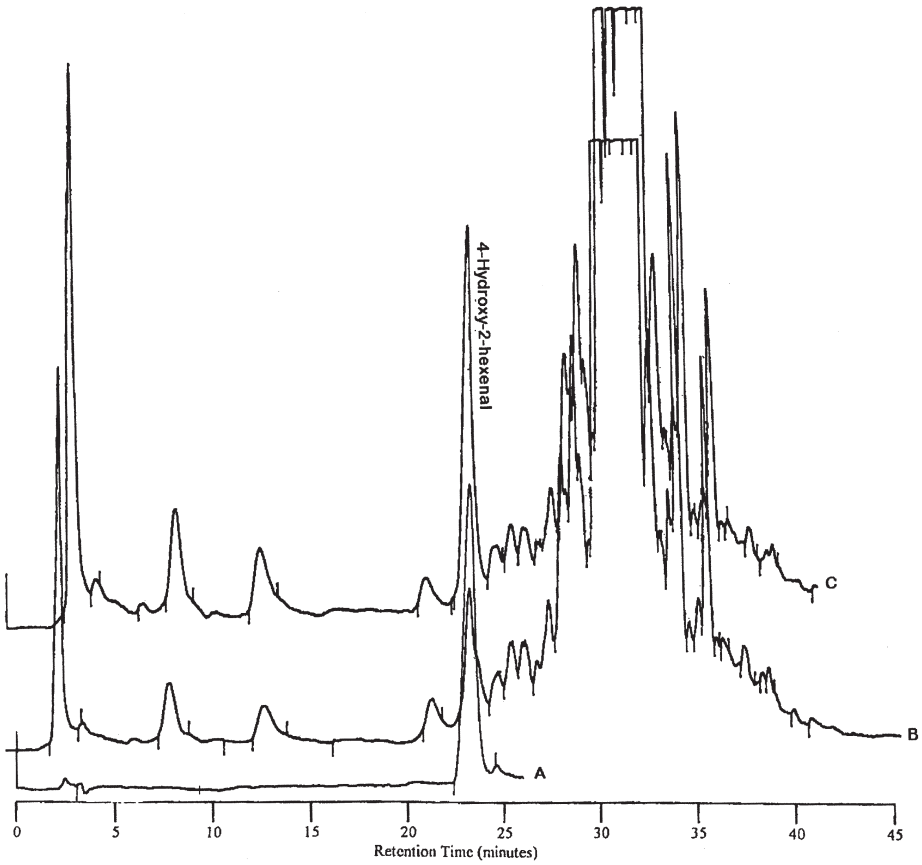

FIG. 3. Co-chromatography by HPLC of DNPH derivatives of 4-hydroxy-2-hexenal (HHE) derived from heated soybean oil with HHE standard. A: HHE standard; B: polar lipophilic carbonyl compounds from soybean oil heated 8 h at 185°C in the presence of air; C: co-chromatogram of oil-derived polar lipophilic carbonyl compounds with HHE standard. Separation conditions are given in Figure 2. For abbreviations see Figure 1.

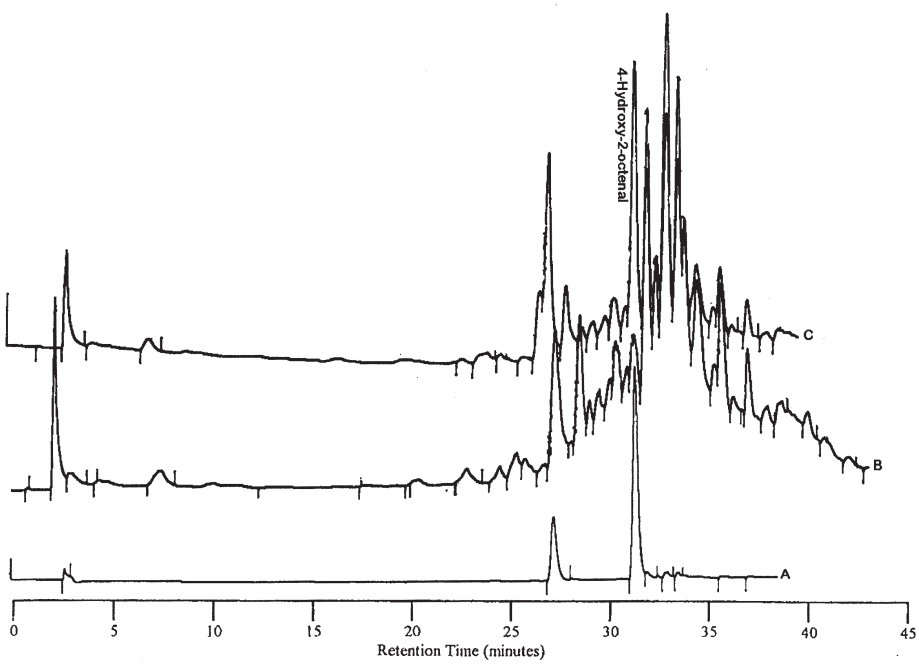

FIG. 4. Co-chromatography by HPLC of DNPH derivatives of 4-hydroxy-2-octenal (HOE) derived from heated soybean oil with HOE standard. A: HOE standard; B: polar lipophilic carbonyl compounds from soybean oil heated 8 h at 185°C in the presence of air; C: co-chromatogram of oil-derived polar lipophilic carbonyl compounds with HOE standard. Separation conditions are given in Figure 2. For abbreviations see Figure 1.

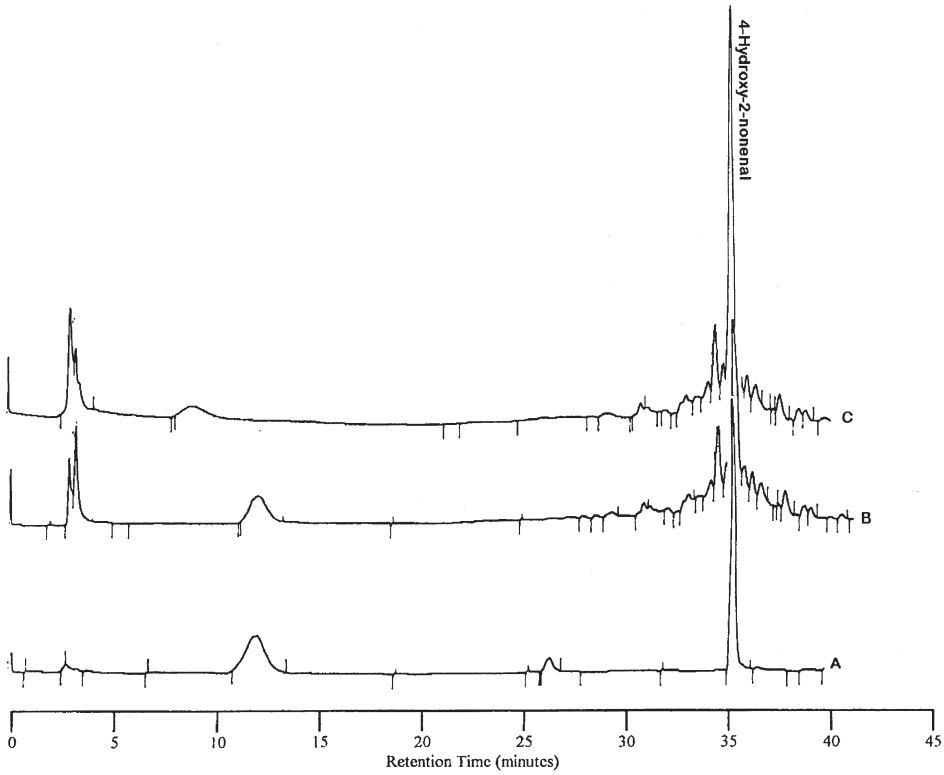

FIG. 5. Co-chromatography by HPLC of DNPH derivatives of 4-hydroxy-2-nonenal (HNE) derived from heated soybean oil with HNE standard. A: HNE standard; B: polar lipophilic carbonyl compounds from soybean oil heated 8 h at 185°C in the presence of air; C: co-chromatogram of oil-derived polar lipophilic carbonyl compounds with HNE standard. Separation conditions are given in Figure 2. For abbreviations see Figure 1.

The most abundant nonpolar compound found in the heated soybean oil was 2,4-decadienal, an aldehyde associated with a frying odor, at a concentration almost four times greater than the next most abundant compound. 2,4-Decadienal, as well as hexanal and heptenal, are major compounds reported to be formed by the autoxidation of linoleic acid (18:2 *c*9, *c*12); and, 2,4-heptadienal and octanal are major oxidative degradation products of linolenic (18:3 *c*9, *c*12, *c*15) and oleic (18:1 *c*9) acids, respectively (41). Soybean oil contains 38–57% linoleic acid, 21–38% oleic acid, and 5–9% linolenic acid (42). It was noted that a greater concentration of the less volatile, longer-chain carbonyl compounds remained in the oil than the more volatile, shorter-chain compounds such as butanal, butanone, pentanal, pentanone, hexenal, and hexanal. These volatile compounds only partially remained in the oil at the end of the 8-h heating period. In a separate experiment, when the volatile compounds were collected during heating of soybean oil with identical conditions, the short-chain volatile compounds were found in much higher concentrations in the volatile phase than in the oil (Seppanen, C.M., and A.S. Csallany, unpublished data).

Figure 7 shows the average peak area per gram oil (mean peak area ± SEM, n = 5 replicates) for the major polar lipophilic aldehydes and carbonyl compounds, separated by HPLC, found in thermally oxidized soybean oil. The concentrations of the identified polar compounds, HHE, HOE and

TABLE 2
Recovery of Added Pure Aldehyde Standards to the Aldehydes Derived from Heated Soybean Oil from Co-chromatography[a]

	45% MeOH/ 55% water	50% MeOH/ 50% water	55% MeOH/ 45% water	60% MeOH/ 30% water	68% MeOH/ 32% water
4-Hydroxy-2-hexenal		107.9%		101%	102.2%
4-Hydroxy-2-octenal		**	94.9%		
4-Hydroxy-2-nonenal	92.1%	94.9%	93.3%		

[a]Calculation: $a/(b + c)$, where a = area from the co-chromatography of compound from oil plus standard; b = area of compound from oil; c = area of standard. Values represent means of multiple samples. **No recovery data for HOE in 50% MeOH. Tentative identification by co-elution.

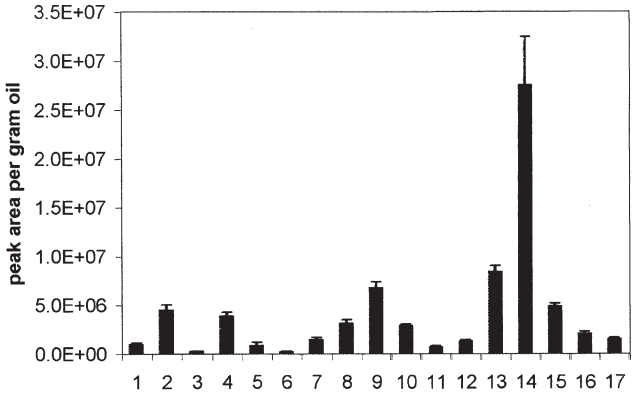

FIG. 6. Distribution of nonpolar lipophilic aldehyde and related carbonyl compound DNPH derivatives in soybean oil heated 8 h at 185°C in the presence of air separated by HPLC. Values represent mean ± SEM for $n = 4$ oil samples. Compounds are identified in Figure 1. For other abbreviations see Figure 1.

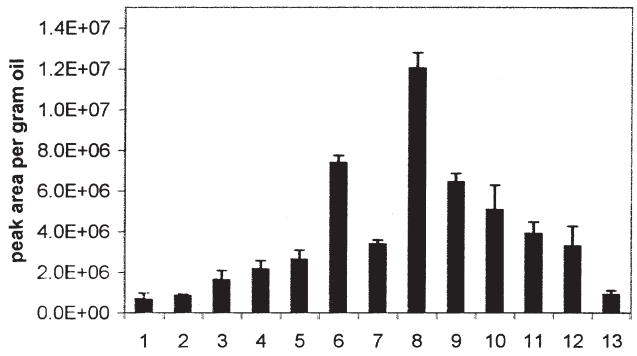

FIG. 7. Distribution of polar lipophilic aldehyde and related carbonyl compound DNPH derivatives in soybean oil heated 8 h at 185°C in the presence of air separated by HPLC. Values represent mean ± SEM for $n = 5$ oil samples. Compounds are identified in Figure 2. For other abbreviations see Figure 1.

HNE, are listed in Table 3. The most abundant polar carbonyl compound was identified as HNE at a concentration of 2.45 ± 0.15 μg/g of heated oil (21.49 ± 1.34 nmol/g heated oil). About five times more HNE than HHE was found in soybean oil heated at 185°C for 8 h, which may reflect the initial concentrations of linoleic acid, a precursor to HNE, and linolenic acid, a precursor to HHE, in soybean oil.

Few reports are in the literature of the detection of HNE in edible oils. HNE has been found in unheated olive, sunflower, and pumpkin seed oils at levels ranging from 0.08 to 7.3 nmol per gram (34). HNE has also been found in unheated soybean oil (26–36 pmol per gram) and in seasame oil (597–786 pmol per gram) (35). Cod liver oil oxidized with Fenton's reagent also contained a measurable amount of HNE, ~3 mmol per gram (36). The levels of HNE determined in heated soybean oil in this experiment were higher than those reported in the literature for unheated oils, and reflect the significant production of secondary lipid peroxidation products due to heating this oil in the presence of air. This is the first report in the literature of the simultaneous measurement of polar and nonpolar secondary lipid peroxidation products, including HNE, HHE and HOE, in heated soybean oil.

TABLE 3
Quantitative Estimate of Nonpolar and Polar Lipophilic Aldehydes and Related Carbonyl Compounds in Soybean Oil Heated 8 h at 185°C[a]

	nmol/g oil	μg/g oil
Nonpolar compounds		
Butanal	1.83 ± 0.25	0.13 ± 0.02
2-Butanone	8.05 ± 1.04	0.58 ± 0.07
Pentanal	0.40 ± 0.12	0.03 ± 0.01
2-Pentanone	6.92 ± 0.79	0.60 ± 0.07
2-Hexenal	0.42 ± 0.14	0.04 ± 0.01
Hexanal	2.61 ± 0.48	0.26 ± 0.05
2,4-Heptadienal	5.48 ± 0.79	0.60 ± 0.09
2-Heptenal	12.11 ± 1.10	1.36 ± 0.12
Octanal	5.20 ± 0.26	0.67 ± 0.03
2-Nonenal	15.00 ± 1.19	2.10 ± 0.17
2,4-Decadienal	56.32 ± 6.58	8.56 ± 1.00
Decanal	8.73 ± 0.57	1.36 ± 0.09
Undecanal	2.77 ± 0.29	0.47 ± 0.05
Polar compounds		
4-Hydroxy-2-hexenal	1.52 ± 0.14	0.17 ± 0.02
4-Hydroxy-2-octenal	4.73 ± 0.80	0.54 ± 0.09
4-Hydroxy-2-nonenal	21.49 ± 1.34	2.45 ± 0.15

[a]Compounds were separated and identified by high-performance liquid chromatography (HPLC). Quantitation was based on 1 ng hexanal = peak area of 2000 and an assumption of a similar molecular extinction coefficient for all compounds (41).

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