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ORIGINAL PAPER

Temperature Dependence of HNE Formation in Vegetable Oils and Butter Oil

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Abstract The temperature dependence of the formation of toxic 4-hydroxy-2-*trans*-nonenal (HNE) was investigated in high and low linoleic acid (LA) containing oils such as corn, soybean and butter oils. These oils contain about 60, 54 and 3–4% of LA for corn, soybean and butter oils, respectively. The oils were heated for 0, 0.5, 1, 2, and 3 h at 190 °C and for 0, 5, 15 and 30 min at 218 °C. HNE concentrations in the oils were analyzed by high performance liquid chromatography (HPLC). The maximum HNE concentrations in heated (190 °C) corn, soybean and butter oils were 5.46, 3.73 and 1.85 μg HNE/g oil, respectively. The concentration of HNE at 218 °C increased continuously for all the three oils, although they were heated for much shorter periods compared to the lower temperature of heating (190 °C). HNE concentration at 30 min reached the maximum of 15.48, 10.72 and 6.71 μg HNE/g oil for corn, soybean and butter oils, respectively. HNE concentration at higher temperature (218 °C) was 4.9, 3.7, and 8.7 times higher than at the lower temperature (190 °C) and 30 min of heating for corn, soybean and butter oils, respectively. It was found that HNE formation was temperature dependant in the tested oils.

Keywords Butter oil · Corn oil · Heating temperature · HNE · 4-Hydroxy-2-*trans*-nonenal · Linoleic acid · N6 fatty acid · Soybean oil

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Introduction

4-Hydroxy-2-*trans*-nonenal (HNE) is known to be a secondary lipid peroxidation product of linoleic acid (LA) and other *n*-6 fatty acids [1, 2]. HNE is a toxic compound, related to atherosclerosis [3, 4], LDL oxidation, stroke, Parkinson's, Alzheimer's and Huntington's diseases [5–10], liver diseases [11], and other diseases.

The toxicity of HNE is due to α,β-unsaturated hydroxyaldehyde structure [12]. These types of aldehydes are of particular interest because they are readily absorbed from the diet [3, 13, 14]. The toxicity arises because these aldehydes are highly reactive substances which can modify proteins, nucleic acids and other biomolecules in vivo [15–18]. At cellular concentrations of 1–20 μM, HNE partially inhibits DNA and protein synthesis [2]. 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 led to cell death. Concentrations such as these may arise in the cells because of in vivo oxidative stress or because of consumption of these oxidation products from foods.

Previous experiments in this laboratory have reported the formation of HNE in soybean oil, an oil high in LA, at frying temperature (185 °C) [19] and its incorporation into fried food from the frying oil [20]. It was shown that the concentration of HNE in the oil of the food was the same as the concentration of the frying oil.

Since HNE is a very toxic compound which is absorbed from the diet and relates to a number of human diseases, information about its formation in various oils at frying temperatures is very important from a public health point of view. In the present study, the formation of HNE was investigated at a higher frying temperature, 218 °C for short

periods of time and compared to the lower frying temperature, 190 °C and longer durations of heat treatment.

Materials and Methods

Chemicals and Materials

Butter (Land O' Lakes, Inc. Arden Hills, MN), soybean oil (J.M. Smucker Co. Orrville, OH) and corn oil (ACH Food Inc. Memphis, TN) were purchased from retail stores (Roseville, MN). 2, 4-Dinitrophenylhydrazine (DNPH) and hexanal standard were purchased from Sigma (ST. Louis, MO) and HNE from Cayman Chemical Co. (Ann Arbor, MI). Hydrochloric acid was obtained from J.T. Bakers Inc. (Phillipsburg, NJ), HPLC-grade methanol from Fisher Scientific (Fair Lawn, NJ), and HPLC-grade water, *n*-hexane and dichloromethane from EMD Chemicals, Inc. (Gibbstown, NJ). Plates for thin layer chromatography (TLC) and No. 1 filter paper were purchased from Whatman Ltd. (Kent, England).

Recrystallization of DNPH

DNPH (10 g) was dissolved in 200 ml methanol and heated at 60 °C. The dissolved DNPH was placed in an ice bath for at least 18 h for crystallization. The crystallized DNPH was filtered by No. 1 filter paper and redissolved in about 200 ml of methanol. The crystallization process was repeated at least two more times and the collected DNPH crystals were placed in a desiccator for 3 days for drying.

Preparation of DNPH Reagent

The freshly recrystallized DNPH (10 mg) was dissolved in 20 ml 1 N hydrochloric acid and heated at 50 °C for 1 h. After cooling, the impurity was washed four times with 10 ml of hexane (total 40 ml) and the hexane layers were discarded. The purified DNPH reagent was used for the production of DNPH derivatives of aldehydes including HNE in the butter oil and vegetable oils.

Preparation of Butter Oil

The butter was heated at 45 °C until it melted completely. The melted butter was filtered through cheese cloth and the filtrate was collected as butter oil for the experiment.

Preparation of Thermally Oxidized Butter Oil and Vegetable Oils

Three-gram samples of butter oil, soybean or corn oil were placed into open test tubes (25 × 125 mm), and continuously heated either at 190 °C for 0, ½, 1, 2 and 3 h, or at 218 °C for 0, 5, 15 and 30 min in a glycerol bath.

Preparation of Standard

For the quantification of HNE, pure hexanal was used as a standard. The 2, 4-dinitrophenylhydrazone derivative of hexanal was prepared by a method developed in this laboratory [13]. The mixture of 800 mg of recrystallized DNPH, 80 ml methanol, 2 ml 6 N hydrochloric acid and 1 ml pure hexanal, was heated for 10 min at 60 °C, and placed into an ice bath for crystallization. For purification, the hexanal-DNPH crystals were filtered through Whatman No. 1 filter paper and they were recrystallized two more times from about 50 ml of methanol. After the final crystallization, the hexanal-DNPH was dried in a desiccator for 3 days and the product was kept at −20 °C in an air tight container.

Preparation of HNE-DNPH Derivatives

The preparation of HNE-DNPH derivatives in butter oil and vegetable oils was conducted as described by the method of Seppanen and Csallany [21]. One gram of the previously heat treated or unheated oil sample was reacted overnight at room temperature with 5 ml freshly prepared DNPH reagent. The DNPH derivatives were extracted with 10 ml of methanol:water 75:25 (v/v) from the oil. This procedure was repeated two more times and the methanol:water extracts were combined. The DNPH derivatives were re-extracted with 10 ml of dichloromethane three times from the combined methanol:water extracts and concentrated with N_2 gas to about 1 ml.

Thin Layer Chromatography

The concentrated DNPH derivatives in about 1 ml dichloromethane were applied to two TLC plates for preseparation of compounds and developed in dichloromethane. The polar and nonpolar aldehydes, and osazone regions were 0.25, 0.75 and 0.50 Rf values, respectively. The polar region, which included HNE, was extracted from TLC plates three times with 10 ml of methanol. The combined methanol extracts were evaporated under N_2 gas to the exact volume of 1 ml and aliquots of 50 µl were injected into the HPLC column.

HPLC Analysis of HNE-DNPH

The HPLC system consisted of a sample injector (712 WISP, Waters, Milford, MA), a solvent delivery system (9050, Varian, Walnut Greek, CA) and a UV–Vis detector

(9010, Varian). The HPLC column was Ultrasphere ODS (5 × 4.6 mm, 25 cm, Beckman, Fullerton, CA). A gradient solvent system was used for the separation of HNE-DNPH from the other polar DNPH derivatives. An isocratic elution with a mixture of 50:50 (v/v) methanol:water was used for 10 min, the graduate elution increased to 100% of methanol in 10 min, and 100% of methanol was used for an addition of 20 min. The absorbance of the HNE-DNPH was monitored at 378 nm.

The retention time of HNE-DNPH, from the oil samples, was identified by comparing with the retention time of pure HNE-DNPH standard. Further identification was carried out by co-chromatography in three different polarity solvent systems [methanol:water, 50:50, 55:45 and 60:40 (v/v)] as described by Seppanen and Csallany [21]. HNE was quantified first by comparing the peak area of HNE-DNPH with the peak area of pure hexanal-DNPH standard and expressed as μg hexanal equivalent/g oil. The hexanal equivalent was converted to μg HNE/g oil by calculation based on the molecular weight differences between hexanal and HNE.

Statistical Analysis

All determinations were performed in duplicate samples. Data were statistically analyzed by analysis of variance and Duncan's multiple range test using SAS software (Statistical Analysis System Institute, Cary, NC). Significant differences were determined at $P < 0.05$ level.

Results and Discussion

The present investigation was conducted to measure the formation of toxic HNE in low LA butter oil, and high LA soybean and corn oils in short term and higher temperature of heating, and compare it to longer term and lower temperature of heating. Butter oil and the vegetable oils were heated at 190 °C from 0 to 0.5, 1, 2 and 3 h and at 218 °C from 0 to 5, 15 and 30 min, and the heated oils were analyzed for their HNE concentrations.

Typical HPLC chromatograms of polar aldehydes and the related carbonyl compounds including HNE in butter

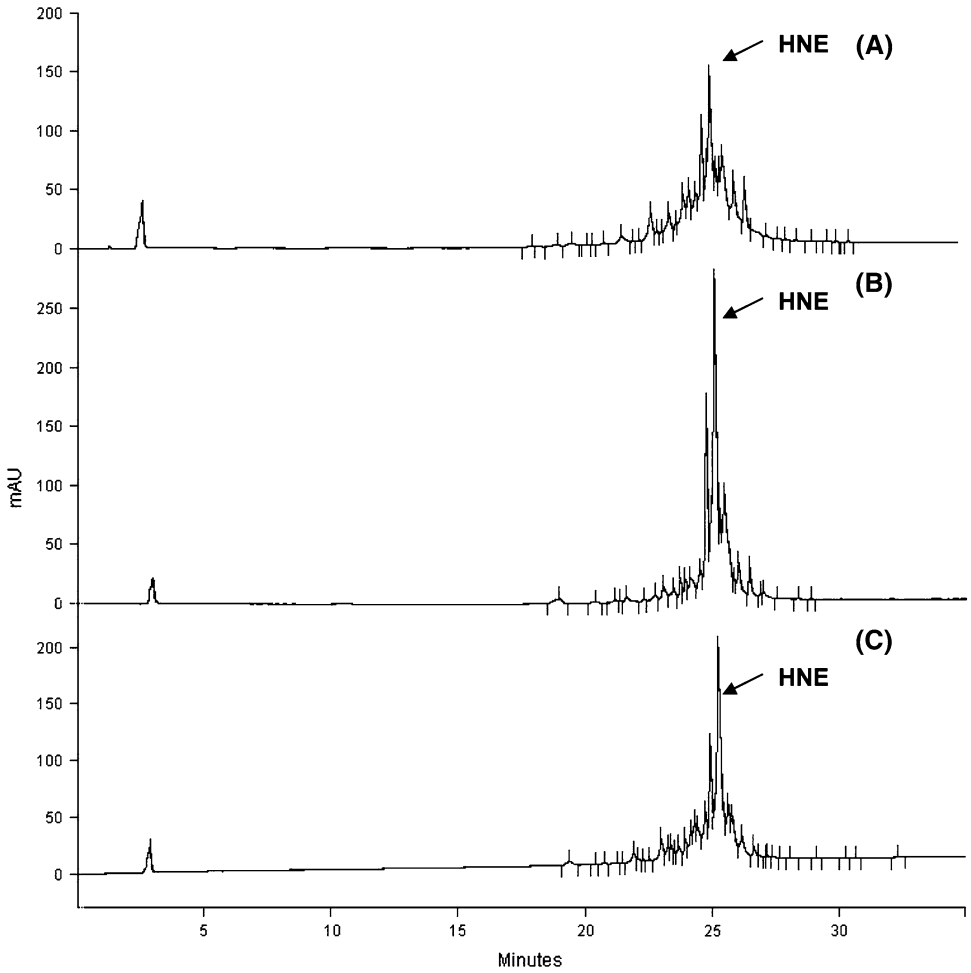

Fig. 1 Typical HPLC chromatogram of the DNPH derivatives of HNE and the other polar aldehydes and related carbonyl compounds extracted from butter oil (**a**), corn oil (**b**) and soybean oil (**c**) heated at 190 °C for 3 h. *HNE* DNPH derivative of 4- hydroxy-2-trans-nonenal

oil, corn and soybean oils heated for 3 hours at 190 °C are presented in Fig. 1. HNE was the major compound found among the polar aldehydes and the related carbonyl compounds in all three oils. Typical HPLC chromatograms of A, B and C, show the successful separation of HNE-DNPH from the other polar DNPH derivatives with retention time at 25 ± 0.5 min. The HNE-DNPH peak area was the largest in corn oil, followed by soybean oil and butter oil. The purity of HNE-DNPH isolated from the oil samples was tested by co-chromatography in three different polarity solvent systems with pure HNE standard according to Seppanen and Csallany [21]. The results (Table 1) demonstrate that the HNE standard and HNE derived from the heated oils were co-eluting in the different polarity solvent systems on the HPLC column.

The HNE concentrations in the three oils, heated at 190 °C from 0 to 3 h are shown in Fig. 2. The concentration of HNE in heated butter oil increased with the heating time and reached 1.85 μg HNE/g oil at 3 h. Although the HNE formation was much lower in butter oil than in corn or soybean oils its formation continuously increased in butter oil and reached a maximum after 3 h of heating. The HNE reached maximum concentration in corn oil after 1 h heating. The 5.46 μg HNE/g oil was observed after 1 h and then decreased significantly to 3.27 μg HNE/g oil after 3 h of heating. The decline of HNE concentration in corn oil after 1 h of heating may relate to its faster decomposition of HNE than its formation. Soybean oil also reached maximum concentration (3.73 μg HNE/g oil) after 1 h heating, and remained in similar concentrations up to 3 h of heating. The relative ratios of HNE formation in corn and soybean oils compared to butter oil at 190 °C during the 3 h of heating period are presented in Table 2.

The formation of HNE in butter oil, corn and soybean oils, heated at 218 °C for 0, 5, 15 and 30 min, is shown in Fig. 3. The concentration of HNE increased in this temperature with the increase of heating time in all three oils. After 5 min of heating, the HNE concentration was the highest in soybean oil (8.08 μg HNE/g oil), followed by corn oil (5.93 μg HNE/g oil) and butter oil (2.13 μg HNE/g oil). However, after 15 min of heating the concentration of HNE in corn oil was the highest among the three oils. After 30 min of heating, HNE concentration in corn oil was still the highest at 15.48 μg HNE/g oil, followed by soybean oil (10.72 μg HNE/g oil) and butter oil (6.71 μg HNE/g oil). The relative ratios of HNE formation of corn and soybean oils compared to butter oil during the heating period of 30 min at 218 °C are presented in Table 3.

Table 1 Recovery of added pure HNE standard to HNE derived from heated oils in co-chromatography[a]

Oils	Solvent systems (methanol:water v/v)		
	50:50	55:45	60:40
Corn oil (%)	104.5	103.5	106.7
Soybean oil (%)	95.6	110.1	109.8
Butter oil (%)	106.8	114.1	107.0

[a] Calculation: a/(b + c), where *a* area of HNE in oil plus standard in the co-chromatography; *b* area of HNE in oil; *c* area of HNE standard

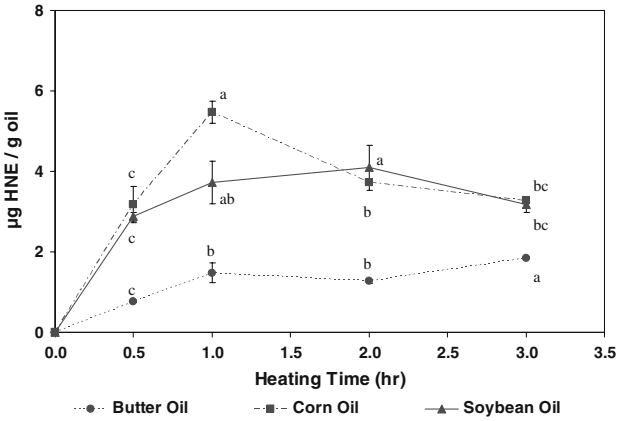

Fig. 2 HNE formation in butter oil, corn and soybean oils heated at 190 °C for 0, 1, 2 and 3 h. a, Values in the same oil with different letters are significantly different at $P < 0.05$

Table 2 Relative ratios of HNE formation in corn and soybean oils compared to butter oil at 190 °C during the heating period of 3 h

Oils	Heating time (h)				
	0	0.5	1	2	3
Corn oil	0	4.1	3.7	2.9	1.8
Soybean oil	0	3.7	2.5	3.2	1.7
Butter oil	0	1.0	1.0	1.0	1.0

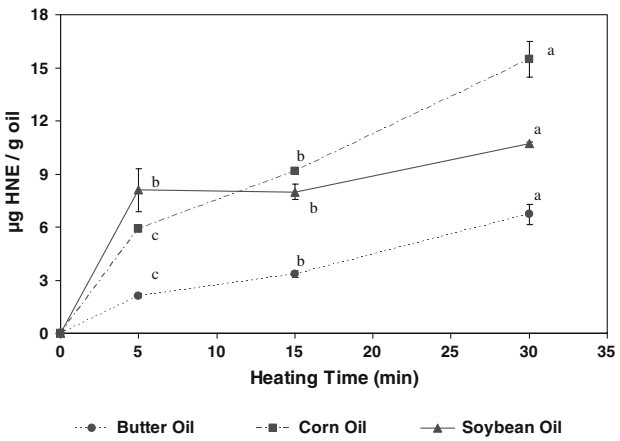

Fig. 3 HNE formation in butter oil, corn and soybean oils heated at 218 °C for 0, 5, 15 and 30 min. a, Values in the same oil with the *different letter* are significantly different at $P < 0.05$

Table 3 Relative ratios of HNE formation in corn and soybean oils compared to butter oil at 218 °C during the heating period of 30 min

Oils	Heating time (min)			
	0	5	15	30
Corn oil	0	2.8	2.7	2.3
Soybean oil	0	3.8	2.4	1.6
Butter oil	0	1.0	1.0	1.0

4-Hydroxy-2-*trans*-nonenal is known to derive from *n*-6 fatty acids, including LA, therefore its formation is affected by the fatty acid composition of the oils [2]. Corn oil contains about 60%, soybean oil about 54% and butter oil about 3–4% LA [22]. As it was suspected, HNE formation at both temperatures (190 and 218 °C) was much lower in butter oil than in high LA containing vegetable oils (Figs. 2, 3).

Comparison of HNE concentrations in the three oils heated for 30 min at 190 and 218 °C are shown in Fig. 4. When the temperature was raised from 190 to 218 °C, HNE formation dramatically increased in all three oils. HNE formation at this higher temperature not only increased but it continued to increase and reached a maximum without decline up to 30 min of heating (Fig. 3). At the end of 30 min heating period at 218 °C, the HNE concentrations were 8.7, 4.9, and 3.7 times higher in butter oil, corn oil and soybean oil, respectively, than at 190 °C (Fig. 4). This indicates that the formation of HNE was highly affected not only by the concentration of LA in the oils and the duration of heating, but also depended on the heating temperature.

Heating times required to reach the concentration of 1 μg HNE/g oil at 218 and 190 °C for butter oil, corn and soybean oils are shown in Fig. 5. The required times were

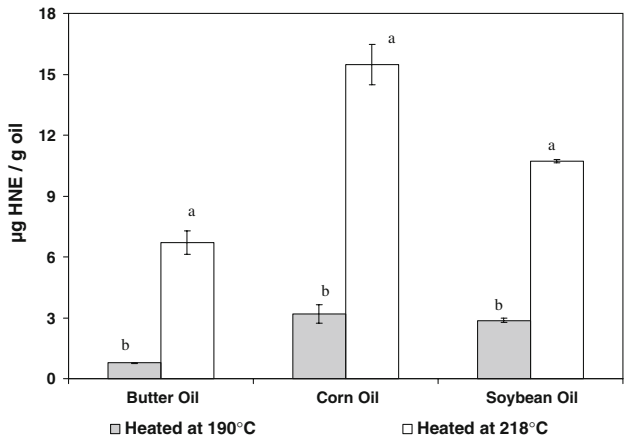

Fig. 4 Comparison of HNE concentrations of butter oil, and corn and soybean oils heated for 30 min at 190 and 218 °C. a, Values in the same oil with *different letters* are significantly different at $P < 0.05$

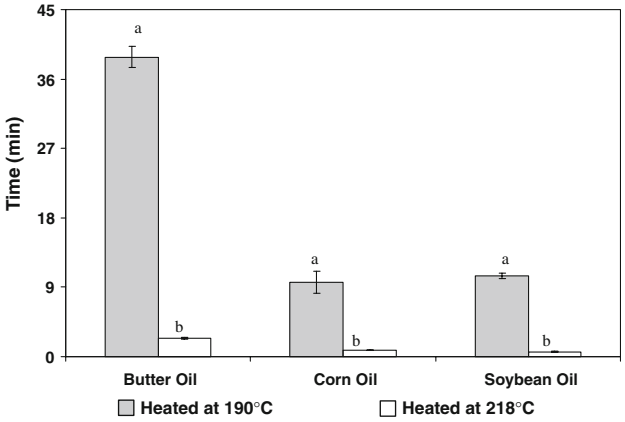

Fig. 5 Comparison of the heating times, required for the formation of 1 μg HNE/g oil, between 190°C and 218°C for butter oil, and corn and soybean oils. a, Values in the same oil with the different letter are significantly different at $P < 0.05$

2.4, 0.8 and 0.6 min for butter oil, corn and soybean oils, respectively at 218 °C and 38.8, 9.6 and 10.5 min at 190 °C, respectively. This demonstrates that the heating times to reach 1 μg HNE/g oil at 190 °C were for butter oil, corn oil and soybean oil 16.2, 11.8 and 16.5 times longer, respectively, than the heating time at 218 °C. The higher heating temperature promoted not only higher HNE production but also faster HNE production for all three oils.

The data supported our speculation that the low-LA-containing butter oil had significantly lower HNE formation at both lower (190 °C) and higher (218 °C) frying temperatures in either short or extended periods of heating compared to the higher-LA-containing soybean and corn oils. However, it was not suspected that a relatively small increase in temperature (only 28 °C) would cause a dramatic increase in HNE regardless of the oil even over short periods of heating compared to the lower temperature. This study indicates that frying temperature for LA containing oils in general should be at the lowest possible temperatures to reduce the formation of the toxic HNE in oils and potential human exposure since HNE incorporates into fried food [20] and it is readily absorbed from the diet [12].

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