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ORIGINAL PAPER

4-Hydroxynonenal (HNE), a Toxic Aldehyde in French Fries from Fast Food Restaurants

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Abstract The toxic lipid peroxidation product, α,β,4-hydroxy-2-*trans*-nonenal (HNE) concentration, was measured in French fries (FF) from six local fast food restaurants. FF were purchased between 2 and 3 pm from all six restaurants. FF were also purchased at 12, 2, 4, 6 pm from one and at 1, 3, 5, 7 pm from another restaurant. Samples were analyzed for total fat, fatty acid distribution and for HNE by high performance liquid chromatography (HPLC). HNE was confirmed by HPLC/MS. HNE concentrations in FF from the 6 fast food restaurants were between 7.83 and 32.15 μg HNE/100 g FF and between 0.9 and 4.9 μg HNE/g extracted fat. HNE concentrations in FF purchased at 12, 2, 4, 6 pm were between 19.07 and 32.15 μg/g of FF and purchased at 1, 3, 5, 7 pm were between 7.47 and 10.21 μg HNE/100 g of FF. Differences in FA distribution were observed in the samples from some restaurants. FF which contained higher levels of linoleic acid (LA) also contained more HNE. It is clear that HNE is produced during the heating process of the frying oils and is incorporated into FF. Frequently consumed foods containing considerable amounts of HNE, a toxic aldehyde, may be a public health concern since HNE toxicity is related to a number of common pathological conditions.

Keywords Fast foods · French fries · Heated oils · HNE · Lipid oxidation

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Introduction

Lipid peroxidation results in the oxidative deterioration of fatty acids (FA). Direct reaction of molecular oxygen with lipids involves the production of semi-stable peroxides from free radical intermediates [1]. The process of lipid peroxidation is involved in the decomposition of FA in fats and oils [2–6]. This process is the major course of deterioration for many foods containing fats and oils leading to quality and nutritional losses [7–9]. The oxidative degradation of FA leads to a variety of secondary lipid peroxidation products. These products include various lipophilic aldehydes such as alkanals, alkenals, alkadienals and hydroxyalkenals and these are readily absorbed from the diet [10–13]. One class of aldehydes, the 4-hydroxyalkenals, generated from lipid peroxidation of unsaturated FA are of special importance because of their reactivity to biomolecules [14, 15]. The cytotoxic and mutagenic α,β,4-hydroxy-2-*trans*-nonenal (HNE) [16] is one of the four 4-hydroxyalkenals that has been found to be formed from the oxidation of n-6 FA, including linoleic acid [12, 15, 17] which is high in polyunsaturated fatty acid (PUFA) oils. HNE toxicity has been related to atherosclerosis [18, 19], liver disease [20], Parkinson's, Alzheimer's, Huntington's diseases and LDL oxidation [21–26]. HNE also can damage DNA by gene mutation and affect cancer-related proteins [27]. It has been suggested in the literature that the toxicity of thermally oxidized oils is due to the non-volatile secondary oxidation products such as hydroxyl and hydroperoxyalkenals [28, 29]. HNE formation was reported by this laboratory in soybean oil after 2 h of heat treatment at 185 °C [30]. This laboratory also reported that due to thermal oxidation of soybean oil at 185 °C the major

polar lipophilic aldehyde is HNE, beside three minor α,β-unsaturated hydroxyaldehydes: 4-hydroxy-2-*trans*-hexenal (HNE), 4-hydroxy-2-*trans*-octenal (HOE) and 4-hydroxy-2-*trans*-decimal (HDE) [31]. This laboratory previously demonstrated that HNE incorporated from the frying oil into the fried potato at similar concentration as it was produced in the frying oil [32]. Since it is known that HNE is readily absorbed from the diet and that large quantities of commercially fried food, mainly French fries (FF), are consumed by the public, our present objective was to measure the concentration of HNE incorporation into FF from the frying oils in various fast food restaurants.

Experimental Procedures

Chemicals and Materials

All solvents used were HPLC grade. Hexanal and 2,4-Dinitrophenylhydrazine (DNPH) were obtained from Sigma (St. Louis, MO); Methanol and HPLC-grade acetone, dichloromethane from Mallinckrodt (Paris, KY); Silica gel TLC plates (AI Sil G, aluminum-backed, 20 × 20 cm, 250 um layer) were obtained from Whatman Ltd. (Maidstone, Kent, England). HPLC-grade hexane and hydrochloric acid from Fisher Scientific (Fair Lawn, NJ); and HPLC-grade water from E Science (Gibbstown, NJ).

Sample Collection

Duplicate large portions of FF were purchased from 6 local fast food chain restaurants between 2 and 3 pm on the same day. On a separate occasion duplicate large portions of FF were purchased from 2 of the above mentioned local fast food restaurants in the Twin Cities, Minnesota. The times of purchases were as follows: restaurant 1; at 12 noon, 2, 4 and 6 pm and restaurant 2; at 1, 3, 5 and 7 pm. All samples were placed immediately into separate plastic bags and stored at −20 °C until analysis.

Extraction of Fat from French Fried Potatoes

Duplicate 10 g samples from each of two portions of large FF purchased from each restaurant (a total number of 4 samples) were analyzed. Samples were homogenized in a blender for 30 min with 20 g anhydrous Na_2S0_4 and 50 mL hexane. The hexane supernatant was removed, and the potato/Na_2S0_4 slurry was extracted two subsequent times with 50 mL of hexane. The hexane portions were combined and evaporated under vacuum. The oil extracted from the FF was weighed and immediately analyzed for HNE.

Measurement of HNE and Other Polar Lipophilic Aldehydes and Related Carbonyl Compounds in the Extracted Oil Samples

The method described by Seppanan and Csallany [19] was used to analyze the oil for HNE and other polar aldehydic secondary lipid peroxidation products. Briefly, 1 g aliquots of the oil extracted from the FF were reacted in duplicate with 5 mL DNPH reagent overnight at room temperature to form hydrazine derivatives with the aldehydic secondary oxidation products. The DNPH reagent was prepared by combining 10 mg recrystallized DNPH with 20 mL of 1 N HCl. The DNPH derivatives were extracted from the oil first with methanol/water (75:25 vol/vol) and then with dichloromethane. The lipophilic DNPH derivatives were then separated into three groups by TLC on silica gel developed with dichloromethane to the polar carbonyl compound derivatives, the nonpolar carbonyl compound derivatives and the osazones. The group of lipophilic polar carbonyl hydrazones, which included HNE, were eluted from the TLC plates with methanol, then the solvent was evaporated by N_2 gas to 1 mL. Aliquots (10 µL) of the concentrated DNPH derivatives were separated and quantified by HPLC on a reversed phase C18 column [Ultra sphere ODS, 25 cm × 4.6 mm i.d., 5 µm particle size (Altex, Berkeley, CA)] with a Seppac guard column 2 cm × 2 mm i.d., (ChromTech, Apple Valley, MN). The group of lipophilic polar carbonyl hydrazones, which includes HNE, was eluted at a flow rate of 0.8 mL/min with methanol water (50:50 vol/vol) followed by a linear gradient to 100 % methanol for a total elution time of 40 min. Absorbance was monitored at 378 nm. The disposable syringes used for sample injection were equipped with a 0.2 µm polyvinylidene difluoride 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. HNE was quantified using authentic pure HNE-DNPH external standards. Identification of HNE in the extracted oils of FF from the 6 fast food restaurants was determined by comparison to the retention time and co-chromatography with pure HNE-DNPH standard. Verification of HNE was also established by liquid chromatography-mass spectroscopy (LC/MS).

Liquid Chromatography-Mass Spectroscopy (LC/MS) Analysis of HNE-DNPH Adduct

Samples of the polar lipophilic DNPH adducts from FF were collected between 32 and 33 min from the HPLC column corresponding to the retention times of pure HNE-DNPH, and the pooled samples were subjected to LC/MS analysis. This analysis was completed by a previously published technique [33]. A 5 µL aliquot of the pooled samples

was injected into a Waters Acquity ultra-performance liquid chromatography system (Milford, MA) and separated by a gradient of mobile phase ranging from water to 95 % aqueous acetonitrile containing 0.1 % formic acid over a 10 min run. LC eluate was introduced into a Waters SYNAPT QTOF mass spectrometer (Milford, MA) for accurate mass measurement and tandem MS (MS/MS) analysis. Capillary voltage and cone voltage for electrospray ionization was maintained at −3 kV and −35 V for negative mode detection, respectively. Source temperature and desolvation temperature were set at 120 and 350 °C, respectively. Nitrogen was used as both the cone gas (50 L/h) and the desolvation gas (600 L/h) and argon as the collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range *m/z* 50–1000) and monitored by the intermittent injection of the lock mass leucine encephalin [$(M - H)^- = 554.2615$ *m/z*] in real time. Mass chromatograms and mass spectral data were acquired and processed by MassLynx software (Waters) in centroid format. The presence of HNE-DNPH in the samples was confirmed by a comparison with the authentic pure standard and MS/MS fragmentation.

Fatty Acid Distribution

Fatty acid distributions in fats extracted from FF from all 6 fast food restaurants were determined by GC of the fatty acid methyl esters (FAME). Approximately 100 mg of extracted fats were methylated using boron trifluoride-methanol (BF_3), as described previously by Metcalf *et al.* [34] prior to analysis by GC. Analysis of FAME was performed on a HP 5830A GC with flame ionization detection and a Carbowax capillary column, 15 m, i.d of 0.53 mm and film thickness of 1.2 IJ (All Tech Econo Cap# 119563, Deerfield, IL, USA).

Helium was used as the carrier gas at a velocity of 70 mL/min as well as the make-up gas at a flow rate of 30 mL/min. FAME dissolved in hexane were injected at split mode of injection. The injector, oven and detector temperatures were 230, 220 and 250 °C, respectively.

Statistical Analysis

Differences between the groups were compared by ANOVA and Tukey's HSD tests. $P \leq 0.05$ was considered as the statistical significant level.

Results and Discussion

In the present experiment, FF from fast food chain restaurants were analyzed for the concentrations of HNE, a toxic secondary oxidation product of heat-treated omega 6 fatty acids such as linoleic acid. This fatty acid is in high concentration in certain vegetable oils used for commercial frying.

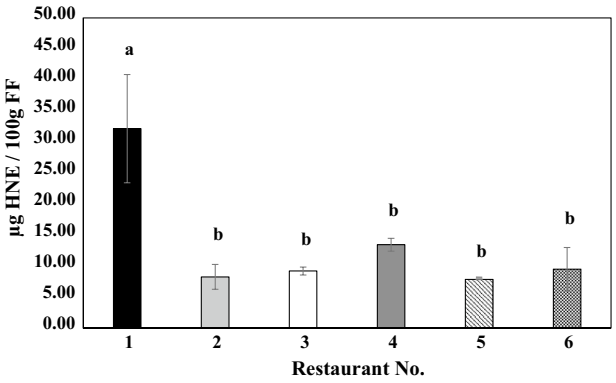

Fig. 1 HNE concentrations in French fries from 6 different local fast food restaurants. Restaurants No. 1, 2, 3, 4, 5 and 6. Results show HNE µg/100 g FF

In the Twin Cities of Minnesota, FF from 6 fast food chain restaurants were purchased at 2 and 3 pm on the same day. The time of purchase was chosen so to have the oils in the restaurants heated for several hours before obtaining the FF. The HNE concentrations found in FF are shown in Fig. 1 and they averaged 13.52 µg HNE/100 g FF. It can be seen in Fig. 1 that FF from restaurant No. 1 contained a significantly higher level, 32.1 µg HNE/100 g FF, than FF from the rest of the 5 restaurants with the lowest level 7.83 µg HNE/100 g FF. Weight percent fat content of the various FF samples from the 6 restaurants is demonstrated in Table 1. The average of fat concentration in FF from the 6 different Twin Cities fast food restaurants was 12.37 %, the range was 11.06–13.70 %. Fat concentration in FF from No. 1 and 3 restaurants were the highest, but the rest of the FF, from restaurants No. 2, 4, 5 and 6, had similar levels (11.06–11.67 %). The difference between the highest and the lowest levels were 2.64 %. Since all the fast food restaurants use commercial mass produced prefried frozen FF from various sources, the variations in fat absorption during frying may be due to some of the differences used in their technologies, such as blanching, air cooling, dewatering, frying, deoiling, freezing and packaging. Variations in the type of potatoes do not seem to differ between prefried frozen FF; they all use Russet potatoes most of the time. The surface area of prefried frozen FF seems to be uniform; therefore the frying time between restaurants may also be involved in causing some of the differences in percentage fat absorptions.

Table 2 shows the concentration of HNE/g extracted fat in FF from the 6 restaurants. FF from restaurant No. 1 had a significantly higher level of HNE/g fat than FF from the 5 other restaurants. Fat extracted from the FF contained an

Table 1 Weight percent of fat in French fries from 6 different local fast food restaurants

Restaurant No.	Extracted fat (%)
1	13.70
2	11.06
3	14.62
4	11.67
5	11.52
6	11.66
Average	12.37

Table 2 HNE concentrations in the extracted fats of French fries from 6 different local fast food restaurants

Restaurant No.	μg HNE/g extracted fat (mean ± SD)	Significant difference ($p < 0.05$)	Average μg HNE/g extracted fat from 6 restaurants
1	2.35 ± 0.64	a	1.06
2	0.74 ± 0.18	b	
3	0.63 ± 0.04	b	
4	1.15 ± 0.09	b	
5	0.68 ± 0.00	b	
6	0.81 ± 0.30	b	

average of 1.06 μg HNE/g of fat, the range was between 0.68 and 2.35 μg HNE/g fat. Since the formation of HNE in an oil is dependent on the temperature and the heating time in addition to its linoleic acid concentration [17, 31], two restaurants were selected to measure the possible changes of HNE concentration in FF purchased at different times of the same day when oils may be heated in the restaurants at different lengths of time. The restaurants selection was based on previously measured fat and HNE concentrations in their FF. Restaurant No. 1 had the highest and restaurant No. 2 had the lowest percentage of fat in its FF and the highest and almost the lowest μg HNE/100 g FF concentration respectively. FF were purchased from both of these restaurants on the same day as follows: restaurant No. 1 at 12 noon, 2, 4 and 6 pm, and restaurant No. 2 at 1, 3, 5, and 7 pm. The delay of 1 h in purchasing times between restaurants No. 1 and 2 were necessary since we wanted all the samples to be collected on the same day. The 2 h differences between the collection of samples from each restaurant was designed to measure the fluctuation of HNE concentrations in FF starting between the noon hours and the early evening. During this period it is believed that most of the FF are purchased by the public. In both of these restaurants the frying times possibly started before the noon hours and may continue with some intervals during the early evening. It is postulated that most of the FF are consumed by the public during the noon and evening hours and therefore during these periods the oils in the frying vessels are heated most continually.

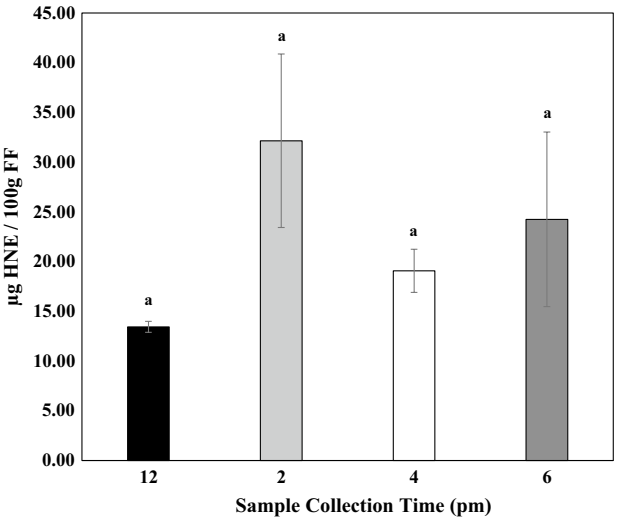

Fig. 2 HNE concentrations in French fries purchased from local fast food restaurant No. 1 at 12, 2, 4 and 6 pm. Results show HNE μg/100 g FF

Results from restaurant No. 1 are shown in Fig. 2 and ranged between 19.07 and 32.15 μg HNE/100 g FF and between 1.07 and 2.35 μg HNE/g fat (Table 3). The average value was 22.23 μg HNE/100 g FF and 1.68 μg HNE/g fat. Results from restaurant No. 2 are shown in Fig. 3 and FF HNE concentrations ranged between 7.47 and 10.21 μg HNE/100 g FF and between 0.73 and 0.94 μg HNE/g fat. Average value was 8.71 μg HNE/100 g FF and 0.82 μg HNE/g fat (Table 3).

It can be seen that in both restaurants, fluctuations took place in HNE concentrations in FF between the different times of purchasing, but these differences were not found to be significant within the same restaurant. It seems that the fluctuations in HNE concentrations in FF during the 6 h time periods, in both restaurants, were caused by the addition of unheated fresh oil which does not contain measurable amounts of HNE [28]. The time-to-time addition of unheated oil, which is replacing the oil absorbed by the potatoes during frying, seems to produce a somewhat steady state situation in HNE concentration in the oil and therefore in FF during the frying period.

It is interesting to note that the average concentration (22.23 μg/100 FF) for FF from No. 1 restaurant was 2.55 times higher than the average concentration (8.71 μg/100 g FF) for FF from No. 2 restaurant. Differences could mainly be due to the selection of the frying oil and the relative amount of heating time before the oils is completely changed in the frying vessel. For comparison, it is noted that this laboratory reported the incorporation of HNE into

Table 3 HNE concentrations in extracted fats of French fries from restaurant No. 1 and 2 obtained at different times of the day

Restaurant No.	Collection time (pm)	μg HNE/g extracted fat (mean ± SD)	Significant difference ($p < 0.05$)	Average μg HNE/g extracted fat from each restaurant
1	12	1.07 ± 0.04	a	1.68
	2	2.35 ± 0.64	a	
	4	1.43 ± 0.16	a	
	6	1.88 ± 0.68	a	
2	1	0.94 ± 0.27	a	0.82
	3	0.74 ± 0.18	a	
	5	0.73 ± 0.09	a	
	7	0.85 ± 0.42	a	

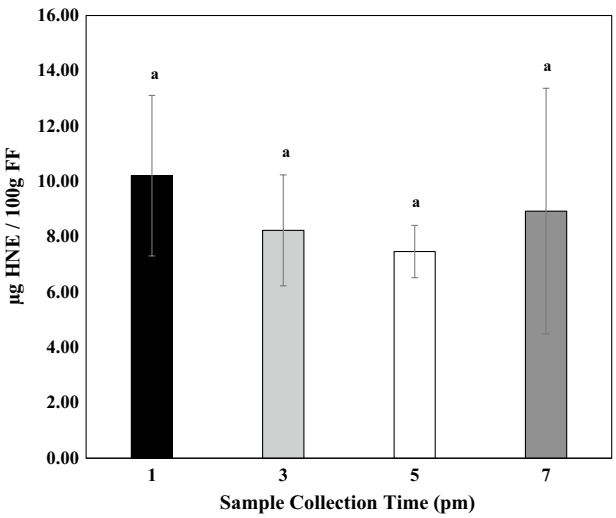

Fig. 3 HNE concentrations in French fries purchased from local fast food restaurant No. 2 at 1, 3, 5 and 7 pm. Results show HNE μg/100 g FF

fried potato from the frying oil. Fresh, not prefried frozen potatoes were fried in soybean oil, preheated for 6 h at 185 °C, and it was found that 4.90 μg HNE incorporated into 100 g fried potato [32]. The level of HNE in the oil extracted from the potato was found to be similar (0.51 μg HNE/g oil) to the level of HNE in the frying oil (0.57 μg HNE/g oil) [32]. In the present study, similar but somewhat higher levels of HNE were found in commercial FF from the 6 fast food restaurants except in No. 1 restaurant. The increased levels may also be due partly to that commercial fast food restaurant use prefried frozen potatoes beside differences in the oil and the heating times.

The fatty acid distributions in the fat extracted from FF from the 6 different fast food restaurants are shown in Table 4. The linoleic acid concentrations from restaurants No. 1, 3, 4 and 5 were similarly high (between 46.8 and 54.9 %). However, FF from restaurant No. 6 contained 35.3 % and from restaurant No. 2 contained only 24.7 %

Table 4 Fatty acid distributions of fat extracted from French fries from 6 different local fast food restaurants

Restaurant No.	Fatty acids (%)				
	Palmitic	Stearic	Oleic	Linoleic	Linolenic
1	12.4	5.9	23.4	52.3	5.9
2	5.0	4.8	60.7	24.7	3.4
3	12.0	5.6	27.5	46.8	8.0
4	10.7	3.4	32.8	49.2	3.1
5	11.3	3.9	25.5	54.9	4.4
6	11.2	8.0	39.3	35.3	4.0

linoleic acid. The type of oils used for frying in the 6 fast food restaurants was not revealed to the authors. In general that most restaurants use vegetable oils or a blend of vegetable oils for frying. The fatty acid distribution show that restaurant No. 2 used a high oleic and low linoleic oil or oils blend, contrary to restaurant No. 1 which used a high linoleic and low oleic acid oil or oil blend. Consequently the average concentration of HNE was much higher in restaurant No. 1 then in restaurant No. 2 where the linoleic acid concentration was low. Interestingly, restaurant No. 5 seemed to use similar blends of oils as No. 1, but its HNE concentration in FF was significantly lower than in restaurant No. 1. This may represent a difference in the length of heating time of the oil in the frying vessel since HNE formation from linoleic acid is temperature and heating time related [17, 31]. The average concentrations of HNE/100 g FF from the 6 restaurants was 13.52 μg, but there was significantly higher HNE concentration, 32.15 μg/100 g FF in FF from restaurant No. 1 than the rest of the 5 restaurants, whose average level was 9.79 μg/100 g FF (Fig. 1). While the daily variations of HNE concentrations in FF within the same restaurant were not significant (Figs. 2, 3), the actual average levels were 2.55 times higher in restaurant No. 1 than in restaurant No. 2, these were 22.23 and 8.71 μg HNE/100 g FF, respectively. It seems that besides the type of oil, the linoleic acid concentrations, some other

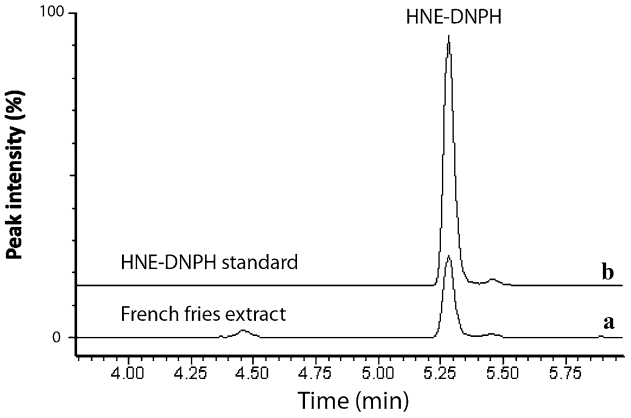

Fig. 4 LC/MS analysis of HNE-DNPH in fat extracted from French fry from local fast food restaurant (**a**), HNE-DNPH standard (**b**)

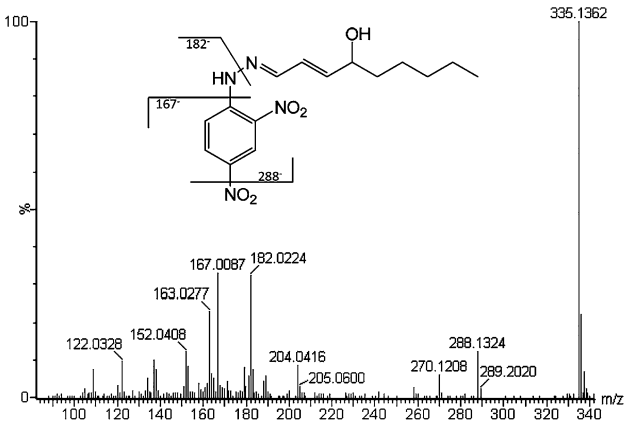

Fig. 5 Structural confirmation: MS/MS analysis of HNE-DNPH fragmentation in fat extracted from French fries from local fast food restaurant

factors were also involved in the production of the higher HNE concentrations. These could be the treatment of frozen FF, heating time of oil in the fryer, frequency of fresh oil addition, antioxidant concentration of oil, and some changes in heating temperature. Palmitic and stearic acids (Table 4) are saturated fatty acids and they are not precursors for HNE, however, linolenic acid is responsible for the formation of another minor toxic α,β-unsaturated hydroxy aldehyde formation in thermally treated oils, namely 4-hydroxy-2-*trans*-hexenal (HHE), which is similar to HNE in structure function and toxicity [17], but this minor compound was not measured in the present study.

Identification of HNE in the extracted fat from FF was made by HPLC comparison to the retention time of pure HNE-DNPH standard and co-chromatography with pure HNE-DNPH as described in the method section [31]. Further verification of the identity of HNE extracted from the various FF samples was confirmed using LC/MS analysis and by fragmentation (Figs. 4, 5).

Besides the endogenous formation of HNE, this toxic compound can be found in various foods due to oxidation of dietary PUFA [35–37]. It is mentioned in the introduction that this laboratory reported the formation of HNE in heat treated PUFA oils [30, 31] and its incorporation into fried food [32]. It seems that one could be exposed, through various oxidized lipids in foods, to significant concentrations of HNE, which could be a toxicological concern.

The oral toxicity of HNE has been reported by several investigators. Acute toxicity of HNE from a single dose (10–1000 mg/kg BW) was observed by Nishikawa *et al*. [38]. They observed liver cell necrosis at all levels of treated animals after 14 days and death from a dose of 1000 mg/kg after a few hours, with kidney tubular necrosis. HNE specific propano adducts in the forestomach DNA in rats treated with one oral dose of HNE (500 mg/kg BW) was reported by Wacker *et al*. [39]. Kang *et al*. recently reported the hepatoxicty and nephrotoxicity produced by HNE following 4 weeks of oral dose from 0.5 to 12.5 mg/kg BW [40]. A recent study reported the *in vivo* fate of oral administration of HNE using radioactive isotopes [41]. They have shown the metabolism and the incorporation of dietary radioactive and stable HNE isotopes into various major organs including the liver, kidney and six other tissues, among them the brain and the heart.

Conclusion

In this study, it has been demonstrated that HNE, a toxic aldehyde which is produced during the heat treatment of frying oils, was incorporated into commercially available FF purchased from 6 fast food restaurants in the Twin Cities, MN. It is believed that frequently consumed fried foods containing considerable amounts of HNE, which is readily absorbed from the diet and incorporated into tissues, may be a public health concern since HNE toxicity has been related to a number of common pathological conditions in the literature.

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