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ORIGINAL PAPER

The Toxic Aldehyde, 4-Hydroxy-2-*trans*-nonenal (HNE) Formation in Natural and Imitation Mozzarella Cheeses: Heat Treatment Effects

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Abstract The formation of 4-hydroxy-2-*trans*-nonenal (HNE), a toxic aldehyde formation, was investigated in heat treated imitation Mozzarella cheeses which are made with vegetable oils and in natural Mozzarella cheeses which contain dairy fats. The cheeses were heat treated at 204 °C for 30 and 60 min, and at 232 °C for 15 and 30 min. The HNE formations were much higher in imitation cheeses than in natural cheeses due to both heat treatments. Average HNE concentrations in imitation cheeses, after 30 min of heat treatment at 204 °C, were 110.3 ng HNE/g cheese and it increased to 877.1 ng HNE/g cheese when the temperature was raised to 232 °C. In natural cheeses, the average HNE concentration was much lower only 13.4 ng HNE/g cheese after 204 °C heat treatment for 30 min and it increased only to 182.8 ng HNE/g cheese using 232 °C. Since imitation cheeses are made with vegetable oils which contain much higher levels of linoleic acid, a precursor for HNE, than dairy fat, it is not surprising that heat-induced lipid peroxidation results in increased HNE formation in imitations cheeses compared to dairy fat containing cheeses, which are low in linoleic acid.

Keywords 4-Hydroxy-2-*trans*-nonenal (HNE) · Toxic aldehyde formation · Lipid peroxidation · Cheeses

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Introduction

4-Hydroxy-2-*trans*-nonenal (HNE) is known as a secondary lipid peroxidative product of n6 fatty acids, including linoleic acids [1, 2]. It is a toxic compound and has been reported to be related to atherosclerosis, LDL oxidation [3, 4], stroke, Parkinson's, Alzheimer's and Huntington's diseases [5–10], disease of the liver [11] and other diseases.

Because the toxicity of HNE, (it may induce various health problems in humans), it is important to investigate its formation in various foods due to heat treatment.

HNE concentrations ranging from 3.75 to 95.20 μmol/kg were reported in ham, bacon and sausages after smoking [12] and this laboratory reported 42.5 μg HNE/g oil in soybean oils after heating at 185 °C for 6 h in the presence of air bubbling [14].

HNE is derived from the peroxidation of n6 fatty acids mostly from linoleic acid [13, 14]; this implies that oils containing higher concentrations of n6 fatty acids produce more HNE due to heat treatment than oils low in n6 fatty acids. Vegetable oils, including corn and soybean oils, contain high percentages of n6 fatty acids while animal fats, including milk fat, contain much lower quantities of n6 fatty acids compared to vegetable oils. Soybean oil contains about 54 %, corn oil about 60 % and sunflower oil about 68 % linoleic acid.

Animal fats such as lard and beef tallow and milkfat contain less than 10 % of linoleic acid [15]. Therefore HNE formation, due to heat treatment, is expected to be higher in vegetable oils than in animal fats. Imitation Mozzarella cheese is replacing natural Mozzarella cheese in many instances to lower the cost. Imitation cheeses are prepared with vegetable oils, therefore HNE formation due to heat treatment is expected to be higher than in natural cheeses. In the present study, HNE formation was

examined in two imitation Mozzarella cheeses and in two natural Mozzarella cheeses exposed to various temperatures and heating times.

Materials and Methods

Chemicals and Materials

Two different low-moisture part-skim Mozzarella cheeses were purchased from a local store (Roseville, MN) and two different imitation cheeses were provided by Dr. Metzger (South Dakota State University). Hexanal and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma (ST. Louis, MO). HNE standard was obtained from Cayman Chemical Co. (Ann Arbor, MI). Hydrochloric acid was purchased 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). Thin layer silica gel plates (20 × 20 cm, 250 μm layer, Al SIL G) for chromatography (TLC) and No. 1 filter paper were purchased from Whatman Ltd. (Kent, England).

Preparation of DNPH Reagent

The reagent was prepared daily, according to the method of Seppanen and Csallany [16], by combining 10 mg DNPH, recrystallized three times from methanol, with 20 mL 1N 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.

Preparation of Hexanal-DNPH Standard

Pure hexanal-DNPH was prepared by the method developed in this laboratory [17]. The mixture of 800 mg of recrystallized DNPH, 80 mL methanol, 2 mL 6N hydrochloric acid and 1 mL reagent grade hexanal, was heated for 10 min at 60 °C, and placed into an ice bath for crystallization. The hexanal-DNPH crystals were filtered through Whatman No. 1 filter paper and recrystallized two more times from ~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 under N_2 gas.

Heat Treatment and Solvent Extraction of Natural and Imitation Cheeses

One hundred grams of each cheese sample, in duplicate, was placed on the surface of aluminum foil on a petri dish in a preheated oven and heated for 0, 30, and 60 min at 204 °C for 0, 15 and 30 min at 232 °C. Each heated cheese sample was mixed with 200 mL hexane and blended in a laboratory blender for about 10 min, filtered through a Whatman No. 1 filter paper and the hexane filtrate was collected. The residue was mixed with 100 mL hexane and blended for 5 min and after filtration the hexane was collected. The combined hexane extracts were used for HNE analysis.

Preparation of DNPH Derivatives of HNE from Heat-Treated Cheese Samples

The preparation of DNPH derivatives of HNE from heat-treated cheese samples was conducted as described by the detailed method of Seppanen and Csallany [16]. Briefly, 10 ml of hexane extracts, from the heat treated or the control unheated cheese samples, were reacted overnight with 5 mL freshly prepared DNPH reagent at room temperature. The DNPH derivatives were extracted with 10 mL of 75:25 (v/v) methanol: water, from the hexane extract and the DNPH reagent reaction mixture. This extraction procedure was repeated two more times and the 75:25 (v/v) methanol:water extracts were combined. From the combined methanol:water extracts, the DNPH derivatives were extracted four times with 10 mL of dichloromethane and the combined dichloromethane extracts were evaporated under N_2 gas to about 1 mL.

Thin Layer Chromatography (Pre-Separation of DNP Derivatives)

The whole concentrated DNPH dichloromethane extract, about 1 mL, was applied to two TLC plates and developed with dichloromethane. On the TLC plates the polar and nonpolar aldehydes, and osazone regions (Rf = 0.25, 0.75 and 0.50, respectively) were separated. The polar aldehyde fraction region which included the HNE DNPH derivative was cut from the TLC plate into small pieces and extracted three times with 10 mL of methanol. The combined methanol extracts were centrifuged at 1360×*g* for 15 min to remove residual silica. The clarified supernatant fractions were combined and concentrated under N_2 gas to the exact volume of 1 mL. Aliquots of 50 μL were injected into the HPLC column in duplicate for the HNE analysis.

HPLC Analysis

The HPLC system consisted of a solvent delivery system (9050, Varian, Walnut Greek, CA), a sample injector (712 WISP, Waters, Milford, MA), Ultrasphere ODS HPLC column (5 × 4.6 mm, 25 cm), (Beckman, Fullerton, CA) and a UV–VIS detector (9010, Varian). The integration of

peaks shown on chromatograms was conducted by Star Workstation software (Varian, Walnut Greek, CA) installed on the computer connected to the UV–VIS detector. For polar aldehyde analysis, an isocratic elution with 55:45 (v/v) methanol: water was used for 10 min, a gradient elution increased to 100 % of methanol over 20 min and held at 100 % of methanol for an additional 10 min. The absorbance of the polar aldehydes was monitored at 378 nm. Rejection of peaks was set to 2,000 area counts. The detection limit of the HPLC system was previously determined to be 12 ng hexanal (measured as its DNPH derivatives) per 50 μL injection.

The peaks of the HNE from the HPLC chromatograms were identified by comparing the retention time of the isolated HNE with the retention time of the peak of pure standard. Identification of the isolated HNE was also carried out by co-chromatography with pure standard in three different polarity solvent systems as described in the method developed in this laboratory [17]. Solvent systems used for co-chromatography for DNPH derivatives of HNE were methanol: water, 50:50, 55:45 and 60:40 (v/v). The percent recovery of added pure aldehyde-DNPH to the isolated sample from the co-chromatography was calculated from the increase in the peak area due to the added pure HNE-DNPH to the original peak area of the isolated HNE-DNPH from the heat treated cheese samples. HNE-DNPH derivative was quantified by comparing their peak area with the area of known amount of pure hexanal-DNPH standard and expressed as μg hexanal equivalent/g cheese. One ng of pure hexanal was measured to be equivalent of 4,500 area counts by the HPLC system used in the experiment. The isolated HNE concentrations were calculated from the hexanal equivalents based on the molecular weight differences between hexanal and HNE. The HNE concentrations were expressed as ng HNE/g cheese. In total, two different natural and two different imitation cheeses, in duplicate, were analyzed. The HPLC injections were in duplicate for each duplicate cheese sample.

Statistical Analysis

All measurements were conducted in duplicate. The average and standard error of the mean (SEM) of HNE concentration were calculated for each heating time period, and for each cheese sample.

Results and Discussion

The formation of HNE in natural low-moisture part-skim Mozzarella and imitation Mozzarella cheeses were investigated due to heat treatment at 204 °C up to 60 min and at 232 °C up to 30 min. These temperatures are commonly used in home and commercial applications. Typical HPLC chromatograms of polar aldehydes, including HNE, from heat treated natural cheese A and imitation cheese B are shown in Fig. 1. For detection of HNE peak of samples, the retention time (Rt) on the HPLC column was compared with pure HNE standard and they were both shown at 28.9 ± 0.4 min Rt. Co-chromatography was conducted for identification in three different polarity solvent systems. Recoveries from co-chromatography using three different polarity solvent systems were the following: 88.3 % using methanol:water 50:50 (v/v), 91.9 % using 55:45 (v/v) and

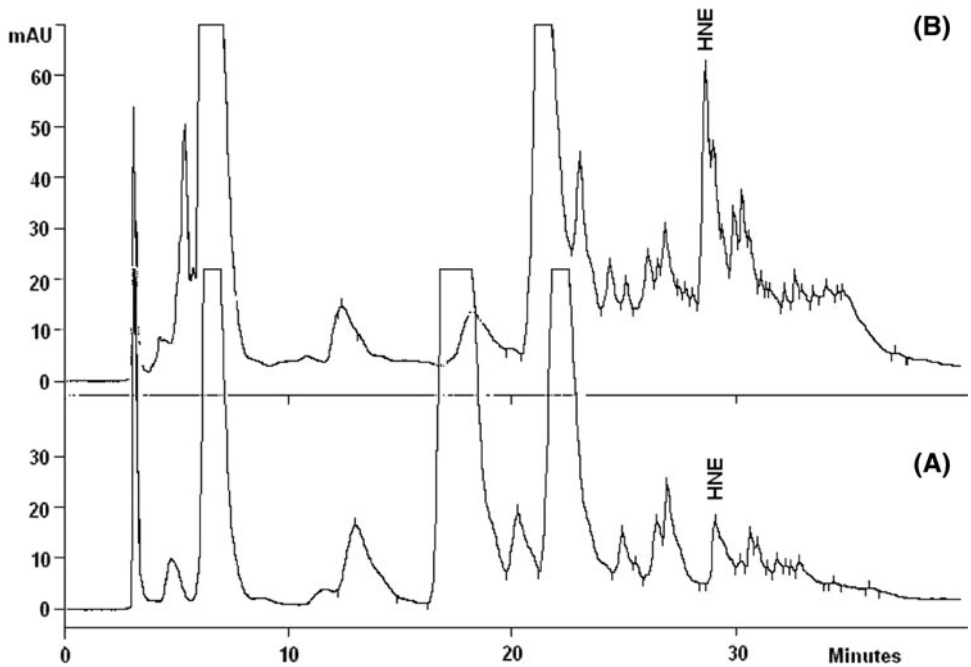

Fig. 1 Typical HPLC chromatograms of polar aldehydes, including 4-hydroxy-2-*trans*-nonenal (HNE), from natural low-moisture part-skim Mozzarella cheese heat treated at 232 °C for 30 min (**a**) and imitation Mozzarella cheese heat treated at 204 °C for 60 min (**b**)

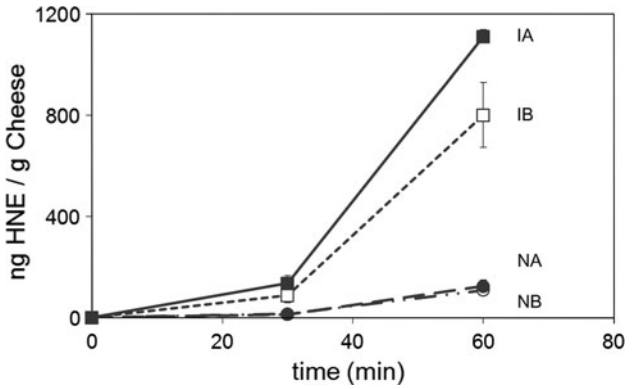

Fig. 2 HNE concentration in natural low-moisture part-skim Mozzarella and imitation Mozzarella cheese samples heat treated at 204 °C for 0, 30 and 60 min. Imitation cheeses IA, IB and (from two different sources) and natural low-moisture part-skim Mozzarella cheeses NA and NB

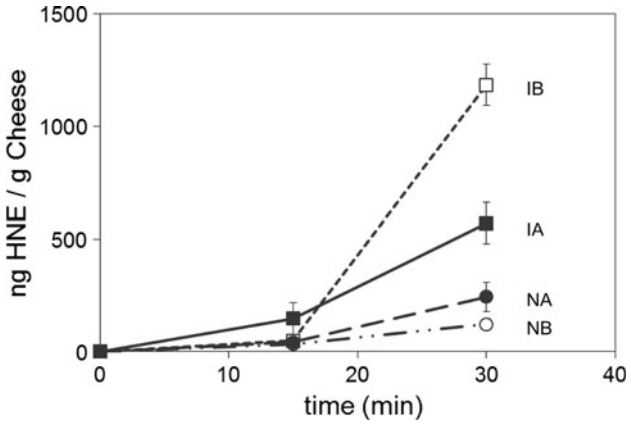

Fig. 3 HNE concentration in natural low-moisture part-skim Mozzarella and imitation Mozzarella cheese samples heat treated at 232 °C for 0, 15 and 30 min. Imitation cheeses IA and IB and natural low-moisture part-skim Mozzarella cheeses NA and NB

94.6 % using 60:40 (v/v). As shown in Fig. 1 the relative area of HNE peak was much larger in imitation cheese (b) than on natural cheese (a).

Figure 2 illustrates the HNE concentrations in imitation and natural cheeses treated at 204 °C for 0, 30 and 60 min. HNE concentration increased in all cheeses with the length of heating time. The imitation cheeses reached higher HNE concentration than natural cheeses already after 30 min and much higher levels after 60 min of heat treatment. Cheese samples A and B are indicating different sources for cheese samples and duplicate or triplicate HNE analyses. For imitation cheese samples IA and IB, HNE concentration after 30 min heating time were 86.5 and 134.0 ng HNE/g cheese, respectively. For natural cheeses NA and NB, HNE concentrations were 14.2 and 12.6 ng HNE/g cheese, respectively. After 60 min of heat treatment the HNE concentration increased 8–10 times higher in imitation cheeses compared to natural cheeses. Imitation cheese IA was 1,120 and IB was 780 ng HNE/g cheese. Natural cheese NA and NB average was 116 ng HNE/g cheese. The high increase of HNE concentration in imitation cheeses was expected since these cheeses are made with vegetable oils which are high in linoleic acid, a precursor of HNE [14]. This laboratory has previously reported higher HNE formation in high linoleic vegetable oils than in butter oil due to heat treatment [18]. Figure 3 shows the HNE concentration in imitation cheeses and natural cheeses treated at higher temperature at 232 °C up to 30 min. HNE concentrations at this temperature rapidly increased after only 15 min heating time indicating the accelerated rate of heat induced peroxidation and decomposition of polyunsaturated fatty acids (PUFA), including linoleic acid which is responsible for the increase of HNE in the samples. HNE concentrations increased much more in the imitation cheeses than the natural cheeses at this

Fig. 4 Average HNE concentrations, μg HNE/g cheese, in natural low-moisture part-skim Mozzarella and imitation Mozzarella cheeses heat treated at 204 and 232 °C for 30 min

temperature resulting in a similar pattern as found before at 204 °C. Imitation cheese IA and IB showed very large increases of HNE concentration, at 30 min compared to concentration at 15 min of heat treatment. HNE concentrations in natural cheeses NA and NB increased to much lower levels at 30 min, compared to concentrations at 15 min heat treatment. The difference in HNE concentration between IA and IB samples at 232 °C, due to 30 min heat treatment, is possibly due to the different types of vegetable oils used for the manufacture of these imitation cheeses. It seems that IB sample fatty acid composition was different from IA sample, most probably the linoleic acid concentration was in higher level in IB sample since this N6 fatty acid is a precursor of HNE. Figure 4 shows the average HNE concentrations of the two imitation and the two natural cheeses at 204 or 232 °C after heat treatment for 30 min and illustrates the effect of temperature

difference on fatty acid peroxidative degradation resulting in HNE formation. As shown on the figure, HNE concentrations were much higher in both imitation and natural cheeses treated at the higher temperature of 232 °C than at 204 °C. Average HNE concentrations in imitation cheeses were after 30 min heat treatment at 204 °C, 110.3 ng HNE/g cheese and it increased to 877.1 ng HNE/g cheese when the temperature was raised to 232 °C. In natural cheeses, the average HNE concentration was much lower, only 13.4 ng HNE/g cheese after 204 °C heat treatment for 30 min and it increased to only 182.8 ng HNE/g cheese using 232 °C. Since imitation cheeses are made with vegetable oils which contain much higher levels of linoleic acid, a precursor for HNE, than dairy fat it is not surprising that heat induced lipid peroxidation results in increased HNE formation in imitations cheeses compared to dairy fat containing cheeses, which are low in linoleic acid. The large increase of HNE formation at higher temperature agrees with the result previously reported for high linoleic acid vegetable oil and butter by this laboratory [18]. Results showed significant temperature dependence in the oxidative degradation of fatty acids and therefore the increased formation of HNE.

Conclusions

The formations of HNE, a toxic aldehyde, were found to be in much lower concentrations in natural low-moisture part-skim Mozzarella cheeses which contain dairy fats than in imitation Mozzarella cheeses containing vegetable oils due to heat treatments for 15, 30 and 60 min at both 204 and 232 °C. The higher linoleic acid concentration in heat treated imitation cheeses compared to heat treated natural cheeses seems to be responsible for the increase in HNE formations in imitation cheeses. Based on the present results of this study it is suggested that all cheeses, and especially imitation cheeses and imitation cheese containing foods, should be heated at the lowest possible temperatures and the shortest heating time, to lower HNE a toxic aldehyde formation. Since this aldehyde has been shown to be readily absorbed from the diet into the gut and implicated in various pathological conditions, the chronic consumption of this toxic compound could be negatively related to human health. However, because to our knowledge no study has yet published on the health related effects of dietary long term continuous HNE ingestion from food, more research is needed to establish its toxic effect.

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