4-hydroxynonenal in foodstuffs: heme concentration, fatty acid composition and freeze-drying are determining factors

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Short refereed paper

4-Hydroxynonenal in foodstuffs: heme concentration, fatty acid composition and freeze-drying are determining factors

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4-Hydroxynonenal (HNE) is a product of lipid peroxidation. It has been often used as a biomarker of endogenous lipid peroxidation and its concentration is increased in several diseases. But HNE is not only formed during lipid peroxidation occurring in the body. Some authors have shown that it is also present in oxidized oils and in meats. The aim of this study is to compare the effect of food composition (heme iron, fatty acid composition) or freeze-drying on HNE formation in foodstuffs. The methodology is based on extraction/purification procedure followed by HPLC separation with UV detection. As HNE is chemically very reactive and binds easily to proteins, we used radiolabeled HNE to calculate extraction efficiency, so total HNE can be estimated as only free HNE can be measured. The concomitant presence of both heme iron and omega 6 fatty acids, such as linoleic acid, is important for HNE formation in foodstuffs. Freeze-drying increases this formation.

Keywords: 4-Hydroxynonenal, foodstuffs, heme, fatty acids, freeze-drying

Introduction

Oxidative stress and resulting lipid peroxidation are supposed to be involved in the pathogenesis of numerous diseases including inflammatory, cardiovascular and neurodegenerative diseases and cancer. 4-Hydroxynonenal (HNE), a major secondary oxidation product of lipid peroxidation of omega 6 fatty acids present in biological membranes in cells and tissues, is believed to be a 'second messenger of oxidative stress' because it may also be involved in the pathogenesis of these diseases. For a long time, this compound has been considered only as a mere lipid peroxidation derived compound, with genotoxic and cytotoxic properties. More recently, it has also been shown to play a real biological role, acting as a signaling molecule under normal and pathological conditions, particularly in cell cycle regulation.[1,2] The literature concerning HNE has been focused mainly on its endogenous formation and its consequences. However, several authors have shown that it is present in various foodstuffs, including oils and meats and some authors have proposed that this compound would be useful as a marker of food quality as far as rancidity is concerned.[3,4] In the present study, we measured HNE in various meats and in cooked cured pork products but also in rat diets formulated to contain different amounts of heme iron. We developed a methodology using radiolabeled HNE to quantify extraction recovery. The effect of fatty acid composition, heme content and of freeze-drying are discussed, together with the consequences of HNE in foodstuffs on human health.

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Materials and Methods

Chemicals

4-HNE was purchased in its protected form (4-HNE-dimethylacetal) from Alexis Corp. and hydrolyzed with HCl 1 mM just before use. [4-^{3}H]-HNE diethylacetal was synthesized at CEA, Service des Molécules Marquées CEN (Saclay, France) according to a method developed in the laboratory.[5] All solvents were HPLC

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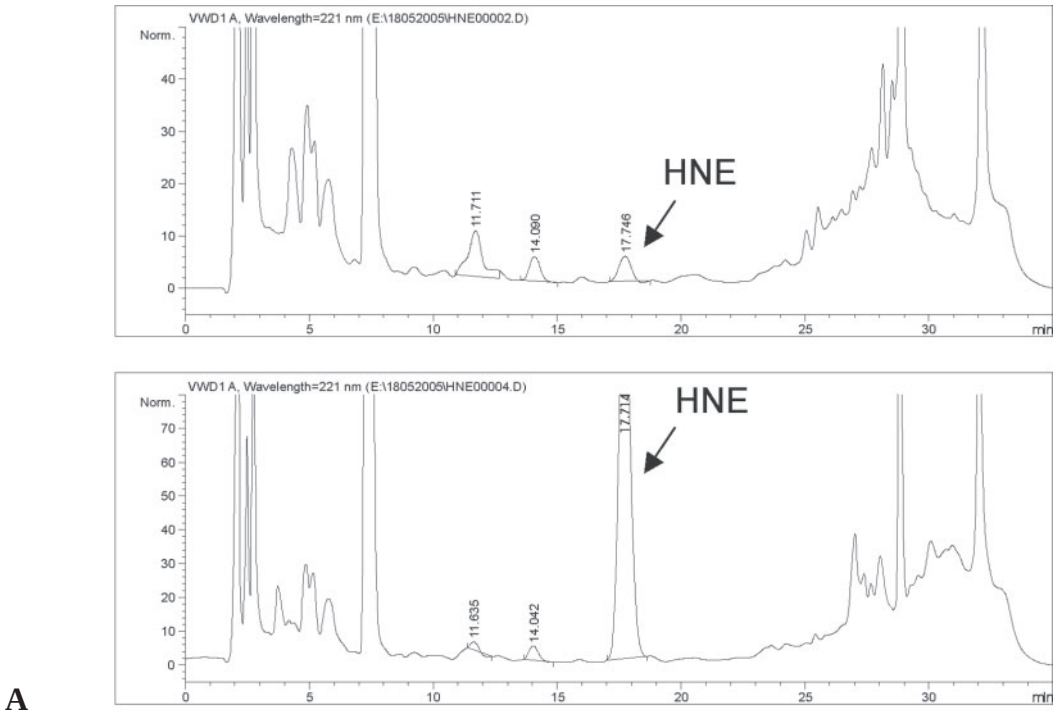

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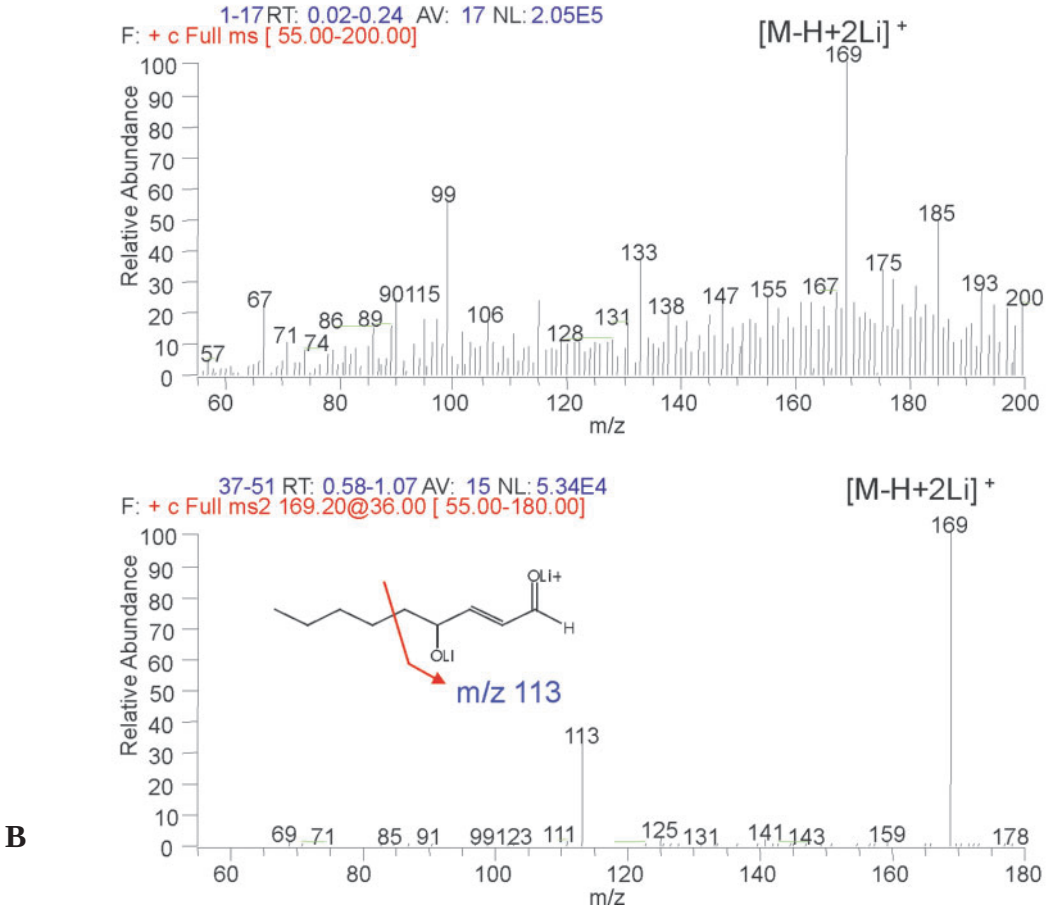

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Fig. 1. (A) HPLC analysis of blood sausage extract. Top: blood sausage sample analyzed for HNE; Bottom: *idem* + HNE standard. (B) Mass spectra of HNE extracted from blood sausage.

grade. Ethyl acetate, chloroform, methanol and glyceryl triheptadecanoate were obtained from Sigma.

Samples

Meat samples (chicken breast without skin and beef ribs) and cooked or cooked cured pork products (pork blood sausage with meat pieces, liver pâté, ham and salami) were purchased in a local supermarket. They were cut into small pieces and the fatty parts were removed.

Rat diet samples were homogenized in water directly. These rat diets were based on a modified AIN-76 diet in a powdered form. They were formulated to contain varying concentrations of heme as hemoglobin or myoglobin by the addition of freeze-dried beef, chicken or black pudding at 60% meat of the total diet, while the control diet contained only casein as protein source.[6] They all contained 5% safflower oil. For the study of freeze-drying, an aliquot of each sample was freeze-dried in a lyophilizer and stored at –20°C until analysis.

Heme assay

The amount of heme in meat was determined according to Van den Berg *et al.*[7] Salami and ham were analyzed for heme as hematin by the method of Hornsey.[8]

Measurement of total fatty acid methyl ester molecular species (FAME) by gas-liquid chromatography

Samples were weighed and then crushed using an Ultra-Turrax in 2 ml of methanol/5 mM EGTA (2:1, v/v). Aliquots corresponding to an equivalent of 0.5 mg of tissues were evaporated, the dry pellets were dissolved in 0.5 ml of NaOH (0.1 M) overnight and proteins were measured with the Bio-Rad assay. Lipids corresponding to an equivalent of 1 mg of tissue were extracted in chloroform/methanol/water (2.5:2.5:2.1, v/v/v), in the presence of the internal standard glyceryl triheptadecanoate (1 µg). The chloroform phase was filtered over glass wool and evaporated to dryness. The lipid extract was transmethylated with 1 ml of acetyl chloride in methanol (1:20, v/v) for 90 min at 55°C. After evaporation to dryness, the FAMEs were extracted with 2 ml of hexane in the presence of 2 ml of water. The organic phase was evaporated to dryness, and dissolved in 50 µl of ethyl acetate. Aliquots of FAME (1 µl) were analysed by gas-liquid chromatography[9] on a 5890 Hewlett Packard system using a Famewax RESTEK fused silica capillary column (30 m x 0.32 mm i.d, 0.25 mm film thickness) and equipped with a flame ionisation detector. Oven temperature was programmed from 110°C to 220°C at a rate of 2°C per min and the carrier gas was hydrogen (0.5 bar). The injector and the detector were set at 225°C and 245°C, respectively.

4-HNE analysis

Food or rat diet samples (1–2 g) were homogenized in 30 ml water with an Ultra-Turrax for 2 min and then centrifuged (10,000 rpm/10 min). The residue was homogenized and centrifuged again. The supernatants were pooled and lipophilic compounds were extracted twice with 60 ml of dichloromethane. Organic phases were evaporated under reduced pressure. The extract was delipidated using a partition between 16 ml isooctane saturated acetonitrile and 4 ml acetonitrile saturated isooctane. Acetonitrile was evaporated under reduced pressure and the dry extract containing HNE was dissolved in 500 µl acetonitrile/water (50/50, v/v) to be further analyzed by HPLC.

The HPLC system was an Hewlett-Packard pump HP1100 equipped with a reverse phase ODS2 Spherisorb column (5 µm, 10 x 4.6 mm). The flow rate was 1 ml/min. 4-HNE detection was achieved with an UV detector set at 221 nm (Fig. 1A) and radiolabeled HNE was detected using an online radioactivity detector Flo-One A500. A standard curve was used to quantify HNE. In order to calculate the recovery of HNE in the samples, tritiated HNE was added before the extraction and radioactivity was analyzed by radio-HPLC. The presence of HNE was confirmed by mass-spectrometry analysis (Fig. 1B).

Results and Discussion

Several methods have been previously developed to measure HNE, with or without a derivatization step.[3,4,10] In the present study, we chose to measure HNE directly but we used radiolabeled HNE, added before the homogenization step, in order to evaluate HNE that would be bound to the food matrix. Actually, HNE is a very reactive chemical compound, that can make covalent links to various nucleophilic compounds, such as DNA bases or some amino-acids. The effect of the food matrix can change depending on the food itself, as recovery with blood sausage reaches 60% while recovery with chicken meat drops to 12% (results not shown). As the covalent link between HNE and the macromolecules may be reversible, it seems important to be able to measure ‘bound’ HNE and not only ‘free’ HNE.

HNE and heme concentration

Rat diets were formulated to maximize, with the use of safflower oil which is rich in the polyunsaturated fatty

Table 1. Iron, heme and 4-hydroxynonenal contents in rat diets and meat samples

	Iron (mg/kg)	Heme (mg/kg)	HNE (mg/kg)
Rat diets			
Control diet	140	ND	0.14
Chicken diet	140	ND	0.79
Beef meat diet	140	220	3.31
Black pudding diet	950	5900	66.40
Meat samples			
Chicken meat	10	ND	ND
Beef meat	20	180	ND
Liver pâté	240	210	0.11
Blood sausage (I)*	130	1040	12.51

ND, not detectable; HNE, 4-hydroxynonenal.
*Blood sausage (I) Table 1 and (II) Table 2 came from different suppliers.

acid (PUFA) linoleic acid and poor in natural antioxidants, the peroxidative processes linked to the presence of oxidant compounds (iron or heme iron) in order to study the colon cancer promoting properties of these diets,[6] as lipid peroxidation may be involved in colon carcinogenesis. All those diets, were balanced for iron with iron citrate except the black pudding diet in which black pudding is very rich in iron as heme.

Heme iron, rather than 'free' iron is associated with HNE formation in rat diets and in foodstuffs (Table 1). Hemoglobin and myoglobin are rich in histidine. HNE easily forms adducts with histidine, which can affect the redox stability of the heme proteins by increasing their oxidation status, thereby increasing lipid oxidation and producing more HNE.[11,12] As rat diets are rich in linoleic acid, which gives HNE when oxidized, the phenomenon is very important with black pudding diet which amounts 66 ppm of HNE.

It is important to note that heme and especially HNE content may vary dramatically between samples of meat and particularly cooked and processed meat. In the present study, blood sausage HNE content varied more than 15 times between the two different samples coming from different suppliers (Tables 1 and 2, blood sausages I and II). This can be explained by a different composition, including NaCl[13] and antioxidant content, or different storage conditions (duration, temperature, *etc*.).[14] Some authors have already reported such variability in HNE concentration in smoked ham.[15]

HNE, freeze-drying and fatty acid composition

Freeze-drying increases HNE formation, especially in foodstuffs containing nitrosylated heme which is present in cured products (Table 2). This can be explained by the low water activity *(a_w)* found after freeze-drying. Some authors reported that freeze-dried beef was more stable to oxidation at an a_w of 0.27 than at a lower a_w.[16]

Results from Table 2 show that both heme and omega 6 fatty acid concentrations are important for HNE formation. Omega 6 PUFAs are associated with HNE formation because HNE comes from omega 6 fatty acid oxidation. Chicken meat that contains no detectable heme and middle concentrations of both linoleic and arachidonic acids has no detectable HNE formation. Beef meat that contains low omega 6 fatty acids and middle heme concentration has no detectable HNE formation either, even when freeze-dried. On the contrary, ham and salami that have a high concentration of linoleic acid and a middle concentration of heme show very significant HNE formation (more than 6 ppm), but only after freeze-drying. Blood sausage that has a high concentration of both heme and linoleic acid, shows a significant formation of HNE (2.4 ppm), even without freeze-drying (0.82 ppm). So the mechanisms involved in HNE formation in foodstuffs surely involve heme iron as pro-oxidant, and omega 6 fatty acids as precursors; probably other compounds such as antioxidants could decrease this formation.

Consequences

Consumption of red meat and blood sausage is associated with increased colorectal cancer risk as shown by

Table 2. Heme, omega 6 fatty acid and 4-hydroxynonenal contents in fresh and freeze-dried meat samples

Meat	Heme	Omega 6 fatty acids (nmol/mg)		HNE (mg/kg)	
	(mg/kg)	Linoleic acid	Arachidonic acid	Freeze-dried	No treatment
Chicken meat	ND	36.2	22.2	ND	ND
Beef meat	180	20.6	7.1	ND	ND
Ham	80	107.6	3.7	6.19	ND
Salami	88	221.0	8.5	6.73	ND
Blood sausage (II)*	540	270.2	7.6	2.44	0.82

ND, not detectable; HNE, 4-hydroxynonenal.
*Blood sausage (I) Table 1 and (II) Table 2 came from different suppliers.

epidemiological studies.[17,18] Animal studies show that heme promotes preneoplastic lesions in cancer initiated rats.[6] Omega 6 fatty acids also promote preneoplastic lesions in such rats, while omega 3 fatty acids have a rather protective effect.[19] As HNE is one of the major oxidation product of omega 6 fatty acids and comes only from omega 6 fatty acid oxidation, one can hypothesize that HNE may play a role in colon cancer development. Very recently, we have shown that fecal waters from rats fed heme and an omega 6 rich diet could be more cytotoxic, when added to the culture medium, to mouse colon wild-type cells as compared to premalignant cells that had already been mutated for Apc gene, thereby conferring a selective advantage to those mutated cells.[20] Apc mutation is an early event in human colon cancer development. HNE has been found in those fecal waters. Moreover, when added under the same conditions to the culture medium, HNE mimics the effects of those fecal waters.[20] HNE may then contribute to the effect of heme iron and omega 6 fatty acids on colon cancer development. The effect other lipid peroxidation products, such as 4-hydroxyhexenal, that comes specifically from omega 3 fatty acid oxidation, is now under investigation.

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