

A Diet with 3% of Energy from a Mixture of Omega-3 Fatty Acids Significantly Increases *in Vivo* Lipid Peroxidation in Postmenopausal Women

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How to cite this paper: Csallany, A.S., Ainslie-Waldman, C.E., Young, L.R., Chen, C., Kurzer, M.S. and Raatz, S.K. (2016) A Diet with 3% of Energy from a Mixture of Omega-3 Fatty Acids Significantly Increases *in Vivo* Lipid Peroxidation in Postmenopausal Women. *Food and Nutrition Sciences*, 7, 1099-1111.

<http://dx.doi.org/10.4236/fns.2016.712105>

Received: September 2, 2016

Accepted: October 16, 2016

Published: October 20, 2016

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Abstract

Dietary omega-3 (n – 3) polyunsaturated fatty acids (PUFA) are recommended by public health organizations to reduce the risk of cardiovascular disease, and several epidemiological studies have suggested there is an inverse association between n – 3 intake and human cancers. However, n – 3 are susceptible to an increase in lipid peroxidation in the human body. As part of a crossover dietary intervention study of a diet (20% of energy from fat) with or without an additional 3% of energy from a mixture of n – 3 (with 5.36 g α -linolenic acid and 1.45 g eicosapentaenoic acid and docosahexaenoic acid per 2000 kcal per day), we measured total *in vivo* lipid peroxidation in healthy postmenopausal women (n = 15). Our results indicated that the diet with 3% of energy from n – 3 significantly increased the urinary concentrations of total polar lipophilic aldehydes and related compounds produced via lipid peroxidation ($p < 0.05$) as well as the α , β -unsaturated hydroxy aldehydes 4-hydroxy-2-*trans*-hexenal ($p < 0.05$) and 4-hydroxy-2-*trans*-decenal ($p < 0.05$) compared to the diet with less than 1% of energy from n – 3. This is also the first study to document the presence of 4-hydroxy-2-*trans*-decenal in the urine of individuals consuming n – 3. These results demonstrate that an increase in 3% of energy from dietary n – 3 increases *in vivo* lipid peroxidation.

Keywords

Lipid Peroxidation, n – 3 Fatty Acids, Human, HHE, 4-Hydroxyhexenal, HDE, 4-Hydroxydecenal, α , β -4-Hydroxyaldehydes, HPLC, MS

1. Introduction

Lipid peroxidation is an autocatalytic, free radical-induced process by which polyunsaturated fatty acids (PUFA) are degraded into lipid hydroperoxides and reactive lipophilic aldehydes and ketones. This process can occur in food products as well as in biological membranes, where the production of lipophilic α , β -unsaturated hydroxyaldehydes such as 4-hydroxy-2-*trans*-hexenal (HHE) and 4-hydroxy-2-*trans*-nonenal (HNE) may result in cellular damage or death [1] [2]. The work of Esterbauer and colleagues established that such 4-hydroxyalkenals were highly reactive and could bind with most amino acids, and that HNE had been implicated in the etiology of atherosclerosis, diabetes, and neurodegenerative diseases [2]-[4]. In addition to vitamin E deficiency and impaired endogenous antioxidant systems, a diet high in PUFA would be expected to increase total lipid peroxidation within an organism [5]-[9]. Previous research from our laboratory demonstrated that heating methyl linolenate, formed from the omega-3 ($n - 3$) PUFA α -linolenic acid (ALA, 18:3 $n - 3$), yielded detectable quantities of HHE [10], but limited human research had documented its production via *in vivo* lipid peroxidation following $n - 3$ consumption [11].

As a secondary analysis of an NIH-funded, dietary crossover study evaluating $n - 3$ intake and breast cancer risk [12], our objective was to evaluate *in vivo* lipid peroxidation in response to a diet with or without foods rich in $n - 3$ (e.g., salmon, tuna, flaxseed oil, and walnuts). Briefly, each study participant ($n = 15$) consumed meals providing 3% or less 1% of energy from $n - 3$ for eight weeks; energy needs were computed to maintain body weight based on the Harris-Benedict equation. After a washout period of six to eight weeks, each study participant completed the other diet. Diets were similar with respect to macronutrient distribution, except for the differences in $n - 3$. The diet with 3% of energy from $n - 3$ provided approximately 5.36 g ALA and 1.45 g eicosa-pentaenoic acid (EPA, 20:5 $n - 3$) and docosahexaenoic acid (DHA, 22:6 $n - 3$) from food sources per 2000 kcal per day [12].

Previously, this laboratory developed a method based on the work of Esterbauer [13] to identify and quantify the urinary excretion of polar and nonpolar aldehydic products of *in vivo* lipid peroxidation in animal models and validated this method in humans [5] [14]-[16]. Increased *in vivo* lipid peroxidation was measured in rats fed a standard diet compared to fasted rats, suggesting that the formation of these compounds was at least partially influenced by diet [14]. Further research demonstrated that rats were subjected to a vitamin E deficiency, consuming a diet high in PUFA (including cod liver oil), or administered that carbon tetrachloride exhibited increased urinary excretion of lipophilic aldehydes and related compounds [15]. Administration of vitamin E or probucol significantly reduced the increased *in vivo* lipid peroxidation observed in a rat model of diabetes, indicating that antioxidants inhibited the formation of several reactive aldehydes [5]. However, no research has been conducted on the response of *in vivo* lipid peroxidation to a well-controlled human feeding study including a mixture of ALA, EPA, and DHA. We hypothesized that the diet with 3% of energy from $n - 3$ would significantly increase *in vivo* lipid peroxidation in general and the excretion of HHE in particular.

2. Material and Methods

2.1. Chemicals and Supplies

2,4-Dinitrophenylhydrazine (DNPH), hexanal (98%), pentan-2-one (97%), hept-2-enal (97%), hepta-2,4-dienal (90%), decanal, deca-2,4-dienal, methanol, and water were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid and methylene chloride were obtained from J.T. Baker (Philipsburg, NJ). Acetone was purchased from Fisher Scientific (Fair Lawn, NJ), and hexane was purchased from EMD Chemicals (Gibbstown, NJ); butanone, octanal, non-2-enal, and DNPH-derivative of butyraldehyde from Supelco Analytical (Bellefonte, PA). DNPH-derivatives of 4-hydroxy-2-*trans*-octenal (HOE) and 4-hydroxy-2-*trans*-decenal (HDE) were generously provided previously by Dr. Esterbauer's laboratory, University of Graz (Graz, Austria). HHE and HNE were purchased from Cayman Chemicals (Ann Arbor, MI). *L*-ascorbic acid was procured from Eastman (Rochester, NY). All chemicals and solvents were high-performance liquid chromatography (HPLC)-grade and filtered and degassed prior to use.

2.2. Instrumentation

The HPLC system included a Varian 9010 solvent delivery system (Varian, Walnut Creek, CA), a Waters WISP 710B sample injector (Waters, Milford, MA), an Ultrasphere ODS C18 reversed-phase column (25 cm × 4.6 mm i.d., 5 μm particle size) (Beckman, Fullerton, CA) equipped with a 7.5 × 4.6 mm guard column (Alltech Associates, Deerfield, IL), and a Varian 9050 variable wavelength UV-VIS detector (Varian, Walnut Creek, CA). The integration of peaks was completed with Varian Star Chromatography Workstation (Varian, Walnut Creek, CA) software installed on a computer connected to the detector.

2.3. Study Population and Diets

Complete inclusion and exclusion criteria have been provided by Young *et al.* [12]. Healthy postmenopausal women with a body mass index between 19 and 32 kg/m² were eligible to participate in the original study. The subjects (n = 15) were randomly assigned to an isoenergetic diet with 20% of energy from fat with (+n - 3) or without (-n - 3) 3% of energy from n - 3 daily for eight weeks and subsequently consumed the other study diet for the same period of time following a six- to eight-week washout period. The +n - 3 diet macronutrient composition (% energy) per 2000 kcal was as follows: 317 g carbohydrate (62%), 77 g protein (15%), 54 g fat (23%), 25.2 g fiber, 210 mg cholesterol, 14.8 g saturated fatty acids (SFA), 16.2 g monounsaturated fatty acids (MUFA), 16.1 g PUFA, 6.82 g n - 3, 5.36 g ALA, 0.46 g EPA, and 0.99 g DHA. The -n - 3 diet macronutrient composition (% energy) per 2000 kcal was as follows: 329 g carbohydrate (65%), 76 g protein (15%), 46 g fat (20%), 23.9 g fiber, 203 mg cholesterol, 13.4 g SFA, 13.9 g MUFA, 13.3 g PUFA, 0.88 g n - 3, 0.85 g ALA, 0 g EPA, and 0.03 g DHA [12]. Baseline dietary intake was collected via food frequency questionnaire for 14 of the 15 subjects. Plasma phospholipid n - 3 concentrations were significantly higher

when subjects followed the +n – 3 diet compared to the –n – 3 diet [12]. The study was approved by the US Army Medical Research and Materiel Command's Human Subjects Research Review Board and the University of Minnesota Committee for the Use of Human Subjects in Research (study #0307M49927), and the study was conducted in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki)*.

2.4. Twenty-Four Hours Urine Collection Protocol

As part of the original study, each subject provided two consecutive 24-hour urine collections during the final week of each diet [12]. The samples were kept in an opaque, 3.5 L jug with 1 g ascorbic acid at refrigerator temperature throughout the collection. The collections were pooled, and aliquots were stored at –80°C prior to analyses. Urinary creatinine was measured for each sample by rate reflectance spectrophotometry using an Ortho Clinical Diagnostics Vitros analyzer at the University of Minnesota Medical Center, Fairview, Diagnostic Laboratories.

2.5. Preparation of DNPH Reagent and DNP-Hydrazone Standards

The DNPH reagent was prepared daily following the method in Kim *et al.* [14]. 12.5 mg of DNPH, recrystallized three times, was mixed the 25 mL of 1 N hydrochloric acid at 50°C for approximately one hour. Following cooling, the reagent was rinsed four times with hexane to remove any impurities. DNP-hydrazone standards were prepared from pure standards (e.g., hexanal, HHE) following the method of Kim *et al.* [14].

2.6. Formation and Isolation of DNP-Hydrazones of Urinary Aldehydes and Related Compounds

Three mL of urine samples in duplicate were reacted with an equal volume of DNPH reagent overnight with agitation at room temperature. A reagent blank and acetone-DNPH standard were prepared by reacting an equal amount of DNPH reagent or 1% acetone/water (v/v), respectively, and DNPH reagent. The following day, the reaction mixtures were extracted three times with 10 mL methylene chloride, and the organic phases were separated via centrifugation for 10 minutes at 1360 ×g. The sample extracts were pooled and evaporated to 500 µL under N₂ gas. Each sample was applied to two thin-layer chromatography (TLC) plates, and the plates were developed with methylene chloride at room temperature for approximately one hour for primary separation.

Nonpolar and polar compounds were isolated by comparison to the R_f values of acetone-DNPH (0.55) and DNPH reagent blank (0.23). The polar carbonyl compounds were found between the origin and R_f 0.23, while the nonpolar carbonyl compounds were found between R_f 0.55 and the solvent front. Osazones were isolated between the acetone-DNPH and DNPH reference bands and were discarded from the following analyses. The polar and nonpolar carbonyl compounds from the TLC plates were cut and eluted three times with 10 mL 100% methanol. The pooled extracts were evaporated to approximately 5 mL under N₂ gas and centrifuged for 20 minutes at 1360 ×g to

remove any silica. The clear supernatants were evaporated to less than 1 mL and made up to exactly 1 mL with 100% methanol.

2.7. Identification of DNP-Hydrazones

Aliquots of the polar and nonpolar DNP-hydrazones were analyzed separately via HPLC with two different solvent systems [10] [14]. Prior to injection into the HPLC system, the methanol extracts were filtered through a 0.45 μm filter. Aliquots of polar carbonyls (100 μL) were injected into the HPLC system, using isocratic elution for 10 minutes with 55% methanol/45% water (v/v), followed by a linear gradient of 55% methanol/45% water (v/v) for 20 minutes and 100% methanol (v/v) for 10 minutes. The analysis of nonpolar carbonyls was identical, but the initial isocratic gradient was 75% methanol/25% water (v/v). The absorbance of polar and nonpolar aldehydes and related carbonyl compounds was monitored at 378 nm, and the rejection of peaks was set to 2000 area counts. The detection limit of the system was 1 ng hexanal-DNPH per 50 μL injection. Peaks were identified by comparison of retention times to known standards. Each sample was injected at least twice, and paired samples were run in the same series, whenever possible.

2.8. Liquid Chromatography-Mass Spectrometry (LC/MS) Analysis of HDE-DNPH Adduct

For representative subjects' following the +n - 3 diet (n = 8), DNP-hydrazones were pooled in duplicate, and two samples were collected between 32 and 35 minutes of the polar HPLC method described above. LC/MS analysis was completed by following a previously published method [17]. 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 ten minute 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 cone gas (50 L/h) and desolvation gas (600 L/h) and argon as 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 leucineenkephalin ($[\text{M} - \text{H}]^- = 554.2615$ 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 HDE-DNPH in the samples was confirmed by a comparison with the pure standard and MS/MS fragmentation.

2.9. Statistical Analysis

Data were log-transformed and analyzed in SAS v9.2 (SAS Institute Inc., Cary, NC) via

paired Student's t-test. The cutoff for significance was set at $p = 0.05$.

3. Results

3.1. Subject Characteristics

A summary of baseline characteristics for the subjects is shown in **Table 1**. The average age of the subjects was 56.21 ± 5.72 years old, and the average BMI was 27.70 ± 3.38 kg/m². Of the 3% of energy consumed from n – 3 fatty acids during the low-fat +n – 3 diet, approximately 80% was comprised of ALA with the remainder from EPA (6.9%) and DHA (14.9%) [12].

3.2. Total Polar and Nonpolar DNP-Hydrazones

Figure 1 shows the total area under the individually measured peaks from the polar and nonpolar HPLC systems. Following the +n – 3 diet, urinary total polar DNP-hydrazones were significantly increased more than two-fold compared to the diet with less than 1% of energy from n – 3 ($p < 0.05$). In contrast, there was a modest increase in urinary nonpolar DNP-hydrazones in the +n – 3 compared to the –n – 3 diet, but the difference did not reach statistical significance ($p = 0.78$).

Table 1. Baseline demographic and dietary characteristics of women participating in n – 3 crossover feeding study.

Baseline characteristic (n = 15)	Mean ± SD
Race/ethnicity, n (%)	
African-American	1 (6.67%)
Hispanic-White	1 (6.67%)
White	13 (86.67%)
Age, y	56.21 ± 5.72
Body mass index, kg/m ²	27.70 ± 3.38
Baseline diet (n = 14)	Mean ± SD
Total energy, kcal	1785 ± 668
Total fat, g (% energy)	66.06 ± 26.18 (33.41% ± 4.16%)
Saturated fat, g (% energy)	22.10 ± 9.34 (11.18% ± 2.24%)
Monounsaturated fat, g (% energy)	24.65 ± 10.16 (12.43% ± 1.85%)
Polyunsaturated fat, g (% energy)	14.35 ± 10.16 (7.28% ± 0.69%)
18:2n – 6, g	12.87 ± 4.69
18:3n – 3, g	1.19 ± 0.46
20:4n – 6, g	0.086 ± 0.048
20:5n – 3, g	0.021 ± 0.037
22:6n – 3, g	0.055 ± 0.037

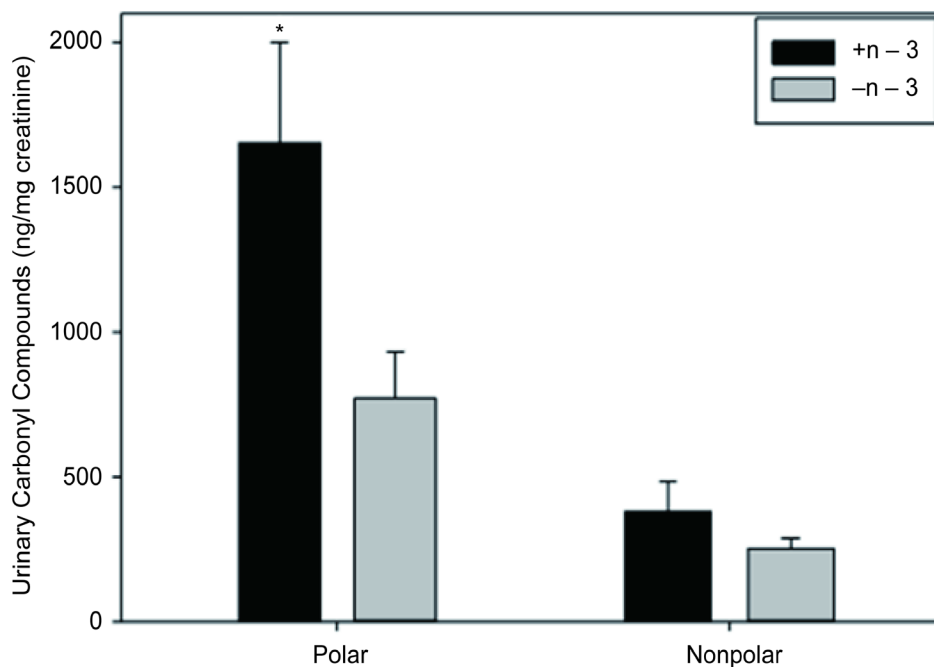


Figure 1. Total urinary polar and nonpolar lipophilic aldehydes following each 8-week diet. * $p < 0.05$.

3.3. Individual Polar and Nonpolar DNP-Hydrazones

A representative chromatogram of polar aldehydes is shown in **Figure 2**. The mean values for individual polar and nonpolar DNP-hydrazones are shown in **Figure 3** and **Figure 4**, respectively. The urinary concentrations of HHE, HDE, and unidentified compounds E and F were significantly higher following the +n - 3 diet compared to the -n - 3 diet ($p < 0.05$). The difference in HNE concentrations did not reach statistical significance ($p = 0.44$). Although all nonpolar compounds quantified in the urine were slightly increased in the +n - 3 versus -n - 3 diet, none of the differences in concentrations reached statistical significance, likely due to the small number of subjects ($n = 15$) and overall low concentrations of urinary nonpolar aldehydes. **Figure 5** shows LC/MS traces confirming the presence of HDE in the urine samples of women consuming n - 3.

4. Discussion

The present experiment is the first to measure a significant increase in total polar aldehydes and two of the α , β -unsaturated hydroxy aldehydes HHE and HDE in the urine of individuals following a low-fat diet with 3% of energy from n - 3. These results confirm our hypothesis that an increase in dietary n - 3 increases *in vivo* lipid peroxidation. The highly unsaturated n - 3 are susceptible to lipid peroxidation and the formation of selected secondary lipid peroxidation products including polar aldehydes, as shown in previous work by this laboratory [10] [15].

The present study is the first to quantify total urinary lipophilic polar and nonpolar

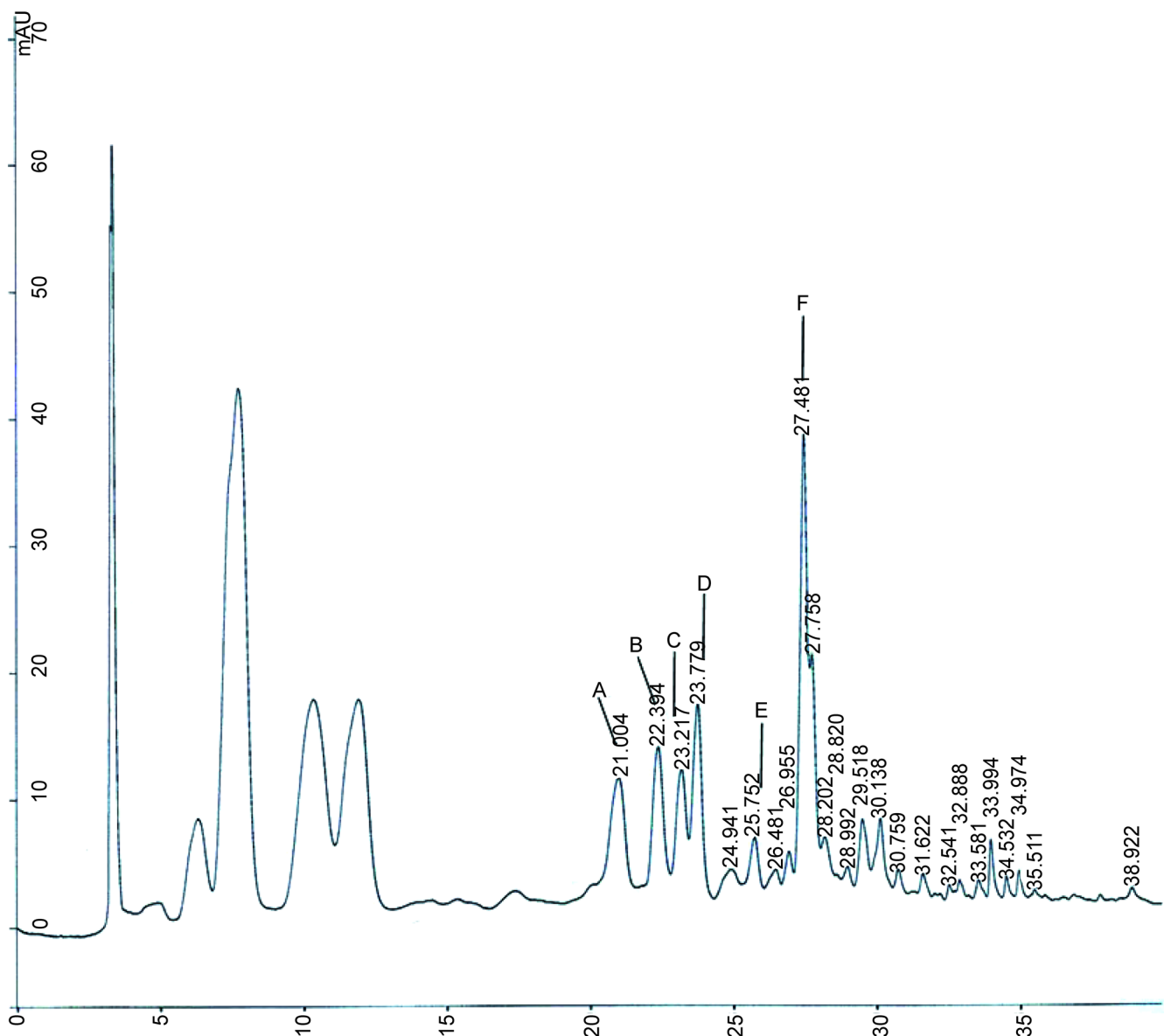


Figure 2. Representative HPLC chromatogram showing polar 2,4-dinitrophenylhydrazones isolated from human urine. A = 4-hydroxy-2-*trans*-hexenal (HHE), B = 4-hydroxy-2-*trans*-octenal (HOE), C = 4-hydroxy-2-*trans*-nonenal (HNE), D = 4-hydroxy-2-*trans*-decenal (HDE), E = unidentified compound, F = unidentified compound.

aldehydes and related carbonyl compounds from the *in vivo* lipid peroxidation of *n* – 3 in humans. This is also the first to report the presence of HDE from human urine (Figure 5). We documented significant increases in HHE, HDE, and two unidentified lipophilic polar carbonyl compounds in women consuming 3% of energy from *n* – 3 for eight weeks. This increase in HHE was expected due to its formation from lipid peroxidation of *n* – 3 [10] and confirmed the results of Calzada *et al.* [11] showing a significant increase in plasma HHE following DHA supplementation at 800 or 1600 mg per day in men. Similar to Turley *et al.* [18], we did not detect a significant increase of HNE in individuals consuming the +*n* – 3 diet. This is not surprising given that thermal

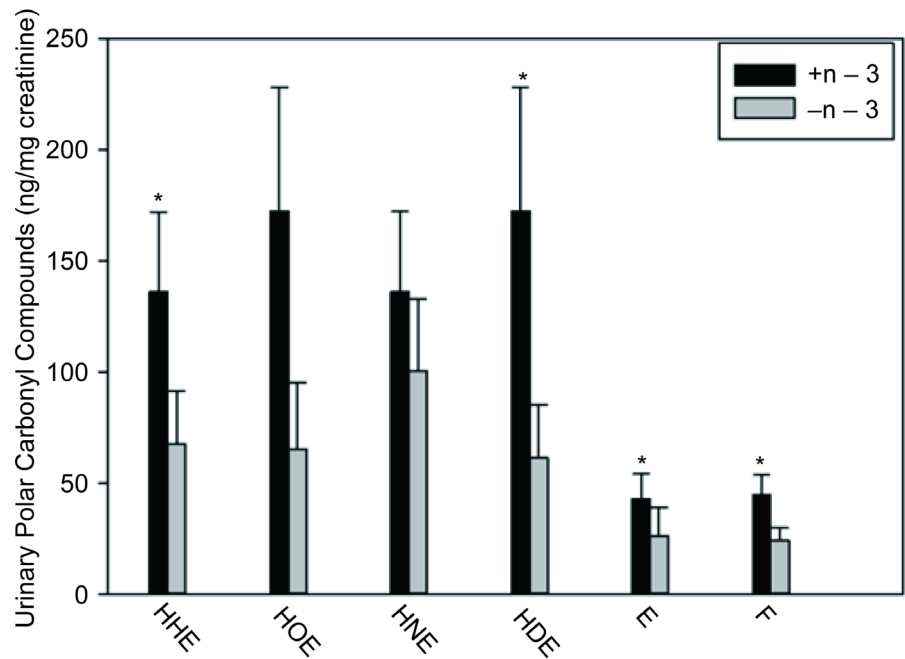


Figure 3. Individual polar urinary carbonyl compounds following each 8-week diet. HHE = 4-hydroxy-2-*trans*-hexenal, HOE = 4-hydroxy-2-*trans*-octenal, HNE = 4-hydroxy-2-*trans*-nonenal, HDE = 4-hydroxy-2-*trans*-decenal, E = unidentified compound, F = unidentified compound. * $p < 0.05$.

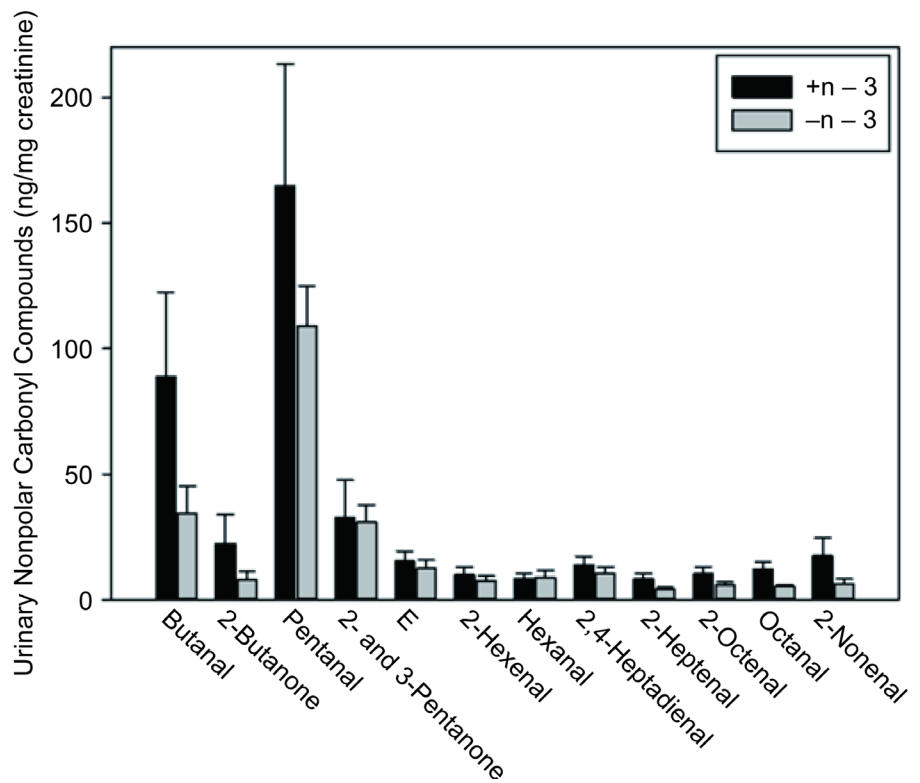


Figure 4. Individual nonpolar urinary compounds following each 8-week diet. E = unidentified compound.

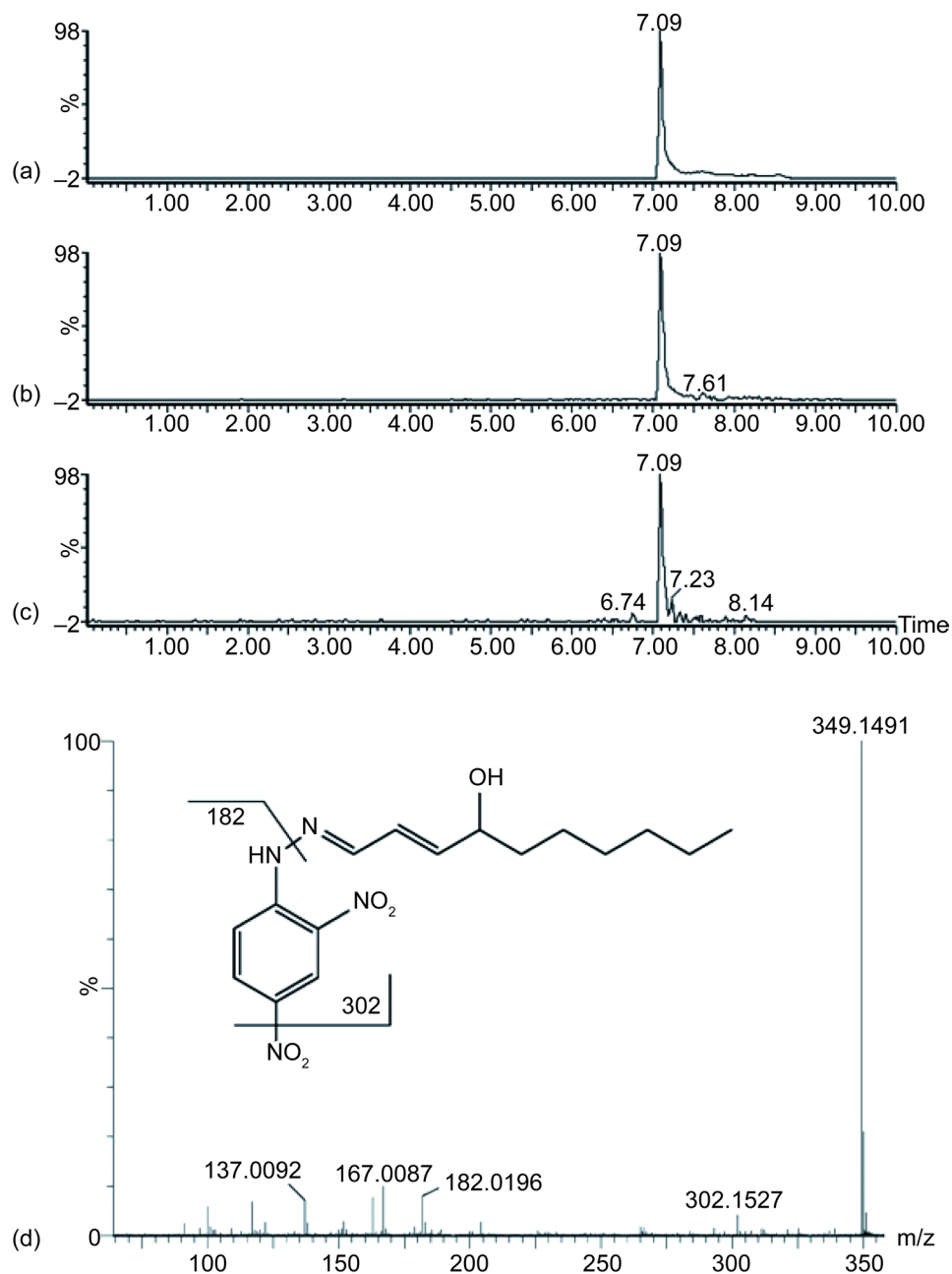


Figure 5. LC/MS trace of 4-hydroxy-2-*trans*-decenal (HDE)-2,4-dinitrophenylhydrazine(DNPH) standard (a), its presence in two pooled +n – 3 urine samples (b and c), and its MS/MS fragmentation (d), with HDE-DNPH structure).

oxidation of ALA does not produce quantifiable levels of HNE, in contrast to linoleic acid (C18:2 n – 6) [10]. As discussed before, this is the first study to document an increase in HDE excretion from *in vivo* lipid peroxidation of dietary n – 3, which was confirmed by LC/MS. A further experiment is needed to identify unknown compounds E and G, but it is plausible that they are longer chain polar lipophilic aldehydes produced from lipid peroxidation of n – 3. Significant changes in total or individual non-

polar lipophilic aldehydes were not found in the present experiment following the n – 3 diet treatment in human subjects. Although not significant, there seemed to be increased concentrations of these compounds following the n – 3 diet, suggesting maybe an overall increase in their production.

Through highly sensitive LC/MS analysis, we were able to confirm the significantly increased concentrations of HDE following the +n – 3 diet. This is the first study to document urinary excretion of HDE from dietary fat intake, as well as its significantly increased concentration following a diet containing 3% of calories from n – 3. These results suggest that HDE is produced from *in vivo* lipid peroxidation of n – 3. Although research examining HDE is very limited, it is plausible that since this compound shares the functional groups of HHE and HNE and differs only in its carbon chain length, it seems probable that it also binds proteins and DNA, potentially resulting in cellular mutations and death.

In conclusion, we demonstrated that a low-fat diet with 3% of energy from n – 3 (primarily as ALA) significantly increased total polar lipophilic aldehydes from *in vivo* lipid peroxidation. Future research utilizing highly sensitive techniques to characterize urinary lipid peroxidation metabolites from diets with a range of n – 3 concentrations will help establish at what level n – 3 are no longer beneficial and may become detrimental to human health. It would also be interesting to find out how dietary α -tocopherol administration alleviates the formation of HHE from n – 3.

Acknowledgements

Funding for this work was provided by grants from the Department of Defense (W81XWH-04-1-0448 and W81XWH-06-1-0778), the National Center for Research Resources at the National Institutes of Health (MO1-RR00400), and the USDA Agricultural Experiment Station. CAW and LRY were supported by Award Number T32CA132670 from the National Cancer Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. Salmon of the Americas, Inc., donated salmon fillets for the controlled diets.

Conflicts of Interest

The authors declare no conflicts of interest.

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Abbreviations

ALA: α -linolenic acid;

DHA: docosahexaenoic acid;

DNPH: 2,4-dinitrophenylhydrazine;

EPA: eicosapentaenoic acid;

HDE: 4-hydroxy-2-*trans*-decenal;

HHE: 4-hydroxy-2-*trans*-hexenal;

HNE: 4-hydroxy-2-*trans*-nonenal;

HOE: 4-hydroxy-2-*trans*-octenal;

HPLC: high-performance liquid chromatography;

LC/MS: liquid chromatography-mass spectrometry;

n - 3: omega-3 fatty acids;

LDL: low-density lipoprotein;

MUFA: monounsaturated fatty acids;

PUFA: polyunsaturated fatty acids;

SFA: saturated fatty acids;

TLC: thin layer chromatography.



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