

# Stability of n-3 fatty acids in human fat tissue aspirates during storage<sup>1-3</sup>

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**ABSTRACT** The content of n-3 ( $\omega$ 3) polyunsaturated fatty acids in fat tissue is an indicator of their long-term consumption. Therefore, a method for determining n-3 fatty acids in human fat tissue microbiopsies was validated and the stability of n-3 fatty acids in biopsies was checked under various conditions of storage. Methyl esters were prepared from 25 to 35 mg adipose tissue and separated by capillary gas chromatography. Recovery of added eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was 98-105%. The change after storage of fat samples at room temperature or at 4, -20, or -80 °C for 3 mo averaged +3.3% for EPA and +2.1% for DHA, with no effect of temperature. Storage at +20 or -80 °C for 7 mo yielded no perceptible change in EPA, DHA, or five other n-3 polyunsaturated fatty acids. EPA and DHA concentrations in adipose tissue aspirates are remarkably stable and deserve attention as biomarkers in epidemiological studies. *Am J Clin Nutr* 1993;57:884-8.

**KEY WORDS** Fat tissue, n-3 fatty acids, eicosapentaenoic acid, docosahexaenoic acid, human, biochemical indicators

## Introduction

The long-chain (n-3 or  $\omega$ 3) polyunsaturated fatty acids found in fatty fish have manifold effects on human metabolism (1-6). However, epidemiological studies on the health effects of these fatty acids are hampered by the absence of reliable and accurate methods to assess long-term intake.

Previous studies have shown that the proportion of the major n-6 polyunsaturated fatty acid, linoleic acid, in subcutaneous adipose tissue is an objective and valid index of the habitual dietary intake of this fatty acid over the past 1-3 y (7-9). Although several authors have reported concentrations of very-long-chain n-3 polyunsaturates in human fat tissue (10-14) and their relation with intake (15, 16), they are not yet widely applied as indicators of dietary exposure in epidemiology. One reason could be technical difficulties: the proportion of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in human adipose tissue is two orders of magnitude lower than that of linoleic acid. In addition, these fatty acids are very prone to oxidation and their stability in fat tissue samples is not known.

The present article reports the stability of n-3 polyunsaturates in stored aspirates of human fat tissue. In addition, the precision and accuracy of the assay have been investigated.

## Materials and Methods

### Samples

For routine control purposes, a control pool was made by supplementing 630 g lard with two fish-oil capsules (Super Epa, Health Craft, London) each containing 164 mg EPA (20:5n-3) and 127 mg DHA (22:6n-3) as analyzed in our laboratory. This produced concentrations of 0.6 mg EPA and 0.4 mg DHA per gram lard.

The stability of EPA and DHA during storage was studied in two independent experiments. For the first study, a pool of human fat was made from intraabdominal adipose tissue obtained during gall bladder surgery in five women aged 30-50 y. None of these women was suffering from a debilitating or terminal illness, and none was on a special diet. Immediately after surgery the samples were cooled to 4 °C and brought to the laboratory in an insulator. The five fat samples were pooled, homogenized with a Sorvall Omni mixer (Sorvall, Newton, CT), and centrifuged at 4 °C at 2000  $\times$  g for 10 min. The liquid supernatant layer was collected and stored in 1.5-mL Eppendorf tubes (Eppendorf, Hamburg, Germany). A similar surgical sample was used to investigate the effect of blood contamination.

For a second storage study, a lump of abdominal fat tissue was obtained during surgery. Immediately on arrival at the laboratory, 80 microbiopsies were taken from this lump by using an evacuated blood sampling tube, a 16-gauge needle, and a Luer adaptor (Venoject xx-MN200; Terumo Inc, Tokyo), as described previously (9); 40 samples were stored at -80 °C and another 40 at room temperature (+20 °C). Additional samples were analyzed immediately to provide baseline values.

To study recovery, 100  $\mu$ L fish oil, which contained 16.4% EPA and 12.7% by wt DHA of fatty acid methyl ester, was diluted in 100 mL petroleum ether. One hundred, 200, or 300  $\mu$ L of this solution was then added to 25 mg of the omental fat tissue homogenate described above for the first storage study. The total

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lipid (17) content of human omental fat was found to be  $85.0 \pm 2.1\%$  by wt of tissue ( $\bar{X} \pm SD$ ,  $n = 6$ ). Gluteal fat aspirates were obtained from volunteers as described previously (9).

The human studies were approved by the Committees on Human Experimentation of the departments involved at Wageningen Agricultural University.

#### Gas-liquid chromatography

The fat microbiopsies were transferred to a 5-mL flask or a 10-mL glass Kimax screw-cap tube (Brunschwig Chemie, Amsterdam, The Netherlands). When surgical samples were used,  $\approx 30$  mg fat was transferred to a 5-mL flask or 10-mL glass tube and weighed. The samples were incubated for 10 min at  $80^\circ\text{C}$  with 0.5 mL NaOH (0.5 mmol/L) each and allowed to cool to room temperature (18). Then 0.5 mL 14% (wt:vol)  $\text{BF}_3$  in methanol (catalog # 61105; Pierce Chemical Co, Rockford IL) was added, the samples were incubated at  $80^\circ\text{C}$  for 2 min and allowed to cool, 4 mL  $\text{H}_2\text{O}$  saturated with NaCl and 1 mL hexane were added, and the hexane layer was transferred to a gas chromatography injection vial after 1 min of shaking. For samples weighing 50–100 mg, volumes of NaOH,  $\text{BF}_3$ , and hexane were doubled.

The validity of this direct esterification method was checked by taking 23 40-mg samples from one piece of human omental fat. In five samples a fat extraction according to Folch et al (17) was done before methylation. In the 18 others methylation was performed directly on the fat tissue sample. Mean ( $\pm SD$ ) concentrations were  $0.0374 \pm 0.0009\%$  and  $0.0396 \pm 0.0017\%$  by wt for EPA and  $0.1630 \pm 0.036\%$  and  $0.1692 \pm 0.0465\%$  by wt for DHA, respectively, with no significant differences between methods. Thus, direct esterification of the fat tissue sample without prior extraction appeared to be justified.

Methyl esters were separated by gas-liquid chromatography on a Chrompack-Packard (model 438 AS; Chrompack, Delft, The Netherlands) by using a 25-m wall-coated open tubular fused silica column (inner diameter 0.25 mm) coated with a 0.2- $\mu\text{m}$  film of CP-Wax 58 (Chrompack, Middelburg, The Netherlands). The injection volume was 1.0  $\mu\text{L}$  and the carrier gas hydrogen was 100 kPa; the split ratio was 1:25. Oven temperature started at  $140^\circ\text{C}$ . It was increased by  $5^\circ\text{C}/\text{min}$  for the first 6 min,  $1.75^\circ\text{C}/\text{min}$  for the next 24.5 min, and  $40^\circ\text{C}/\text{min}$  until a final temperature of  $240^\circ\text{C}$ , at which the oven was held for 13.8 min. The injector temperature was  $275^\circ\text{C}$  and the flame ionization detector,  $265^\circ\text{C}$ . Identification of peaks was based on comparison of retention times with those of pure compounds (Sigma, St Louis) on two columns with different polarities (CP Sil 88 and CP Wax 58; Chrompack). The peaks were integrated on an IBM PC AT by a program from Chrompack (PCI-GC release 4.1). The sum of all peaks was set equal to 100%. This may have caused a slight bias because not all peaks may have represented fatty acids. However, the lipid in adipose tissue is made up overwhelmingly of triglycerides and all major peaks could indeed be identified as fatty acids.

## Results

#### Identification, accuracy, and precision

Figure 1 displays a typical chromatogram of fatty acids in a human gluteal fat tissue aspirate. The detector signal was first digitalized and stored and only afterward plotted at the desired

degree of magnification. Therefore, Figure 1 (A, B, and C) represents the same chromatogram replotted at different scales. Figure 1, C shows that the major C12–C18 fatty acids present in fat tissue (10) can be recognized clearly, although these peaks are distorted because of the large amount of material. Five samples of adipose tissue were also analyzed by using 20 times less material (0.5 instead of 1  $\mu\text{L}$  and a split ratio of 1:250 instead of 1:25). This yielded perfectly straight and symmetrical peaks for the major fatty acids (not shown), but the proportions were very similar; the mean relative differences, as a percentage of the respective peak area, were 0% for 12:0,  $-0.1\%$  for 14:0, 1.1% for 16:0 + 16:1, 0.7% for 18:0 + 18:1, and 1.2% for 18:2. Thus, the method optimized for n-3 fatty acids still provided valid estimates of the other major fatty acids present in adipose tissue. The EPA (20:5n-3) and DHA (22:6n-3) peaks became visible only at higher magnification (Fig 1, A and B). The identity of these peaks was confirmed by supplementing with pure standards followed by separation on columns of differing polarity, as described previously (13).

Repeated injection of methyl esters prepared from a pool of lard supplemented with fish oil produced a within-day CV, as determined in one run of 11 injections of the same sample, of 2.5% for EPA (mean content 0.101 % by wt) and 1.9% for DHA (mean content 0.134 % by wt). The interassay CV, determined on the same sample in triplicate analyses on 18 different days was 2.7% for EPA and 2.5% for DHA.

Accuracy was estimated by adding small amounts of fish oil of known EPA and DHA contents to a homogenized sample of human intraabdominal fat tissue (see Methods). Recovery of added EPA and DHA ranged from 98% to 105%. Linearity was estimated by injecting five samples at 20-fold dilution as described above and also by analyzing samples weighing 17 ( $n = 2$ ), 34 ( $n = 3$ ), 50 ( $n = 3$ ), and 68 mg ( $n = 3$ ), all obtained from the same aliquot sample of intraabdominal fat tissue. Mean concentrations (% by wt) of EPA were 0.081, 0.078, 0.074, and 0.075, respectively, and of DHA were 0.099, 0.096, 0.096, and 0.097, respectively. The detection limit of the procedure was  $< 0.005\%$  by wt (Fig 1).

#### Effect of storage

Figure 2 shows the stability of seven n-3 polyunsaturated fatty acids over a period of 7 mo in the second study. In general all fatty acids were stable at  $-80^\circ\text{C}$ , but some showed a very small downward trend at  $+20^\circ\text{C}$ . Alpha-linolenic acid (18:3n-3) showed a minor decline over the first 2 wk and was stable afterward. It is not clear whether this decline was real or was due to a minor bias in the zero time value.

In the first storage study (Table 1) the mean percentage change over a 3-mo period was +3.3% for EPA and +2.1% for DHA. The regression equation for the concentration of EPA as a function of time during fat storage at room temperature was EPA (% by wt) =  $0.034 + 0.00000374 \times \text{time (d)}$ . When other samples were converted into methyl esters immediately and then stored for various periods dissolved in hexane, changes were also minimal; the concentration changes per month of the different fatty acid methyl esters varied between 0.4% and 1.6% over a 3-mo period. For these samples the regression equation for EPA was EPA (% by wt) =  $0.099 - 0.0000136 \times \text{time (d)}$ .

In the second storage experiment mean ( $\pm SD$ ) concentrations at baseline were  $0.04 \pm 0.000$  for EPA and  $0.17 \pm 0.001\%$  by

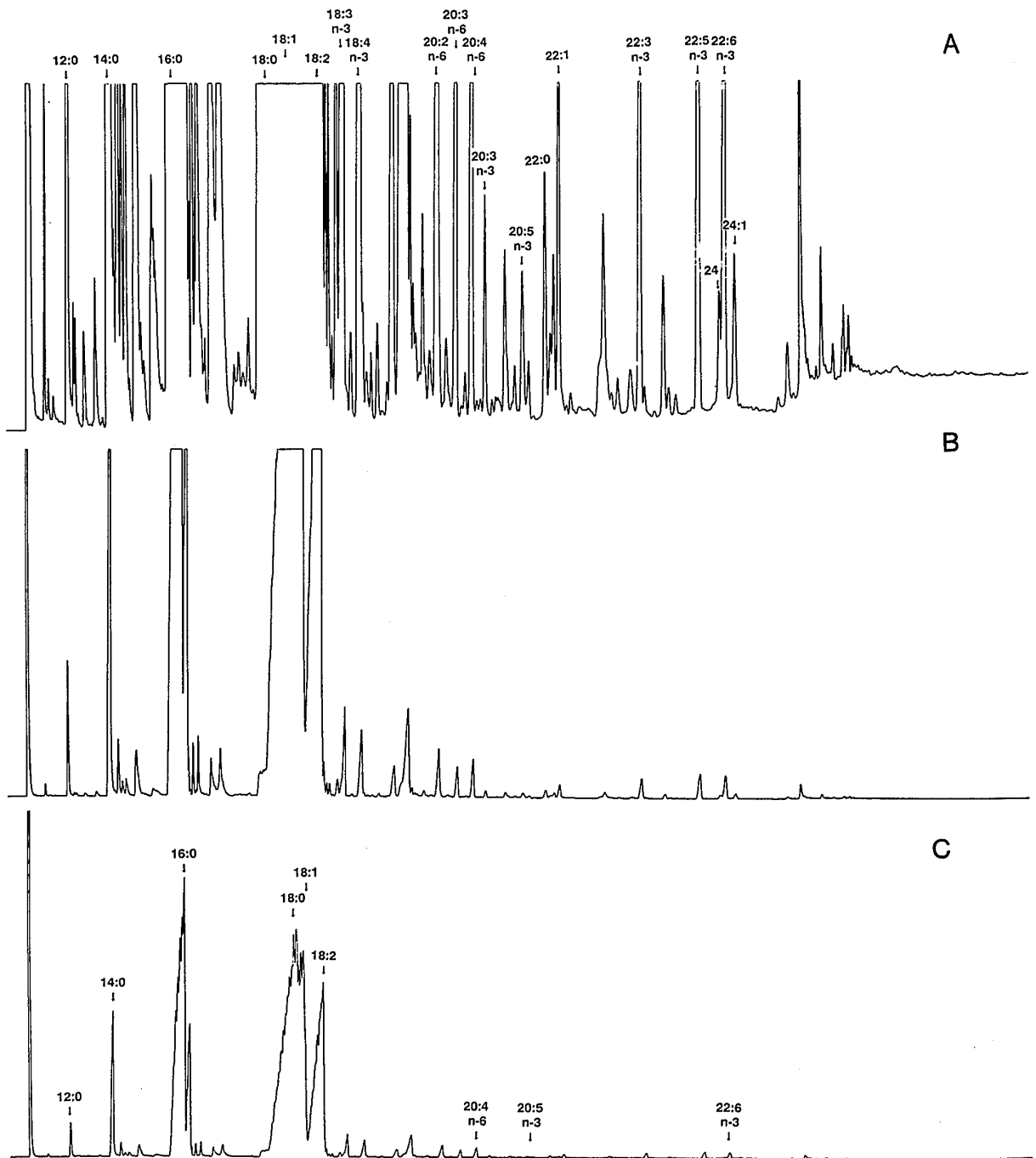


FIG 1. Gas-chromatographic analysis of a gluteal fat biopsy of a healthy human volunteer. A, B, and C represent the same chromatogram plotted at attenuations of 32, 1024, and 4096 times, respectively. The proportion, % by wt fatty acid methyl ester, of 20:5n-3 (EPA) was 0.04 and that of 22:6n-3 (DHA) 0.16.

wt for DHA. These values remained essentially unchanged during storage for 7 mo (Fig 2).

#### *Effect of blood contamination*

We investigated the effect of blood contamination by dividing a piece of human intraabdominal fat obtained during surgery into two parts. Both were homogenized and from each part five samples of  $\approx 3.5$  mg each were taken. To each of the five samples

of the second series,  $\approx 35$  mg whole blood was added and fatty acid composition was measured as described above. Mean concentrations of n-3 fatty acids were little affected by the addition of blood. However, blood contamination resulted in an irregular baseline with many spurious peaks, resulting in a less precise integration of the peaks. The CVs in samples without blood were 2.2% for EPA and 0.7% for DHA; in samples with blood they were 17.4% for EPA and 15.6% for DHA. The amount of

blood added here far exceeded the amount present in a bloody sample of fat tissue. Thus, in actual practice the loss of precision caused by blood contamination is probably minimal.

## Discussion

It is known from other studies that the linoleic acid content of body fat reflects the mean intake in the diet over a period of 2–3 y (7, 8). Similar data are beginning to appear for n–3 polyunsaturated fatty acids (15, 16). We have now shown that n–3 fatty acids can be determined accurately in microbiopsy samples of human adipose tissue and that such biopsies can be stored for  $\geq 7$  mo without perceptible changes in EPA or DHA concentrations. Values for the major fatty acids in fat tissue obtained with the present procedure (Fig 1) were almost identical to those obtained with procedures specifically aimed at quantitating these fatty acids. Thus, the total mass of major fatty acids is estimated correctly, which justifies expression of EPA and DHA as % by wt of total fatty acids. This was confirmed by the linearity studies, in which a fourfold variation in the amount of sample did not change the observed proportions of EPA and DHA.

TABLE 1

Stability of n–3 polyunsaturated fatty acids in human omental fat samples during storage\*

	EPA	22:5n–3	DHA
Initial concentration (% by wt)	0.034 $\pm$ 0.002 <sup>†</sup>	0.265 $\pm$ 0.010	0.154 $\pm$ 0.008
Change (% of initial value/mo) <sup>‡</sup>			
Room temperature	+0.2	+0.8	+0.1
4 °C	+1.3	+0.1	+0.5
–20 °C	+2.0	+1.5	+1.2
–80 °C	+0.9	+0.8	+0.9
Concentration after 3 mo at room temperature (% by wt) <sup>§</sup>	0.033 $\pm$ 0.003	0.273 $\pm$ 0.008	0.155 $\pm$ 0.003

\* EPA, eicosapentaenoic acid; and DHA, docosahexenoic acid.

<sup>†</sup>  $\bar{x} \pm$  SD.

<sup>‡</sup> Calculated from the sample regression coefficient of the regression of fatty acid concentration on time. Ten samples were analyzed at times 0, 1 wk, and 1 and 3 mo for each temperature.

<sup>§</sup> Fatty acid methyl ester/100 g fatty acid methyl ester.

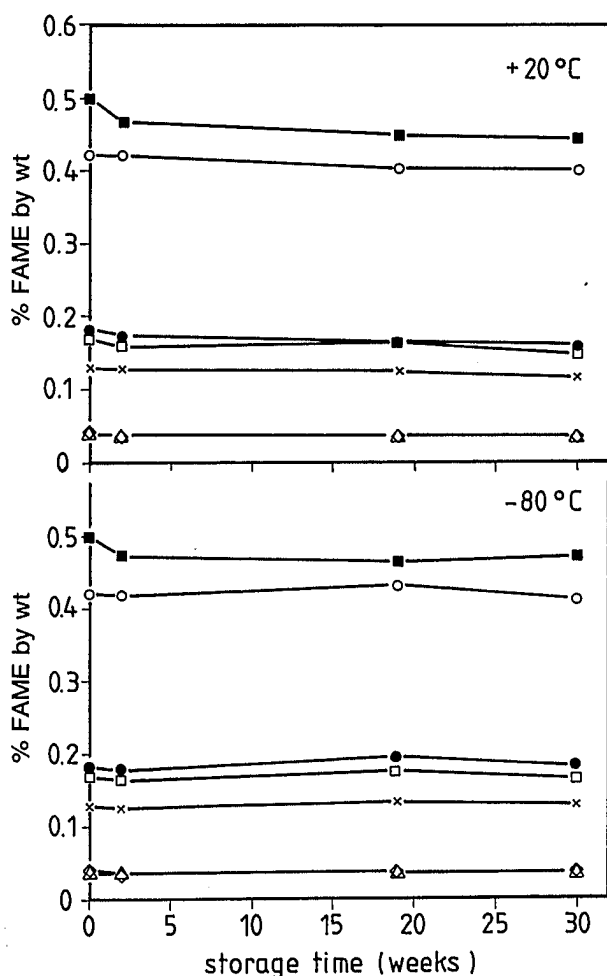


FIG 2. Stability of n–3 polyunsaturated fatty acids in human fat tissue aspirates stored for 7 mo at +20 or –80 °C. Each point represents the mean of 10 independently stored aspirates from one lump of abdominal fat tissue. ■, 18:3n–3; ○, 18:4n–3; ●, 22:5n–3; □, 22:6n–3; ×, 22:3n–3; ◇, 20:5n–3; and △, 20:3n–3. FAMES, fatty acid methyl esters.

Some peaks in the chromatogram may represent lipids other than fatty acids. However, the concentration of such substances in fat tissue is small (10, 19) and removing them from the chromatogram would not change the estimates for EPA or DHA.

The most noteworthy finding of this study was that fat samples could be stored without any precaution even at room temperature for up to 7 mo without losses of the highly polyunsaturated n–3 fatty acids. Addition of antioxidants appeared to be unnecessary. Possibly, the amount of vitamin E present in fat tissue is sufficient to prevent noticeable oxidation (19). The storage studies were performed on samples taken from the supernatant oil that were obtained on centrifugation of human omental fat (Table 1) as well as on aspirates obtained from a lump of human adipose tissue, by using a method similar to that used in vivo (8). Evidently, the handling involved in homogenization, centrifugation, and sampling in the first storage study did not promote measurable degradation or oxidation of EPA or DHA.

When the fatty acids in the tissue biopsies were converted into methyl esters immediately and then stored, the stability of n–3 fatty acids did not appear to suffer. However, further handling, such as separation of fatty acids by thin-layer chromatography, could lead to deterioration. Thus, our findings do not disprove the well-known fact that polyunsaturated fatty acids are prone to spontaneous oxidation.

In conclusion, the concentrations of EPA and DHA in human fat tissue aspirates are stable and can be measured precisely and accurately. These observations emphasize the value of fat tissue aspirates for prospective studies on fatty acids and disease. ■

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