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High sensitivity negative ion GC–MS method for detection of desaturated and chain-elongated products of deuterated linoleic and linolenic acids

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Abstract A sensitive negative chemical ionization (NCI) gas chromatography–mass spectrometry (GC–MS) method for the detection of pentafluorobenzyl (PFB) esters of deuterated fatty acids is described. Deuterated linoleic [18:2n-6 $^2H_4$-9,10,12,13] and linolenic [18:3n-3 $^2H_5$-17,17,18,18,18] acids were converted to chain-elongated and desaturated products during incubations with homogenates prepared from rat liver. The extracted fatty acids were derivatized with pentafluorobenzyl bromide and analyzed in the negative ion mode by GC–MS. The detection limit of the PFB esters in NCI using selected ion monitoring was below 10 femtograms. In general, detection of the PFB derivatives using the negative ion mode was more than three orders of magnitude more sensitive than using a positive chemical ionization (PCI) method with methyl ester derivatives. The PFB esters of the $^2H_4$-18:2n-6 metabolites eluted with their unlabeled analogues, whereas the PFB esters of the $^2H_5$-18:3n-3 metabolites were resolved from the unlabeled compounds on polar capillary FFAP columns. Isotope ratios of the $^2H_4$-18:2n-6 metabolites were used to quantify the deuterated compounds from standard dilution curves generated from the ion abundances of the unlabeled fatty acids. The $^2H_5$-18:3n-3 metabolites were quantified similarly using 18:3n-3. This method is feasible for the study of the in vivo metabolism of deuterated essential fatty acids in whole animals.—**Pawlosky, R. J., H. W. Sprecher, and N. Salem, Jr.** High sensitivity negative ion GC–MS method for detection of desaturated and chain-elongated products of deuterated linoleic and linolenic acids. *J. Lipid Res.* 1992. **33:** 1711–1717.

Supplementary key words pentafluorobenzyl esters • deuterated linoleic and linolenic acid • GC–MS • NCI • desaturation

Linoleic (18:2n-6) and linolenic (18:3n-3) acids are desaturated and chain-elongated to arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acids, respectively, via a series of sequential and alternate enzymatic steps within the membranes of the endoplasmic reticulum in hepatocytes. In vitro desaturation and elongation has been demonstrated with liver homogenates using radiolabeled linolenic and linoleic acid by Brenner (1), Cook (2), Sprecher (3), and others. In an in vivo study with humans, Emken et al. (4) described metabolites of deuterated fatty acids in blood plasma. The fatty acids were analyzed as their methyl esters by GC–MS in the positive chemical ionization mode (PCI).

Fatty acids are commonly detected and quantified as their methyl esters by GC–MS in PCI (4). In general, a substantial increase in sensitivity can be obtained by using fluorinated derivatives followed by detection with negative chemical ionization (NCI). Kawahara (5) introduced the pentafluorobenzyl (PFB) esters of organic acids to provide enhanced detection with electron capture GC. Van Kuijk et al. (6) demonstrated low picogram sensitivity of the detection of the oxygenated derivatives of the fatty acids, 18:1n-9, 18:2n-6, 20:4n-6, and 22:6n-3 in a one-step transesterification reaction. The method used here is an esterification of the saponified total lipid extract with pentafluorobenzyl bromide and diisopropylamine.

Vinyl deuterated fatty acids co-elute with their unlabeled analogues on capillary GC columns. The deuterated fatty acids are then quantified by the increased isotope ratio as determined from ion abundances of the ^2H_n-fatty acids and the ^1H-fatty acids. Compounds labeled with four or five deuterium atoms are preferred for trace analysis detection because ion contributions from the ^{13}C isotopes of the unlabeled fatty acids are minimized. The theoretical abundances of the ions of $^{13}C_4$-20:4n-6 and the $^{13}C_5$-20:5n-3 fatty acids are 0.0017% and 0.00029% of the ion abundances of the ^{12}C fatty acids, respectively. More importantly, fatty acids that are labeled with deuterium on the two terminal carbons yield separate chromatographic peaks that elute prior to their

Abbreviations: GC, gas chromatography; MS, mass spectrometry; NCI, negative chemical ionization; PFB, pentafluorobenzyl; PCI, positive chemical ionization; ME, methyl ester; TLC, thin-layer chromatography.

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unlabeled counterparts. The chromatographic separation eliminates isotopic interferences, resulting in better quantitation with substantially decreased limits of detection. The major advantages of the method are the increased detection limits of the PFB derivatives and the enhanced signal detection limits using the terminally labeled fatty acids. The combination of these two advantages further reduces the amount of isotope needed for whole animal studies.

MATERIALS AND METHODS

Materials

Deuterated linoleic acid (18:2n-6 $^2H_4$-9,10,12,13) was obtained from Medical Isotopes Inc., (Concord, NH) and the deuterated linolenic acid (18:3n-3 $^2H_5$-17,17,18,18,18) was purchased from Cambridge Isotopes (Woburn, Mass). The standards were of greater than 98% chemical purity as determined by TLC and GC-MS analysis. The reagents, NADH, NADPH, ATP, coenzyme-A, malonyl CoA, sucrose, magnesium sulfate, bovine serum albumin, and glutathione were obtained from Sigma Chemical Co. (St. Louis, MO). Magnesium chloride and potassium hydroxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Pentafluorobenzyl bromide and diisopropylamine were obtained from Pierce Chemical (Rockford IL). The 30 m × 0.25 mm DB-FFAP column was purchased from J&W Scientific (Folsom, CA).

Preparation of incubation media from rat liver homogenates

Homogenates of whole rat liver were prepared according to the method of Cook (2). Briefly, adult male rats (Sprague-Dawley) were fed a standard laboratory chow diet ad libitum. The animals were decapitated, livers were removed, and the tissue was homogenized in glass tubes with 0.1 M phosphate buffer (pH 7.4) containing 2 mM reduced glutathione and 25 mM sucrose (4 ml medium per g tissue). The homogenates were centrifuged at 10,000 *g* for 20 min and the pellet was discarded. The protein content of the supernatant was determined by the method of Lowry et. al. (7). The incubation mixtures contained 0.1 M phosphate buffer, pH 7.4, 0.2 mM CoA, 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM NADH, 0.5 mM NADPH, 0.5 mM malonyl-CoA, 25 μM of the deuterated fatty acid (37 picomoles), and 50 μM BSA. The homogenate (94-97 mg protein) was added to the incubation mixture in a final volume of 2 ml. Samples were incubated at 37°C for 15 min in a shaking water bath. The reaction was stopped by the addition of 4 ml of 10% methanolic KOH to saponify the lipids.

Fatty acid extraction and derivatization

The fatty acid methyl ester, 23:0 (2 μg), was added as an internal standard to each of the samples. The methanolic KOH solutions were heated at 65°C for 30 min followed by acidification with 7 N HCl. Samples were extracted three times with a mixture of diethyl and petroleum ethers (1:1, v/v), dried over anhydrous ammonium sulfate, then divided into two fractions and evaporated under N_2. One fraction was derivatized with BF_3 in methanol (14%, w/v) to produce the fatty acid methyl esters according to the method of Morrison and Smith (8). Samples were redissolved in 1 ml of hexane. The other fraction was derivatized with 70 μl of a freshly prepared solution of pentafluorobenzyl bromide, diisopropylamine in acetonitrile (1:10:1000, v/v/v) in sealed screw-capped vials and heated at 60°C for 15 min to produce the PFB esters. The solvent was evaporated under N_2 and the residue was dissolved in 1 ml of hexane for GC-MS analysis.

Preparation of fatty acid standard curves

Two series of standard curves were prepared from dilutions of stock solutions of the deuterated and unlabeled fatty acids. First, standard curves were generated from dilutions of pure standards of 18:2n-6, $^2H_4$-18:2n-6, 18:3n-3, $^2H_5$-18:3n-3, and 20:4n-6 ranging from 10 to 1000 pg of the standard injected and containing 50 pg of 23:0. The samples were derivatized to their PFB esters and run on capillary GC-MS in the NCI mode. Second, curves were generated from serial dilutions of $^2H_4$-18:2n-6 and $^2H_5$-18:3n-3 (10 to 1000 pg of standard injected) that had been added to extracted samples of liver tissue with the internal standard, 23:0 methyl ester (50 pg/μl injected). The samples were derivatized to their PFB esters and analyzed in the negative ion mode.

GC-MS analysis

The GC-MS analysis was carried out on a Hewlett-Packard 5989 mass spectrometer equipped with a 5890 GC. The fatty acid esters, dissolved in hexane, were injected with a 0.5-min delay with a splitless injection technique onto a 30 m × 0.25 mm i.d., 25 μm coated DB-FFAP capillary column interfaced directly into the ion source. The GC oven temperature was programmed from 80° to 185°C at 20°C per min then to 240°C at 10°C per min and held for 30 min. The injector and transfer line were maintained at 250°C and 280°C, respectively. The source temperature was 150°C. Methane (99.99%) was used as the ionization gas with a source pressure of 4×10^{-4} torr, a reading taken from the ionization gauge which itself was mounted external to the source housing. Fatty acids were analyzed in the selected ion mode using the M+1 ion for the methyl ester derivatives in PCI and the M-PFB ion for the PFB derivatives in NCI. Source tuning was optimized for each ionization mode with equivalent electron multiplier potentials for both positive and negative ion analysis. In experiments where limits of detection were ascertained, the selected ion dwell-time was 1000 msec; elsewhere the ion dwell-times were on the

TABLE 1. Ion abundances of the fatty acid methyl and pentafluorobenzyl esters in negative and positive chemical ionization

A. Fatty Acid Derivative	Ion	Mean Corrected Areas[a] (± SE)
	m/z	
Positive ion mode		
$^2H_4$-18:2n-6-ME	299	5.8×10^4 ($\pm$ 130)
$^2H_4$-18:3n-6-ME	297	1.3×10^3 ($\pm$ 64)
$^2H_4$-20:4n-6-ME	323	2.8×10^3 ($\pm$ 98)
$^2H_4$-18:4n-3-ME	296	4.7×10^3 ($\pm$ 43)
Negative ion mode		
$^2H_4$-18:2n-6-PFB	283	1.5×10^8 ($\pm 2.1 \times 10^4$)
$^2H_4$-18:3n-6-PFB	281	1.4×10^6 ($\pm 1.2 \times 10^3$)
$^2H_4$-20:4n-6-PFB	307	5.9×10^6 ($\pm 6.3 \times 10^3$)
$^2H_5$-18:4n-3-PFB	280	1.3×10^6 ($\pm 1.1 \times 10^3$)
B. Fatty Acid Derivative/Ionization Mode		**Absolute Area Counts**
$^2H_4$-18:2n-6-ME/PCI (2.5 pg)	299	1.2×10^3
$^2H_5$-18:3n-3-ME/PCI (2.5 pg)	298	7.9×10^2
$^2H_4$-18:2n-6-PFB/NCI (10 fg)	283	2.4×10^4
$^2H_5$-18:3n-3-PFB/NCI (10 fg)	282	1.8×10^4

A: Comparison of the ion intensity in positive and negative ion modes for various fatty acid metabolites. The values for the corrected area counts, based on three injections, from SIM data for the ions from the methyl and PFB esters are presented for $^2H_4$-18:2n-6, $^2H_4$-18:3n-6, and $^2H_4$-20:4n-6, resulting from a liver homogenate incubation of 25 μM $^2H_4$-18:2n-6; also, $^2H_5$-18:4n-3 resulting from the incubation of $^2H_5$-18:3n-3 with the homogenate. B: Deuterated fatty acid derivatives and ionization mode. The area counts are for 2.5 pg or 10 fg of the fatty acids as either derivative.

[a]Areas corrected for sample losses based on the recovery of the internal standard 23:0.

order of 100 msec for each fatty acid with a 10-msec cycle time.

RESULTS

Sensitivity of the negative and positive ion analysis

The results in **Table 1** present the corrected area counts (corrected area counts are the absolute raw data area counts that have been revised for minor extraction inefficiencies) for the ions of the methyl and PFB esters of the fatty acids for $^2H_4$-18:2n-6, $^2H_4$-18:3n-6 and $^2H_4$-20:4n-6 resulting from an incubation of $^2H_4$-18:2n-6, and also the $^2H_5$-18:4n-3 ion resulting from the incubation with $^2H_5$-18:3n-3. The same amount of each sample (PFB or methyl ester preparation) was injected onto the GC-MS and analyzed as described above. The area counts for the ions of PFB derivatives using NCI were 10^3 to 10^4 more abundant than those of the ions of the methyl ester derivatives analyzed by PCI. The signal to noise ratio of a 10 fg standard of the $^2H_4$-18:2n-6 PFB was 12.3, whereas the lowest detectable amount of the $^2H_4$-18:2n-6-methyl ester in PCI was 2.5 pg.

Table 2 illustrates the change in the ion ratios of $^2H_4$-18:3n-6/18:3n-6 and $^2H_4$-20:4n-6/20:4n-6 that result from an incubation with $^2H_4$-18:2n-6. The increased ion

TABLE 2. Isotope ratios of the various metabolites of deuterium-labeled linoleic acid with and without incubating $^2H_4$-18:2n-6 in a rat liver homogenate

Metabolite	Ionization Mode/Derivative	$^2H_4/_1H$ (SD)
A. Incubation without $^2H_4$-18:2n-6		
$^2H_4$-18:3n-6	PCI/ME	0.0056 (0.003)
$^2H_4$-18:3n-6	NCI/PFB	0.0074 (0.008)
$^2H_4$-20:4n-6	PCI/ME	0.0021 (0.002)
$^2H_4$-20:4n-6	NCI/PFB	0.0032 (0.005)
B. Incubation with $^2H_4$-18:2n-6		
$^2H_4$-18:3n-6	PCI/ME	0.0875 (0.013)
$^2H_4$-18:3n-6	NCI/PFB	0.1196 (0.067)
$^2H_4$-20:4n-6	PCI/ME	0.0042 (0.002)
$^2H_4$-20:4n-6	NCI/PFB	0.0085 (0.005)

The values are computed as ratios of the ion area counts of the peaks from GC-MS analyses after a 15-min incubation. The ratios are given as the mean of three analyses $\pm$ standard deviation.

ratio observed from the incubated samples is due to conversion of the $^2H_4$-18:2n-6 to $^2H_4$-18:3n-6 and $^2H_4$-20:4n-6. The ion ratios from the mean of three trials of the methyl and PFB esters, using negative and positive ion GC-MS analysis, were not equivalent. Both the PFB and methyl ester fatty acids had isotope ratios greater than the theoretical values. The variations in the ratios may be due to proton or hydride exchange occurring in the ion source plasma. The isotope ratios of the fatty acid PFB derivatives were somewhat greater than those for the methyl esters.

Elongation and desaturation of $^2H_4$-18:2n-6

Fig. 1, panels A–D, illustrates the formation of the deuterated fatty acids produced in the liver homogenate incu-

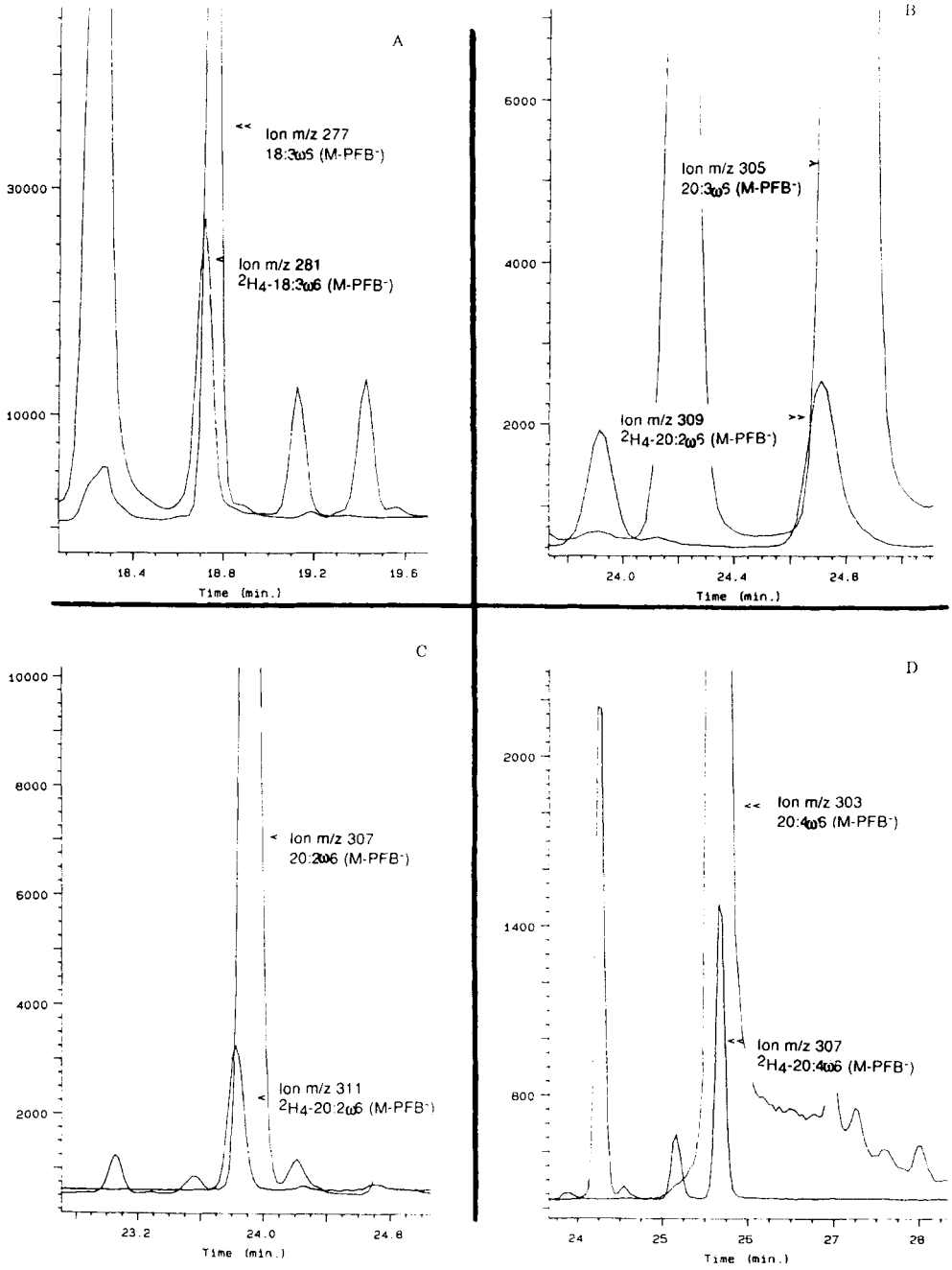

Fig. 1. The NCI GC-MS selected ion monitoring (SIM) tracings of the PFB esters of (A) $^2H_4$-18:3n-6 and 18:3n-6; (B) $^2H_4$-20:3n-6 and 20:3n-6; (C) $^2H_4$-20:2n-6 and 20:2n-6; and (D) $^2H_4$-20:4n-6 and 20:4n-6 from a rat liver homogenate incubation with $^2H_4$-18:2n-6. Peak area counts for the individual metabolites are as follows: $^2H_4$-18:3n-6 (68,459); 18:3n-6 (320,158); $^2H_4$-20:3n-6 (8,875); 20:3n-6 (96,784); $^2H_4$-20:2n-6 (12,333); 20:2n-6 (87,656); $^2H_4$-20:4n-6 (4,756); 20:4n-6 (26,776). (n-6 terminology is used in the figure.)

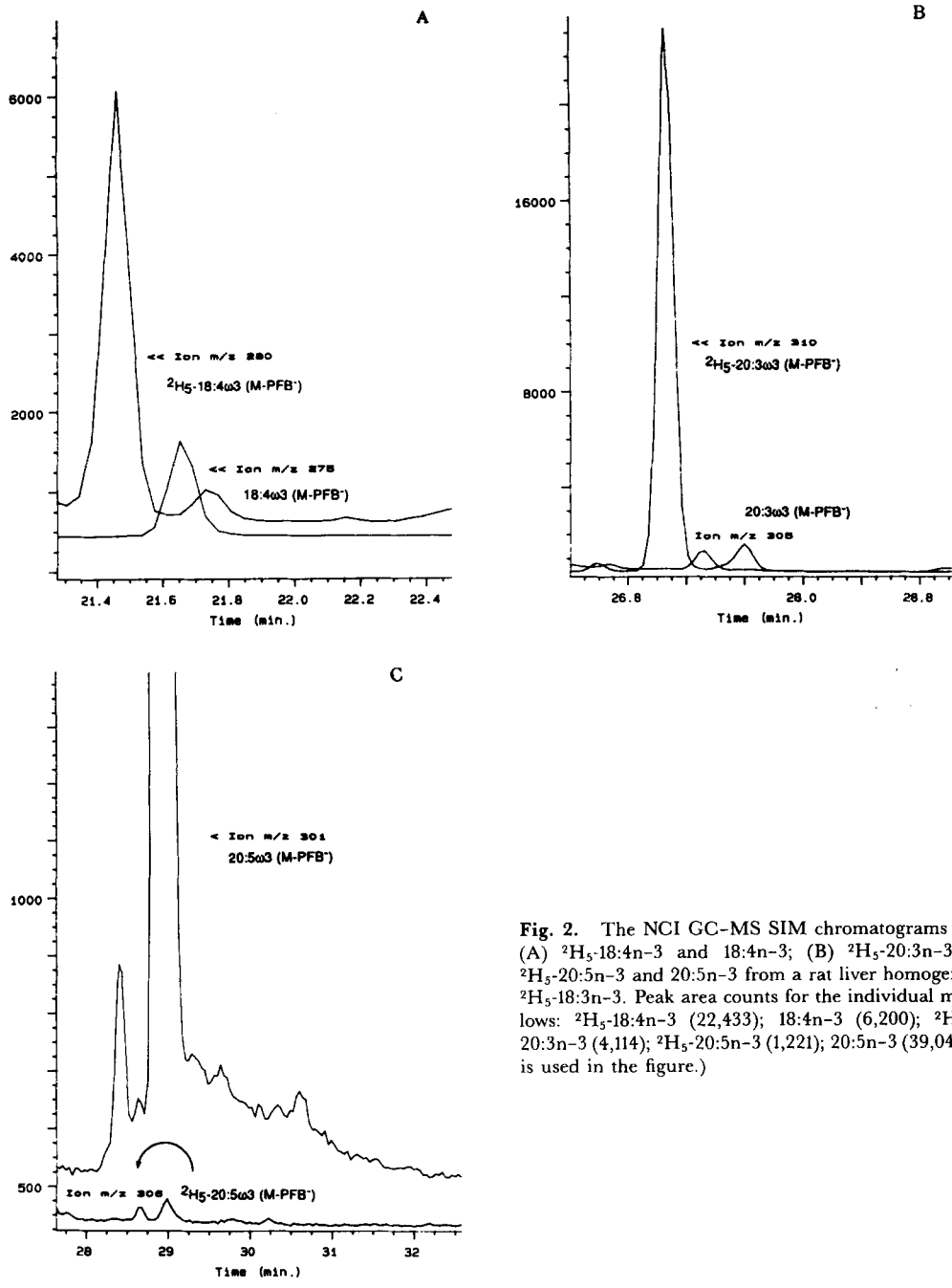

Fig. 2. The NCI GC-MS SIM chromatograms of the PFB esters of (A) $^2H_5$-18:4n-3 and 18:4n-3; (B) $^2H_5$-20:3n-3 and 20:3n-3; (C) $^2H_5$-20:5n-3 and 20:5n-3 from a rat liver homogenate incubation with $^2H_5$-18:3n-3. Peak area counts for the individual metabolites are as follows: $^2H_5$-18:4n-3 (22,433); 18:4n-3 (6,200); $^2H_5$-20:3n-3 (72,366); 20:3n-3 (4,114); $^2H_5$-20:5n-3 (1,221); 20:5n-3 (39,045). (n-3 terminology is used in the figure.)

bations with $^2H_4$-18:2n-6. The fatty acids $^2H_4$-18:3n-6, $^2H_4$-20:2n-6, $^2H_4$-20:3n-6, and $^2H_4$-20:4n-6 are detected and quantified by the increased abundance of the M-PFB$^-$ ions at *m/z* 281, 311, 309, and 307, respectively. The ion abundance ratio of the $^2H_4/^1H$ fatty acid esters is used to quantify the amounts of the deuterated fatty acid from the standard curves as described below. In each case, there was a slight decrease in retention time of the vinyl deuterated fatty acid in comparison to the endogenous form; however, these peaks eluted in the same chromatographic peak envelopes.

Elongation and desaturation of $^2H_5$-18:3n-3

In a manner similar to that of the $^2H_4$-18:2n-6 incubation, the formation of the deuterated n-3 products was observed when $^2H_5$-18:3n-3 was incubated with the liver preparations. These terminally labeled fatty acid metabolites resolved chromatographically from their unlabeled analogues as seen in **Fig. 2,** panels A-C. Neither the $^2H_5$-22:5n-3 nor the $^2H_5$-22:6n-3 fatty acids were detected in the incubations. The amount of $^2H_5$-20:5n-3 formed from these incubations was about 20% of the

TABLE 3. Standard curve data obtained by GC-MS for the PFB derivatives of the deuterated and unlabeled fatty acids 18:2n-6, $^2H_4$-18:2n-6, 18:3n-3, $^2H_5$-18:3n-3, and 20:4n-6

Analyte	Standard Curve[a]		Coefficient (r)
	100 × Slope	Intercept	
Pure standards			
18:2n-6-PFB	2.51	0.09	1.0
$^2H_4$-18:2n-6-PFB	2.43	0.09	1.0
18:3n-3-PFB	3.10	0.01	0.98
$^2H_5$-18:3n-3-PFB	2.83	0.02	0.98
20:4n-6-PFB	2.73	0.07	0.96
Standards in incubation media			
$^2H_4$-18:2n-6-PFB	2.88	0.21	1.0
$^2H_5$-18:3n-3-PFB	2.84	0.03	0.87

[a]A linear regression of the response ratio of (analyte/internal standard) on quantity of analyte (range 10–1000 pg).

amount of $^2H_4$-20:4n-6 formed from $^2H_4$-18:2n-6 in incubations under similar conditions. The deuterated fatty acids were quantitated from dilution curves as described below.

Quantitation of $^2H_4$-18:2n-6 and $^2H_5$-18:3n-3 metabolites from unlabeled fatty acid dilution curves

A semi-quantitative method was used to determine the amounts of the deuterated fatty acid formed from these incubations. Inasmuch as an alternatively labeled internal standard of the individual analytes was not available, the fatty acids were quantified from dilution curves using the response factor of the M-PFB^- ions of the unlabeled standards. Generally, the M-PFB^- ion from the fatty acids of 18 carbons in length had response factors equal to 1.2 ± 0.08 of that of the 20-carbon compounds. The degree of unsaturation did not diminish ion abundances for either the 18- or 20-carbon compounds. The response factor for 18:2n-6 and 18:3n-6 was 1.07 ± 0.02 and for 20:2n-6, 20:3n-6, and 20:4n-6 the response factor was 0.89 ± 0.02.

Standard curves were generated from dilutions of the pure standards of 18:2n-6, $^2H_4$-18:2n-6, 18:3n-3, $^2H_5$-18:3n-3 and 20:4n-6 (10–1000 pg) with a constant amount of the internal standard 23:0 (50 pg). The dilution curves are presented in **Table 3.** The standard curves for the deuterated and nondeuterated 18:2n-6 or 18:3n-3 were the same. Standard curves were then generated for the labeled fatty acids when added to the liver homogenates. The slopes of these curves were the same as those for the pure standards; however, the y-intercept for $^2H_4$-18:2n-6 fatty acid was greater than that of the pure standard. This was due to the ^{13}C isotopic contribution of the endogenous fatty acid from the homogenates. The amount of in vitro formation of the $^2H_4$-18:2n-6 products was calculated from these standard curves using the higher y-intercept value. A similar procedure was used when quantifying the products from $^2H_5$-18:3n-3 incubations. However, the standard curves of the pure material and those from the media incubations had equivalent slopes and y-intercept values.

Application to linoleate metabolism in a rat liver homogenate

To illustrate the utility of this method, $^2H_4$-18:2n-6 was incubated with liver homogenates and the reactions were stopped at 5, 15, 30, and 45 min. The fatty acids, $^2H_4$-18:3n-6, $^2H_4$-20:3n-6, $^2H_4$-20:2n-6, and $^2H_4$-20:4n-6 that were formed were quantified using standard dilution curves as described above. The results of this incubation are presented in **Fig. 3.** The chain-elongated product $^2H_4$-20:2n-6 was formed in the greatest quantities (9500 pmol/mg protein) at 10 min, but diminished to the levels of the other metabolites by 45 min. The desaturated products $^2H_4$-18:3n-6, $^2H_4$-20:3n-6, and $^2H_4$-20:4n-6 were

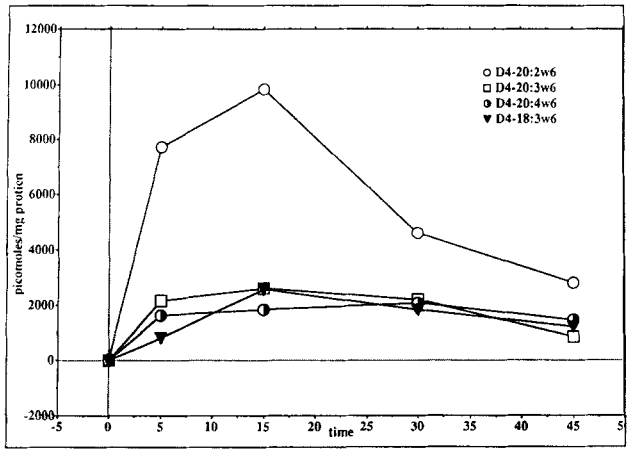

Fig. 3. Conversion of $^2H_4$-18:2n-6 to $^2H_4$-20:3n-6, $^2H_4$-20:2n-6, and $^2H_4$-20:4n-6 during an incubation with rat liver homogenates. The reactions were stopped at 5, 15, 30, and 45 min. The deuterated fatty acids were quantified using standard dilution curves of the unlabeled fatty acids. (n-3 terminology is used in the figure.)

formed in similar amounts (about 2000 pmol/mg protein) confirming findings from other studies that the Δ^6 desaturation is the rate-limiting step in these reactions (2).

DISCUSSION

We have advanced a semi-quantitative negative ion GC–MS method for the purpose of identifying the in vitro production of deuterated fatty acids formed in incubations with rat liver homogenates from either $^2H_4$-18:2n–6 or $^2H_5$-18:3n–3. This method is several orders of magnitude more sensitive than that of positive ion analysis of fatty acid methyl esters. This improvement in sensitivity has resulted in lower detection limits with a corresponding decrease in the amount of deuterated fatty acid required for these studies. The deuterated fatty acids were quantified from standard curves generated from the ion abundances of unlabeled materials. We also observed that fatty acids labeled on the C17 and C18 positions were resolved on polar capillary columns from the unlabeled material, resulting in a further decrease in detection limits and elimination of isotopic interferences.

We observed that the isotope ratios of the fatty acids in either ionization mode were greater than the theoretical prediction. This increase may be due to proton or hydride transfers between the analyte and reagent plasma during the ionization processes. The standard curves for the fatty acids analyzed were linear between 10 and 1000 pg of analyte.

This stable isotope fatty acid PFB ester negative-ion GC–MS method offers several improvements over other GC–MS methods. Because of the increased sensitivity, it makes possible cost-effective whole animal studies using reduced amounts of the isotope.

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