Improved Ozonolysis Method for Analysis of Total n-3 Fatty Acids During Hydrogenation of Fish Oils

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A rapid and precise method for determining the total amount of n-3 fatty acids in small samples of fish oil is presented. The oil is ozonized at −10°C in hexane solution, and the ozonides are subsequently reduced with triphenylphosphine at 40°C. The propanal formed is quantitated by capillary gas chromatography. The method was utilized to follow the industrial hydrogenation of a fish oil, and it was demonstrated that the n-3 double bonds were simultaneously reduced to one-sixth as the iodine value decreased to one-half.

KEY WORDS: Capillary GC, fish oil, hydrogenation, ozonolysis, propanal, triphenylphosphine, n-3 unsaturated fatty acids.

The interest in fish oils and their content of n-3 unsaturated fatty acids has been steadily increasing during the last decade as their key role in nutrition has come to light (1). This has resulted in several efforts to concentrate the amount of active acids by various techniques (2–6) as well as using them in feeding food animals (7). Therefore, fast and reliable methods for determining n-3 unsaturated fatty acids are required. This is true also for studying their chemical reactions, e.g., hydrogenation and polymerization. Although detailed quantitative information may be obtained by capillary gas chromatography (GC) of methyl esters on polar columns (8) and by nuclear magnetic resonance (NMR) technique (9), a rapid method to determine only the n-3 unsaturated fatty acids may be useful. The principle of ozonolysis and subsequent separation of the fragments by GC was introduced by Jart (10), and in the present work ozonolysis is used as a reductive process. Similar methods have been proposed for hydrogenated soybean oil (11) and for linolenic acid methyl ester (12); in the latter method propanal is isolated as a 2,4-dinitrophenyl-hydrazone on thin-layer chromatography (TLC) plates prepared from silica gel G (Merck, Darmstadt, Germany) and quantitated at 362 nm. The purpose of the present work is to develop a rapid and precise method that is usable for analyzing small samples of fish oil.

EXPERIMENTAL PROCEDURES

Analytical principle. The fish oil is treated with ozone, and the ozonides are reduced with triphenylphosphine to aldehydes; propanal is determined quantitatively by GC, allowing the original amount of n-3 unsaturated fatty acids to be calculated. To improve the analytical procedure and to obtain a high yield of ozonides, it is advantageous to use a hydrocarbon solvent (13). Hexane is used because it is easily separated from the propanal. The GC determination is carried out on a nonpolar capillary column (Ultra 2; Hewlett-Packard Co., San Fernando, CA), which allows the removal of high-molecular by-products by occasionally warming to 300°C.

Standard curve. To correct for different split ratios and amounts of sample injected, methyl acetate has been used as an internal standard. In Figure 1 an ideal standard curve is given, i.e., a curve based on the assumption that n-3 fatty acids yield propanal quantitatively. The abscissa is the area ratio between propanal and methyl acetate when using a constant amount ($50 \cdot 10^{-6}$ mL per mL solution) of methyl acetate. The ordinates are calculated as follows: 5.00 mg of fish oil contains 95.5% = 4.775 mg of fatty acids and with the composition given in Table 1; this corresponds to $4.113 \cdot 10^{-6}$ mole = 0.239 mg of propanal. The specific weight is 0.838 and, consequently, the volume will be $285.2 \cdot 10^{-6}$ mL, which is contained in 10 mL solution. The figure shows the amounts of n-3 fatty acids found in four standard samples, B–E that contained various amounts of propanal (Table 2). At point B, the ordinate is calculated as: 26.43% x $11.63 \cdot 10^{-6}/28.5 \cdot 10^{-6}$ = 10.8%, and points C–E are multiples of this value. The standard curve intersects the abscissa axis at point A and not at the origin; this is due to an impurity in the hexane emerging together with propanal in the chromatograms.

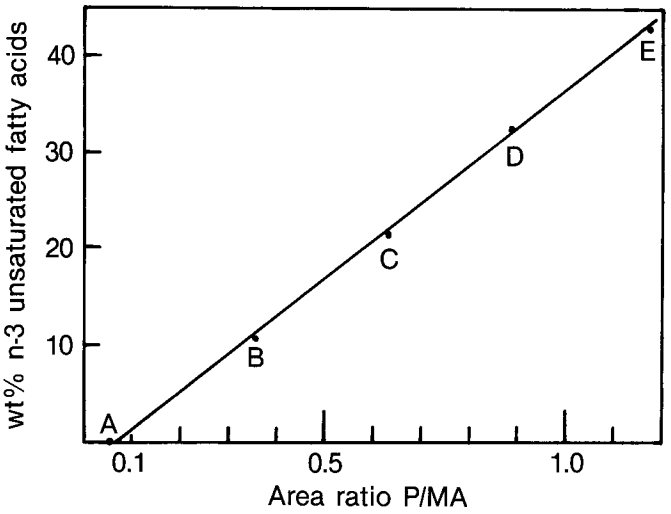

FIG. 1. Standard curve for determining the amount of n-3 unsaturated fatty acids from the P/MA ratio. P, propanal; MA, methyl acetate.

TABLE 1

n-3 Unsaturated Fatty Acids of a Fish Oil Determined by GC of Methyl Esters[a]

Acid	Wt%	Mole · 10^{-6}
16:4	0.55	0.106
18:3	1.31	0.225
18:4	3.68	0.636
20:3	0.17	0.027
20:4	0.81	0.127
20:5	7.73	1.221
21:5	0.40	0.060
22:5	0.90	0.130
22:6	10.88	1.581
Total	26.43	4.113

[a]Column, CP-Sil 88 (Chrompack, Middelburg, the Netherlands).

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TABLE 2

Ratios P/MA for Constructing the Standard Curve[a]

Point	mL of P per mL solution	Area, P	Area, MA	Ratio, (P/MA)
A	0	1,042	24,471	0.043
B	$11.63 \cdot 10^{-6}$	8,608	25,583	0.336
C	$23.3 \cdot 10^{-6}$	15,768	26,769	0.589
D	$34.9 \cdot 10^{-6}$	22,774	27,464	0.829
E	$46.6 \cdot 10^{-6}$	30,258	27,378	1.105

[a]P, Propanal; MA, methyl acetate. All area values are averages of three determinations.

Ozonization and reduction procedure. Ozone was produced in 5 vol% yield in a stream of dry oxygen in an apparatus with three Berthelot tubes, built according to literature (14). The reaction vessel used was a U-tube, constructed from a micro filter with a 15-mm diameter sintered low-porosity disc (Pyrex No. 3780/08; Corning Ltd., Staffordshire, England) and a glass tube inlet. To obtain a high yield of propanal by reduction of the ozonides, it is important to use the optimum reaction time, temperature and ratio between amounts of oil and reduction agent. The best conditions were: 10–15 min at 40–45°C with 5–10 mg of oil per 1 g of triphenylphosphine; the reaction mixture was well stirred.

Recommended method. Two 10-mL volumetric flasks are half-filled with hexane (Merck p.a.; Merck) and placed in an ice-water bath for cooling. Methyl acetate (Merck No. 809711) is taken from the refrigerator, and 0.50 mL is added to one of the volumetric flasks, which is then filled with hexane and returned to the ice-water bath with its stopper replaced. After three min, it is carefully readjusted with hexane. From this solution, 0.50 mL is transferred to the second volumetric flask, which is filled to the mark with hexane and represents the internal standard. The fish oil sample (≈10 mg) is weighed exactly into a 10-mL volumetric flask, which is filled with hexane. The flow of oxygen is adjusted, and the ozone apparatus is connected to the U-tube placed in an ice-water bath. Then 5 mL of sample solution is transferred into the U-tube, which is finally fitted with a moist potassium iodide indicator strip. Ozone is supplied until indicator shift, and the ozonized sample is quantitatively transferred into a 10-mL volumetric flask.

Triphenylphosphine (1 g) and 0.200 mL of internal standard are added, and the flask, containing a small magnet, is filled with hexane. It is placed in a 40°C water bath and stirred with the magnet for 10 min with the stopper well fastened. The reduced sample is cooled in an ice-salt bath, and a part of the clear upper phase is transferred into 2-mL crimp-vials. The samples may be analyzed at once, or they may be stored in a freezer up to 72 h. The following chromatographic conditions are suitable: chromatograph (5890A-II; Hewlett-Packard Co.) with a 50 m × 0.32 mm × 0.52 μm Ultra 2 column from Hewlett-Packard and the following oven temperature program and conditions: 30°C for 4 min, from 30 to 100°C at 70°C/min; at 100°C for 0.5 min, and cooling in 4 min; injector temperature at 200°C; flame-ionization detector temperature at 250°C; column pressure 50 kPa; and hydrogen as carrier gas with a split ratio of 1:10. When 8–10 analyses have been performed, the column should be cleaned up for 10 min at 300°C. Three measurements are carried out per sample, and the ratios between the propanal and methyl acetate areas are calculated. After adjusting the actual oil sample weight in relation to 10 mg, the amount of n-3 fatty acids is read from the corrected standard curve. Individual standard curves should be prepared because the reduction yield may vary.

Hydrogenated fish oil. Samples were drawn during the process from an industrial hydrogenation plant (using nickel catalyst). Iodine values according to Wijs (15) and n-3 unsaturated fatty acid analysis were carried out (Table 3).

RESULTS AND DISCUSSION

Table 4 shows results from triplicate chromatograms of three independent ozonization analyses of a sample of fish oil; Table 1 shows its content of n-3 unsaturated fatty acids by chromatography of their methyl esters. The overall average in Table 4 is 24.41%, with a standard deviation of 0.42. This corresponds to a reduction yield of more than 92% of the theoretical yield of 26.43% (Table 1). On this basis, values taken from the standard curve in Figure 1 should be multiplied by 1.083 before reporting.

In our experience, the results thus corrected show the same accuracy for the total amount of n-3 unsaturated acids as the more complex procedure *via* the methyl esters. In the latter procedure, moreover, problems may arise in obtaining quantitative esterification, keeping the column in adequate condition, obtaining the separation needed and identifying the peaks correctly.

The ozonolysis procedure does not require delicate gas-chromatographic columns, and an analysis only requires 30 min. The method does not separate the fatty acids but, for many practical purposes, will be sufficient. In one experiment the industrial hydrogenation of fish oil was followed as given in Table 3. It can be seen that by the time the original iodine value was reduced to one-half, the n-3 double bonds had reacted considerably faster and were reduced to one-sixth.

TABLE 3

Analysis of Fish Oil During Industrial Hydrogenation with a Nickel Catalyst

Step no.	1	2	3	4	5	6	7
Iodine value	120.8	116.0	109.6	103.0	92.2	80.5	56.7
n-3 Acids (wt%)	19.99	17.31	16.20	14.60	10.37	7.52	3.18

TABLE 4

Triplicate Chromatograms from Three Independent Ozonization Analyses of a Sample of Fish Oil[a]

Amount (mg) weighed out	Gas chromatographic peak area		Ratio (P/MA)	Amount (mg) corrected	n-3 Fatty acids (wt%)
	P	MA			
10.24	18,423	23,398	0.7874	0.7689	24.76
	19,046	23,714	0.8032	0.7844	25.29
	21,256	26,632	0.7981	0.7794	25.12
9.37	19,566	28,407	0.6888	0.7351	23.58
	18,210	26,325	0.6917	0.7382	23.69
	19,694	28,509	0.6908	0.7372	23.66
9.75	21,717	29,397	0.7387	0.7576	24.37
	21,196	28,330	0.7482	0.7674	24.70
	20,471	27,571	0.7425	0.7615	24.50

[a]Abbreviations as in Table 2.

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