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Nervonic acid *versus* tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain $n-3$ polyunsaturated fatty acid methyl esters

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ABSTRACT

Tricosanoic acid (23 0) and *cis*-15-tetracosenoic acid (nervonic acid, 24·1$n-9$) were compared as choices suitable for use as internal standards in the quantitation of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids Experiments conducted included (a) comparison of the flame ionisation detector responses of the two fatty acid methyl esters, (b) estimation of accurately weighed quantities of EPA and DHA using both 23 0 and 24 1 separately as internal standard, (c) determination of EPA and DHA contents of commercially available fish oil ethyl ester capsules using the two as internal standard The results suggest that both 23 0 and 24 1 methyl esters behaved similarly in the flame ionization detector of the gas chromatograph and are comparable internal standards for use in quantitation of EPA and DHA. This includes the analysis of ethyl ester mixtures as long as interesterification of sample with solvent methanol is complete The relatively poor solubility of the saturated 23 0 is countered by its greater stability A possible drawback of 24 1 could be the presence of more than one positional isomer in either a 24·1$n-9$ standard or in the actual sample In principle any fatty acid could serve as an internal standard as long as the limitations involved in the use of each are taken into account

INTRODUCTION

The most common way to quantitate the $n-3$ polyunsaturated fatty acids (PUFA) in fish oil is by gas chromatography of their methyl esters [1]. Since, in theory, there could be some sample loss at almost every step during ester preparation, the preferred practice should be to add an internal standard (I.S.). Optimally, the I.S. should have physicochemical properties sufficiently similar to the compound of interest so that both would behave identically during the work-up procedure [2]. In the past, researchers have used different fatty acids, often saturated fatty acids of varying carbon chain lengths, as I.S. for the analysis of long-chain PUFA in lipid samples. Gerber *et al.* [2] found that the use of tricosanoic acid (23:0) for measurement of arachidonic acid (20:4$n-6$) in plasma was unsatisfactory due to its poor extractability compared to plasma lipids and have suggested the use of dihomo-γ-linolenic acid (20:3$n-6$) as the I.S. Hibino *et al.* [3], however, contend that 23.0 could be a suitable choice of I.S. for quantitation

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of biologically important PUFA. Recently, Emig and Ackman [4] recommended the use of 23:0 as an I.S. in the analysis of the $\omega - 3$ polyunsaturated fatty acids in marine oil products. Tricosanoic acid was chosen because its relative retention time on contemporary bonded Carbowax 20M capillary columns does not coincide with any other fatty acid. In addition it is not susceptible to oxidation and its response under optimised conditions is similar to the fatty acids of interest [5]. Ackman *et al.* [6] have applied this method to the determination of eicosapentaenoic (EPA; 20:5$n - 3$) and docosahexaenoic acid (DHA; 22:6$n - 3$) contents of encapsulated fish oil products. The interlaboratory trial of an AOAC protocol [7] for the determination of EPA and DHA in fish oils and concentrates via capillary gas chromatography (GC) and with 23:0 as the internal standard has been carried out and assessed as successful [8] However, comments, questions and unforseen difficulties were an inevitable fallout from this trial, with a tendency to blame the method for all deficiencies.

The AOAC project has now been further extended in our laboratory to see whether *cis*-15-tetracosenoic acid (nervonic acid, 24:1$n - 9$), in view of its greater solubility, could prove to be a suitable substitute for 23.0 as an I.S. The flame ionization detection (FID) responses of the two fatty acid methyl esters (23.0 and 24:1) were compared. Accurately weighed amounts of EPA and DHA and its contents in commecially available $\omega - 3$-enriched ethyl ester capsules were determined using both 23:0 and 24:1 separately as the I.S. The advantages and disadvantages of the use of either as I.S. are discussed

EXPERIMENTAL

cis-15-Tetracosenoic (nervonic acid) was purchased from Serdary Research Lab. (London, Canada). Pure methyl tricosanoate (Sigma, St. Louis, MO, U S.A.) and ethyl esters of EPA and DHA (Walgreen Labs. and Norsk Hydro) were available in the laboratory. The position of the double bond in the nervonic acid sample was determined by ozonolysis followed by analysis on GC instrumentation [9]. This lot of fatty acid was found to contain 96% $n - 9$ isomer, the rest being $n - 11$ (2.5%) and $n - 13$ (1.5%) isomers; 24·1$n - 7$ was insignificant. Ethyl esters of EPA and DHA which were found to be 95% pure were further purified by semi-preparative high-performance liquid chromatography (HPLC) using a C_{18} Bondapak column (10 cm × 0 8 cm I.D , particle size 10 μm). Methanol–water (95:5, v/v) at a flow-rate of 1.5 ml/min was used as the mobile phase. The pure ethyl esters were transesterified to give methyl esters by using the procedure described below. All esters were checked for purity by both GC and thin-layer chromatography (TLC) with the Chromarod–Iatroscan TLC–FID system based on a flame ionisation detector [10] All stock solutions were prepared in redistilled isooctane.

For the preparation of methyl esters, the method suggested in the AOAC collaborative study [7] was employed, modified for convenience of scale as fol-

lows. Briefly, to about 25 mg of the sample weighed into a screw-cap test tube, 1.5 ml of 0.5 *M* methanolic sodium hydroxide solution were added The tube was capped tightly, vortexed and heated at 100°C for 5–7 min. The solution was cooled, 2 ml of 14% boron trifluoride–methanol (Supelco, Bellefonte, PA, U.S.A.) were added, and the tube capped, vortexed and block-heated at 100°C for 15–20 min. The solution was cooled to 30–40°C, 1 ml of isooctane was added, the contents were vortex-mixed and then shaken vigorously for 30 s while tepid. Saturated sodium chloride solution (5 ml) was added, and the contents were agitated thoroughly. The solution was centrifuged, and the isooctane layer transferred into a second tube. The methanol–water phase was again extracted with 1 ml of isooctane. The isooctane extracts were combined and, when necessary, concentrated before analysis by GC. All operations were carried out under nitrogen.

GC analysis was carried out on a Perkin-Elmer 900 gas chromatograph, equipped with a flame ionization detector and digital integrator, using a DB-WAX column (30 m × 0.25 mm I.D., phase thickness 0.25 μm; J. & W. Scientific, Folsom, CA, U.S.A.). The conditions used were: oven temperature, isothermal (200°C); injection port temperature, 250°C; split ratio, 1:58; helium (carrier gas) flow-rate, 1 31 ml/min. For obtaining a reference chromatogram samples of fatty acid methyl esters of menhaden oil and fish oil ethyl ester concentrates were also analysed on a Perkin-Elmer 8420 gas chromatograph, using a Supelcowax-10 fused-silica capillary (30 m × 0 32 mm I D) column. The GC analysis was temperature-programmed from 195 to 240°C at 3°C/min after an initial 8 min at 195°C, and was held at 240°C for 10 min. GC analysis was also run isothermally at 200°C. The other parameters were: split ratio, 1:32; (helium carrier gas) flow-rate, 1.2 ml/min; injection port temperature, 250°C. A Perkin-Elmer LC1-100 laboratory computing integrater was used for recording and integration. One-way and two-way analysis of variance (ANOVA) was conducted using a mini-computer program. The 5% level of significance was maintained.

The 24:1 and 23:0 as I.S. were compared by conducting the following experiments.

Experiment 1: comparison of the FID responses of 23·0 and 24.1 methyl esters

Accurately weighed amounts of 23:0 and 24:1 methyl esters were analysed by GC The experiment was repeated four times, either by weighing the esters directly on a balance (up to 0.00001 g accuracy) or by pipetting from concentrated standard stock solutions. The latter was done in order to reduce error, if any, in weighing, and since this would be the common practice with the I.S. in actual operation. Each sample, a mixture of 23:0 and 24:1 in isooctane (1–2 μl), was injected a minimum of five times manually, using a Hamilton No. 701 syringe. Later, the analysed mixture was further treated with boron trifluoride–methanol as above and reanalysed by GC

Calculations were done in the following manner:

$$\text{actual weight ratio} = \frac{\text{weight of 23:0}}{\text{weight of 24:1}}$$

$$\text{observed GC weight ratio} = \frac{\text{area counts}_{23\,0} \times \text{TRF}_{23\,0}}{\text{area counts}_{24\,1} \times \text{TRF}_{24\,1}}$$

where TRF = theoretical response factor [5].

Experiment 2: calculation of weights of pure EPA and DHA using 23:0 and 24:1 as I.S.

Stock solutions of 23:0 and 24:1 methyl esters in isooctane were prepared in duplicate. Single accurately weighed stock solutions of 20:5$n-3$ and 22:6$n-3$ methyl esters were prepared separately. Samples were prepared with 1.00 ml of each of the PUFA esters and 1.00 ml of either of the two I.S. A total of six mixtures were made, and each mixture was analysed four times by GC.

The calculations were done as follows:

FAME	Area counts (AC)	TRF	AC × TRF	Calculated amount of EPA or DHA based on I.S.
I.S	A	X	AX	
22:6	B	Y	BY	$W \times \frac{BY}{AX} = W_1$
20:5	C	Z	CZ	$W \times \frac{CZ}{AX} = W_2$

where W = weight of internal standard, W_1 = weight of DHA and W_2 = weight of EPA

Experiment 3: determination of EPA and DHA contents in various fish oil ethyl ester capsules using 23.0 and 24:1 methyl esters as I.S.

Three retail samples of fish oil concentrates in ethyl ester form were chosen. Aliquots of 1.00 ml of the I.S. (methyl ester with a concentration of about 4 mg/ml) were pipetted into screw-cap test tubes, and the solvent was evaporated. An accurately weighed amount (about 25 mg) of fish oil ethyl ester from a freshly opened capsule was added to this and then transesterified to methyl esters and analysed by GC. The amount of EPA and DHA, expressed as mg of EPA and DHA fatty acid per g of sample, were calculated in the following manner:

$$\text{EPA or DHA (mg/g)} = \frac{(A_X)(W_{IS})(CF_X)}{(A_{IS})(W_s)(1.08)} \times 1000$$

where A_X = area counts of EPA or DHA, $A_{IS.}$ = area counts of internal standard, CF_X = theoretical correction factor for EPA or DHA calculated based on I.S., W_{IS} = weight of I.S. added to the sample (in mg), W_S = sample weight (in mg) and 1.08 = conversion factor [7].

RESULTS

Table I shows the comparison between the FID responses of 23:0 and 24:1 by GC. It can be seen that the observed mean ratio of actual/observed weight (1.00 ± 0.015) was not significantly different ($p > 0.05$) from the calculated theoretical value (1.010). Table II compares the weights of EPA and DHA calculated from the use of 23:0 and 24:1 as I.S. The weights of both EPA and DHA calculated by using 23:0 as I.S (6.83 ± 0.41 and 4.22 ± 0.14, respectively) and 24:1 as I.S. (6.92 ± 0.39 and 4.25 ± 0.21, respectively) did not differ significantly ($p > 0.05$) among themselves nor in relation to the actual weights taken (6.98 and 4.30, respectively).

Table III gives the results of the EPA and DHA determined in three different fish oil ethyl ester capsules. For all three capsules of different origins, identified as A, B and C, the weights of EPA and DHA calculated using 23.0 as I.S. were not significantly different ($p > 0.05$) from those calculated using 24:1 as the I.S.

DISCUSSION

From the above results, one can conclude that both 23:0 and 24:1 behaved similarly in the FID system of the GC and are comparable and satisfactory I.S. for use in quantitation of EPA and DHA. Earlier, Wilson [11] had made a comparison between 23:0 and 21:0 for use as I.S. in the estimation of erucic acid

TABLE I

COMPARISON OF FID RESPONSES OF 23 0 AND 24 1 BY GC

Values represent mean ± S D ($n = 5$), no significant difference between values by ANOVA ($p > 0.05$). Calculated theoretical response factor ratio = 1 01

Trial	Weight taken (mg)		Ratio actual/observed weights[a]
	23:0	24 1	
1	5 9000	5 7000	1 02 ± 0 01
2	4 0856	6 4160	0 98 ± 0 01
3	4 0856	6.4160	1 01 ± 0 02
4	4 2000	4.6300	0 99 ± 0 01
Average of experimental ratios			1 00 ± 0 015

[a] Calculated from peak areas of GC chromatogram

TABLE II

CALCULATION OF WEIGHTS OF PURE EPA AND DHA USING 23:0 OR 24 1 AS INTERNAL STANDARD

Values calculated by weight are represented as mean ± S D (n = 4); no significant differences between values (obtained by using 23:0 or 24 1 as I S) by ANOVA (p > 0 05)

Trial	Weight of I S (mg)		Calculated weight (mg)		Average calculated weight[a] (mg)	
	23·0	24 1	EPA	DHA	EPA	DHA
1	4 0856	–	6 81 ± 0 15	4 25 ± 0 04		
2	4 0856	–	6 98 ± 0 63	4 23 ± 0 20	6 83 ± 0 41	4.22 ± 0.14
3	3 5280	–	6 69 ± 0 15	4 17 ± 0 15		
4	–	4.6300	6 81 ± 0 28	4 20 ± 0 12		
5	–	4.6300	6 77 ± 0 52	4 19 ± 0 16	6 92 ± 0.39	4.25 ± 0.21
6	–	3.5420	7 17 ± 0 32	4 35 ± 0 32		

[a] Actual weights of EPA and DHA were 6 98 and 4.30 mg, respectively.

TABLE III

DETERMINATION OF EPA AND DHA IN CAPSULES OF FISH OIL ETHYL ESTERS

Values calculated by weight are represented as mean ± S D (n = 4). no significant difference between sets by ANOVA (p > 0 05)

Trial	Ethyl ester retail type	Weight of I S (mg)		Weight of acid (mg/g of capsule)	
		23 0	24 1	EPA	DHA
1	A	4 0856	–	371 3 ± 26 0	236 3 ± 8 3
2		–	4 6300	367 7 ± 7 2	236 3 ± 2 7
3	B	3.5280	–	197.4 ± 10 8	157 3 ± 1.5
4		–	3 5420	200 9 ± 3 8	160 3 ± 1 5
5	C	3.5280	–	420 0 ± 10 5	248 2 ± 5 5
6		–	3 5420	421 4 ± 21 2	253 6 ± 4 8

content in rapeseed Using a packed column he concluded that the results obtained from use of either I.S. were not significantly different. This would be an expected observation as long as the relative response factors of unsaturated and saturated fatty acid methyl esters determined in practice conform to theory [12]. Hence, any fatty acid could serve as I.S. However, consideration of the relative retention times narrows the choice of I.S. [13]. For fish oils with a complex pattern of fatty acids, the retention time of 23:0 on polyethylene glycol-based columns fits well between EPA and DHA (Fig. 1.) Under the same conditions 24:1n − 9 elutes just after 22:6n − 3. One of the main drawbacks with the use of heptadecanoic acid (17:0) as an I.S. for the determination of EPA and DHA in

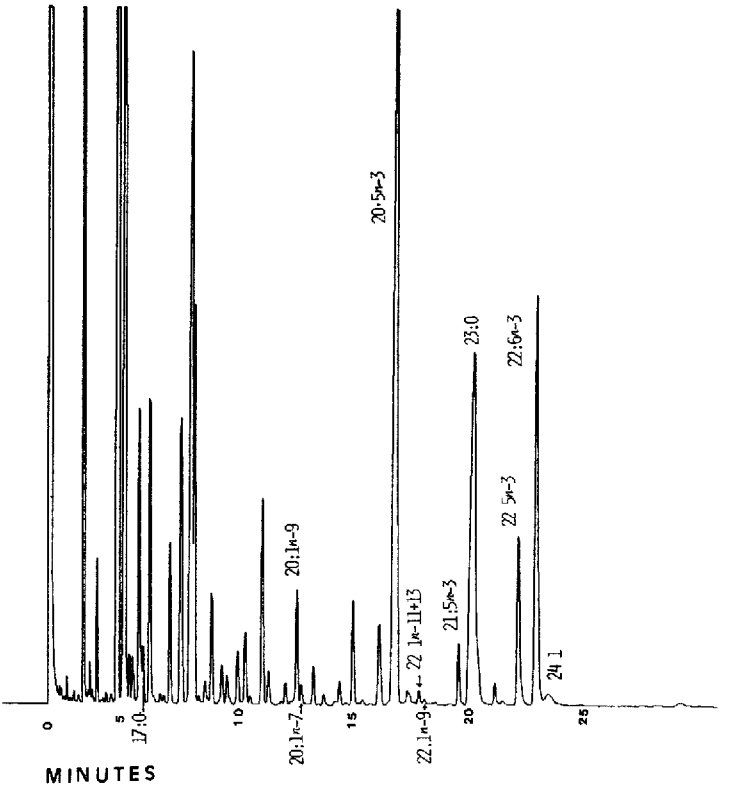

Fig 1. Gas chromatogram of fatty acid methyl esters of menhadan (*Brevoortia tyrannus*) oil containing methyl tricosanoate (23 0) as internal standard Column, Supelcowax-10 fused-silica capillary (30 m × 0 32 mm I D) The GC analysis was temperature programmed from 195 to 240°C at 3°C/min after an initial 8 min at 195°C and was held at 240°C for 10 min The other parameters were: split ratio, 1 32, helium (carrier gas) flow-rate, 1 2 ml/min, injection port temperature. 250°C Abbreviations 17 0 = methyl heptadecanoate, 20 5*n* − 3 = methyl eicosapentaenoate; 21:5*n* − 3 = methyl heneicosapentaenoate, 22 6*n* − 3 = methyl docosahexaenoate, 20 1 = methyl eicosenoate; 22 1 = methyl docosenoate, 24 1 = methyl tetracosenoate (different isomers are indicated by the number showing the position of the double bond)

fish oils is its much shorter retention time compared to EPA ans DHA (Fig. 1), moreover it is a minor ($< 1\%$) component in most fish oils [14]. Nervonic acid, in view of its greater solubility compared to 23:0, could serve as a more suitable I.S for analysis of the PUFA contents in EPA- or DHA-enriched fish oil concentrates as long as the fish oil fatty acids lack 24:1n − 9 in their composition.

One of the negative aspects of using 24:1n − 9 as an I S. is that most original fish oils contain a very small percentage ($< 1\%$) of 24:1, one major isomer being specifically 24:1n − 9 or nervonic acid [15]. The 24:1 is often removed during concentration steps (*cf.* Fig. 2), along with 20:1 and 22:1 [16], but the method should be broadly applicable to raw oils as well as concentrates. Although under the alternative isothermal conditions used the 22:6 and 24.1 were well resolved (*R*

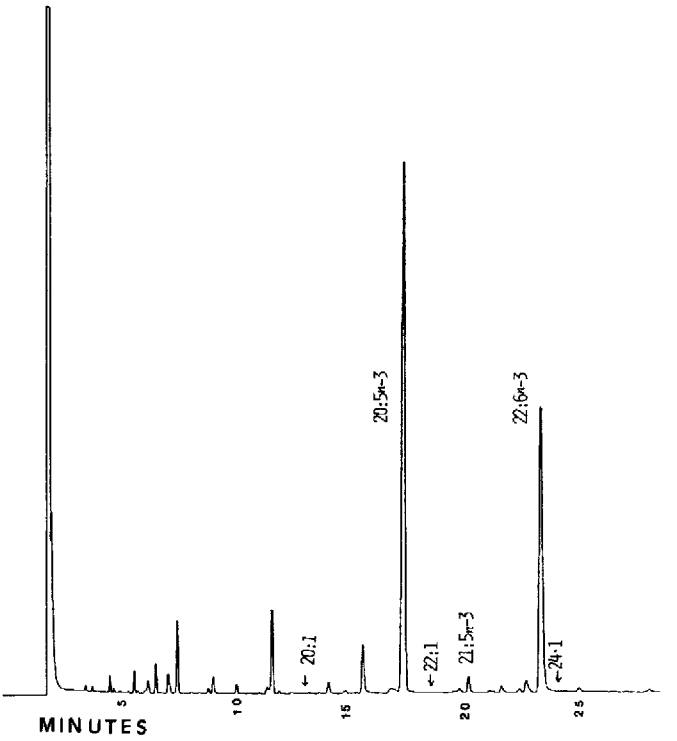

Fig 2. Gas chromatogram of fatty acid methyl esters of a retail fish oil ethyl ester concentrate Column, operating conditions and abbreviations as in Fig. 1 Note absence of 20 1 22 1 and 24 1 The anticipated locations of these fatty acids have been marked.

> 1.0, Fig. 3A), for the normally used temperature programming the resolution between 22:6n − 3 and 24:1n − 9 may not be adequate for accurate quantitation (Figs 1 and 3C). This proximity of 24:1n − 9 to 22:6n − 3 in polyethylene glycol columns could limit its applicability if column efficiency is low or the proportion of 22:6n − 3 is large. Fig 3C illustrates the type of integrator problem arising due to improper resolution between peaks in a real fish oil analysis. The integrator was found to overestimate the 24:1 by a significant amount due to its proximity to the 22:6n − 3 peak on a Supelcowax-10 column under the conditions used. Papas and Tougas [17] have also described such integration problems. This difficulty may not apply if other liquid phases were used. The same Supelcowax-10 column under isothermal conditions gives a better resolution between 22:6n − 3 and 24:1n − 9 (Fig. 3B). In most cases the peak for 24:1 in marine oils, on Carbowax-20M-type columns and with temperature programming, is 24:1n − 9 since the earlier eluting peak for 24:1n − 11 (plus n − 13 and possibly n − 15) falls under 22:6n − 3 [15] Hence, if only 20:5n − 3 and 22:6n − 3 are needed it would be easier and more accurate to run the column isothermally at 200 or 210°C to speed up the analyses.

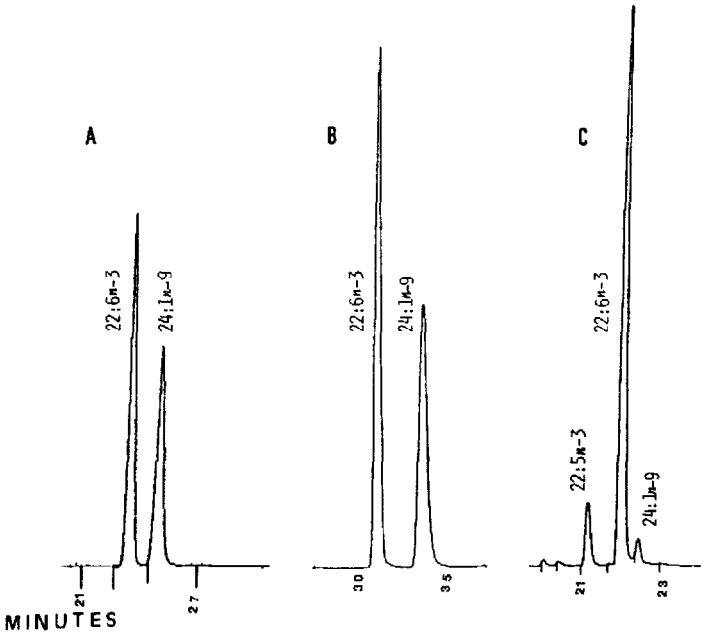

Fig 3. (A, B) Partial gas chromatograms showing resolution between 22 6$n-3$ and 24 1$n-9$. (A) Column DB-WAX fused-silica capillary (30 m × 0.25 mm I.D.) Oven temperature, isothermal (200°C), injection port temperature, 250°C, split ratio, 1 58, helium (carrier gas) flow-rate, 1.31 ml/min. (B) Column, Supelcowax-10 fused-silica capillary (30 m × 0 32 mm I D.) Oven temperature, isothermal (200°C), other conditions as in Fig 1 (C) Partial gas chromatogram of fatty acid methyl esters of cod (*Gadus morhua*) liver oil Column and conditions similar to Fig 1 Abbreviations as in Fig 1

Potentially, 24:1 is relatively less stable than 23:0, however, no relative loss of 24·1 was observed following the further re-esterification. The 23:0 and 24:1 fatty acid methyl ester mixture when treated with boron trifluoride–methanol and re-analysed by GC had the same ratio of actual/observed weights as before.

In the AOAC trial [7] the ethyl ester of 23·0 was provided to accelerate and simplify the determination of 20:5$n-3$ and 22:6$n-3$ in ethyl esters of fish oil fatty acids. The molar ratio of ethanol (in the ethyl ester) to methanol in the procedure followed in this study was heavily weighted in favor of methanol. No extraneous (*i.e* ethyl ester) peaks were detected in chromatograms of methyl esters and therefore interesterification must have been total. The objective of this method is to report 20:5$n-3$ and 22:6$n-3$ *acid* contents of samples and it is therefore convenient to use the methyl ester as an all-purpose internal standard.

In conclusion 23:0 and 24:1 methyl esters are comparable I.S. for use in quantitation of EPA and DHA. The relatively poor solubility of 23:0 is countered by the greater stability of 23:0. The possibility of the presence of positional isomers in commercial 24·1$n-9$ standards, some of which might fall under 22:6$n-3$ depending upon the efficiency and polarity of the column [15], and of 24:1$n-9$ itself in the sample are drawbacks to its use Either fatty acid could serve as I.S. as

long as the limitations involved in the use of each are taken into account. It should also be noted that not all polyethylene glycol-based bonded flexible fused capillary columns give adequate separation between 23:0 and the commonly present heneicosapentaenoic acid (21:5$n-3$). Since 21:5$n-3$ is a common constituent [18] of fish oils (Figs. 1 and 2) this point is easily evaluated, when a new column is installed, with cod liver oil or any other raw fish oil.

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