Validation of a Method for Gas Chromatographic Analysis of Eicosapentaenoic Acid and Docosahexaenoic Acid as Active Ingredients in Medicinal Products[1]

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A gas chromatographic method for the determination of all-*cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and all-*cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) as active ingredients in medicinal products was developed and validated. In accordance with the rules governing medicinal products in the European Community, the method establishes relations between label claims of active ingredients and known reference standards. A routine for examining instrument status is proposed. The relative standard deviation was 1% (n = 26) for determination of the empirical response factors of EPA ethyl ester and DHA ethyl ester relative to the internal standard, C23:0 methyl ester. This experiment included two columns and EPA and DHA standards from two different suppliers and was carried out over a five-month period. Repeatability (n = 6) for low and medium concentrates of glycerides and high concentrates of ethyl esters, expressed as coefficient of variation, was 4, 0.7 and 0.7%, respectively. Accuracy (n = 6) determined as percent recovery was better than 98% for all sample types. Analytical results from a twelve-month stability study of the high concentrate are shown.

KEY WORDS: Capillary gas chromatography, docosahexaenoic acid, eicosapentaenoic acid, fish oil.

Quantitation of all-*cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and all-*cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) has received a lot of attention in the last five years (1–3). The main reason is the many fish oil concentrates intended as dietary supplements of these important n-3 polyunsaturated fatty acids (PUFA) (4–6) now available. Their quality as dietary supplements is directly linked to the content of these two polyunsaturated fatty acids. However, price and label claims may not necessarily contain adequate information for the customer, as specifications are always related to the actual test procedures used. The absolute difference between results from different methods normally increases with increasing concentration of the analyte, resulting in demands for better precision and accuracy of test procedures for the highly concentrated products. Official test procedures (7–9) have been used with packed columns and wall-coated open tubular capillary columns. With these methods the area percentage achieved for each fatty acid in question is reported as the actual content. Unresolved peaks, different responses, injection techniques and instrument set-up may cause different results in different laboratories.

Fish oil concentrates occur both in the form of glycerides and ethyl esters, and may contain impurities from the manufacturing process that are not detected under these chromatographic conditions (*e.g.*, solvents and polymers). The complexity of fish oils and the instability of PUFA made it desirable to have a dedicated method for samples derived from fish oils, especially for the quantitative determination of EPA and DHA.

In 1987, Einig and Ackman (1) proposed a gas-liquid chromatographic (GLC) method with use of a wall-coated open tubular capillary column with split injection and tricosanoic acid as an internal standard. The AOCS Official Method Ce 1b-89 was based on the same principles, but used theoretical instead of empirical response factors. Analysis of n-3 concentrates with this method almost exclusively showed lower values than claimed on the label for samples obtained from retailers (4). Recently, a method equivalent to the AOCS method was adopted by the AOAC (5).

Marketing n-3 concentrates as drug formulations requires that toxicological studies, clinical studies and stability studies are performed with the product. For such products governmental authorities set demands for validation of the analytical test procedures used in quality control and stability studies (10–13). One of the test procedures in our laboratory is the determination of EPA and DHA in the different n-3 concentrates for documentation as medicinal products. The European Community puts demands on test procedures for determination of active ingredients in medicinal products. One of these is that there must be a relationship between label claims and known reference standards of the active ingredients (13).

A test procedure to be used in such circumstances should clearly differentiate between two samples having the same fatty acid profile but different contents of undetectable components, for instance, solvents and polymers. The precision has to be documented and should be equal to or less than 2% for products with total EPA and DHA greater than 50% and equal to or better than 5% for products with total EPA and DHA less than 50%. The method also should be able to detect all peaks equal to or greater than 0.05% of total peak area with sufficient accuracy.

This paper outlines the steps in method development, elucidating factors having effects upon the quantitation of EPA and DHA and shows results validating the final test procedure.

EXPERIMENTAL PROCEDURES

Materials. The high concentrate (ethyl ester mixture) was produced at Norsk Hydro a.s. (Porsgrunn, Norway). Medium and low concentrates were produced at J. C. Martens a.s. (Sandefjord, Norway). Standards of 5,8,11,14,17-EPA ethyl ester were made from the high concentrate by preparative high-performance liquid chromatography (HPLC) (>96%) or purchased from Idemitsu Petro Chemical Co. Ltd. (Tokyo, Japan) (>99%). Standards of 4,7,10,13,16,19-DHA ethyl ester were made from the high concentrate by preparative HPLC (>92%) or purchased from Idemitsu (>99%). Boron trichloride-methanol

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reagent was purchased from Sigma Chemical Company (St. Louis, MO). Boron trifluoride-methanol reagent and the quantitative standard mixture (GLC-40) of C16:0ME, C18:0ME, C20:0ME and C22:0ME were purchased from Supelco Inc. (Bellefonte, PA). The 1,1,3,3-tetramethyl guanidine, 2,6-di-*tert*-butyl-4-methyl-phenol (BHT), *n*-hexane (>99.5%) and *iso*-octane (>99.5%) were purchased from Fluka Chemie AG (Buchs, Switzerland). The C16:0ME, C18:0ME, C20:0ME and C20:4ME (arachidonic acid methyl ester) were purchased from NuChek-Prep (Elysian, MN).

GLC. Analytical GLC was carried out on a Supelcowax 10 capillary column, 30 m × 0.25 mm i.d., D_f 0.25 μm (Supelco Inc.). The column was installed in a Carlo Erba Vega Model 6130 gas chromatograph (Carlo Erba Instrumentation, Milano, Italy) with an AS-V570 autosampler with injection in the split mode. The detector was coupled to a VG Multichrom chromatography data system (VG Laboratory Systems Ltd., Altrincham, England). Helium carrier gas (purity >99.9999%) was used at a pressure of 120 kPa, which gave a flow of 0.7 mL/min. A Supelco high-capacity gas purifier and an OMI-1 indicating purifier, both from Supelco, were put in the carrier gas line to remove water and oxygen. The temperature program was isothermal at 170°C for 0.5 min, then 10°/min to 240°C, then isothermal at 240°C for 22 min (170–240°C, 1°/min is recommended for medium and low concentrates). The flame-ionization detector was operated with air pressure of 100 kPa and hydrogen pressure of 60 kPa. The injector temperature was 250°C and that of the detector was 270°C.

Thin-layer chromatography and flame ionization detection (TLC-FID). The purity of the standards was checked by TLC-FID analysis as described by Einig and Ackman (1).

High concentrates. The empirical response factor of EPA ethyl ester relative to tricosanoic acid methyl ester was determined by injecting 1 μL of a mixture of 88 mg EPA ethyl ester standard and 70 mg tricosanoic acid methyl ester in 10 mL *iso*-octane containing 50 mg BHT/L. The empirical response factor of DHA ethyl ester relative to tricosanoic acid methyl ester was determined by injecting 1 μL of a mixture of 55 mg DHA ethyl ester standard and 70 mg tricosanoic acid methyl ester in 10 mL *iso*-octane containing 50 mg BHT/L. Approximately 175 mg of sample and 70 mg of tricosanoic acid methyl ester were dissolved in 10 mL *iso*-octane containing 50 mg BHT/L. The sample solution (1 μL) was injected onto the column. The areas of the internal standard, the EPA and DHA peaks and the empirical response factors were determined as described above. The areas were used to calculate the content of EPA ethyl ester and DHA ethyl ester by the internal standard method.

Medium and low concentrates. About 88 mg EPA ethyl ester standard, 70 mg tricosanoic acid methyl ester and 55 mg DHA ethyl ester standard were dissolved in 10 mL of *n*-hexane containing 50 mg BHT/L. Approximately 450 mg low concentrate (290 mg medium concentrate) and 70 mg tricosanoic acid methyl ester were dissolved in 10 mL *n*-hexane containing 50 mg BHT/L. For sample and standard, 2.0 mL was pipetted into 20-mL pyrex tubes, and the solvent was evaporated with a gentle stream of nitrogen. Then, 1.5 mL of 0.5M NaOH in methanol was added, and the solution was covered with nitrogen. The pyrex tube was sealed with a screw cap with a teflon-lined rubber liner and heated to 100°C for 7 min. The mixture was cooled and 2 mL of BF_3/MeOH reagent was added. The pyrex tube was purged with nitrogen, sealed and heated to 100°C for 30 min. The mixture was cooled to 40–50°C, 1 mL of *iso*-octane was added and vortexed for at least 30 s. Then 5 mL of saturated NaCl solution was added and the sealed tube was vortexed for another 30 s. The *iso*-octane and water phases were allowed to separate. The *iso*-octane layer was transferred to a separate tube. The aqueous phase was extracted with an additional 1 mL of *iso*-octane and combined with the first extract. The *iso*-octane phase was washed twice with 1 mL of distilled water, dried over anhydrous sodium sulfate (Na_2SO_4) crystals and filtered through a Millex-HA 0.45 μm filter (Millipore, Milforth, MA) into the autosampler vial. Then, 1 μL of the sample or standard was injected. The derivatized standard was used for the calculation of empirical response factors for EPA and DHA relative to tricosanoic acid.

Routine optimization of gas chromatograph. The glass liner was silane-treated and filled with silane-treated glass wool. Injection of the GLC-40 mixture containing equal weights of C16:0ME, C18:0ME, C20:0ME and C22:0ME will, from relative theoretical response factors (14), give the area percent composition 24.4%, 24.8%, 25.2% and 25.6% for the C16, C18, C20 and C22 methyl esters, respectively, for an optimized instrument. The instrument is at optimal conditions if the area percentages of the fatty acid methyl esters in the GLC-40 standards are reading within 1% (absolute) of the theoretical values.

Liner and glass wool should be changed and 10 cm of the column on the injector end should be cut off when the responses for EPA and DHA have decreased relative to the internal standard because of contamination of the injector and column inlet. After reinstallation of the column and a clean liner and glass wool, the accuracy can be checked by analyzing the GLC-40 mixture.

RESULTS

Instrument optimization. Split injection on a WCOT column with a polyethylene glycol-type liquid phase (Supelcowax 10) was chosen because sample amount is not a limitation in quality control analyses and because the column is recommended in the literature (15).

Discrimination between high- and low-boiling components in a complex sample is a problem with split injection. Parameters that may affect the relative areas measured for the fatty acids in a GLC analysis are injector design, injection volume, injection temperature (16), injection technique (17–22), detector conditions (23,24) and derivatization conditions (25–28). This implies that different instruments normally give different results. However, optimization against a standard may reduce the difference to an acceptable level.

Injection of 1 μL of the quantitative standard mixture (GLC-40) with a split of 26 mL/min showed a strong discrimination against the higher boiling components in the mixture (Table 1). Analysis of a mixture containing 60.0% of EPA ethyl ester and 35.8% of DHA ethyl ester by weight under these conditions gave an area percent composition of 65.3 and 31.3%, respectively, showing strong discrimination of DHA ethyl ester relative to EPA ethyl

TABLE 1

Area Percentage (A%) from Injection of 1 μL of a Mixture (GLC-40) Containing Equal Amounts of C16:0ME, C18:0ME, C20:0ME and C22:0ME with Split Flow 26 mL/min

C16:0ME		C18:0ME		C20:0ME		C22:0ME	
wt%	A%	wt%	A%	wt%	A%	wt%	A%
25.0	29.7	25.0	25.5	25.0	23.2	25.0	21.6

ester. The effect of varying injection volume and split flow was examined by analyzing the more stable standard mixture (GLC-40) containing equal amounts of the methyl esters of C16:0, C18:0, C20:0 and C22:0 fatty acids. Changing injection volume between 0.50, 0.75 and 1.00 μL showed no effect (data not shown). Changing the split flow from 50 to 200 mL had little effect upon the discrimination (Table 2).

The design of liners may vary from one type of instrument to another. For our instrument, the standard design was a cylindrical glass tube, 8 cm × 2 mm i.d. Other instrument suppliers have liners constructed to give optimal mixing of sample and carrier gas and have documented that they reduce the discrimination in samples containing both high- and low-boiling components (29,30). Our experimental conditions introduced discrimination into the samples, so the injection conditions were changed by inserting silane-treated glass wool into the liner and by setting the split flow to 200 mL/min. The results are shown in Table 3, where it can be seen that the discrimination was virtually eliminated. Table 3 also compares the results with analysis on an identical instrument with the same modification of the liner, but a different column and manual injection (line 2, Table 3), and with on-column injection on the same type of instrument and same column type (line 3, Table 3).

TABLE 2

Composition of Standard Mixture (GLC-40) Expressed as Area Percentage for Different Split Flows

Split (mL/min)	C16:0ME	C18:0ME	C20:0ME	C22:0ME
50	27.48	25.25	24.14	23.23
100	27.61	25.47	24.05	22.87
150	27.35	25.36	24.20	23.08
200	26.37	25.45	24.54	23.64
wt%	25.00	25.00	25.00	25.00

Test of derivatization procedures. In the determination of EPA and DHA in fish oil and of n-3 concentrates, tricosanoic acid methyl ester was used as the internal standard. When analyzing glycerides, a well-characterized ethyl ester concentrate was used as a standard. The standard and the sample were derivatized according to the procedure for low and medium concentrates. When the empirical response factors for EPA and DHA relative to tricosanoic acid methyl ester were calculated, the responses were lower for the derivatized than for the nonderivatized standards. The differences were significantly greater than expected between ethyl and methyl esters from consideration of theoretical correction factors. There are three possible causes for this discrepancy: i) incomplete derivatization; ii) incomplete extraction of polyunsaturated fatty acids relative to the saturated internal standard; and iii) degradation of polyunsaturated fatty acids in the derivatization step. Complete derivatization was confirmed by the absence of ethyl ester peaks in the chromatograms.

In the literature (6,7,25), several organic solvents are used for the extraction of fatty acid methyl esters after derivatization of triglycerides. The effect of extraction conditions upon the determination of fatty acid content was tested. A mixture of methyl palmitate, ethyl stearate, methyl eicosanoate, methyl arachidonate, EPA ethyl ester, DHA ethyl ester and methyl tricosanoate in *iso*-octane (mixture A), and a mixture of 15 mL of 0.5M NaOH in methanol, 20 mL of 12% BF_3 in methanol and 50 mL saturated aqueous NaCl solution (mixture B), were prepared. Mixture B simulated a "dead" reaction mixture after derivatization. Undiluted mixture A was used as the standard for calculations. Several different extraction procedures (Table 4) were examined. The different extraction procedures gave the same results in quantitation of the PUFA.

A mixture of methyl palmitate, ethyl stearate, methyl eicosanoate, EPA ethyl ester, DHA ethyl ester and methyl

TABLE 3

Composition of Standard Mixture (GLC-40) Expressed as Area Percentage for Different Columns and Injection Techniques Compared with Theoretical Values and Weight Composition Given by the Manufacturer

C16:0ME	C18:0ME	C20:0ME	C22:0ME	Column/injection technique
24.57	25.00	25.18	25.24	Supelcowax 10/split[a]
24.30	24.83	25.49	25.38	CP sil 88/split[a]
25.09	24.86	25.12	24.94	DB wax/on column[b]
25.00	25.00	25.00	25.00	wt%
24.37	24.84	25.23	25.56	Theoretical value

[a]Split flow 200 mL/min; carrier gas flow 0.7 mL/min. Supelcowax 10 from Supelco (Bellefonte, PA).

[b]Temperature program: isothermal at 80°C for 2 min, then 30°/min to 170°C, then 10°/min to 240°C, then isothermal at 240°C for 22 min.

TABLE 4

Fatty Acid Composition (mg) in a Mixture Before and After Extraction by Different Procedures (mean of two analyses)

Sample	C16:0ME	C18:0EE	C20:0ME	C20:4ME	C20:5EE	C22:6EE
1[a]	53.8	53.8	50.9	50.0	86.4	64.3
2[b]	52.4	54.2	51.4	50.5	87.2	64.5
3[c]	52.6	54.2	51.8	50.8	87.6	65.0
4[d]	54.5	54.4	51.8	50.4	87.0	64.4
5[e]	52.5	54.6	52.1	50.9	87.7	64.5
6[f]	54.4	55.0	52.1	51.0	87.8	64.9
7[g]	51.5	53.9	51.7	50.2	86.9	64.5
8[h]	50.9	53.9	51.7	50.4	87.0	64.7

[a]1 mL of A.

[b]1 mL of A + 1 mL isooctane.

[c]1 mL of A + 2 mL isooctane.

[d]1 mL A + 7.5 mL B.

[e]1 mL A + 7.5 mL B + extraction with 1 mL isooctane.

[f]1 mL A + 7.5 mL B + extraction with 1 mL isooctane + 2 mL water wash + Na_2SO_4 drying.

[g]1 mL A + 7.5 mL B + extraction with 1 mL toluene.

[h]1 mL A + 7.5 mL B + 2× extraction with 1 mL toluene.

tricosanoate (C23:Me) in *n*-hexane was prepared to examine the relative stability of these fatty acids under the five different derivatization conditions listed here: i) 1.5 mL of 0.5 N NaOH in MeOH at 100°C for 7 min, then 2 mL of 12% BF_3 in MeOH at 100°C for 30 min; ii) 2 mL of 12% BF_3 in MeOH at 100°C for 30 min; iii) 1.5 mL of 0.5 N NaOH in MeOH at 100°C for 7 min, then 2 mL of 10% BCl_3 in MeOH at 100°C for 30 min; iv) 2 mL of 10% BCl_3 in MeOH at 100°C for 30 min; and v) 2 mL of tetramethylguanidine/MeOH (1:4, vol/vol) at 100°C for 15 min.

Samples of 2 mL were pipetted into 20-mL pyrex tubes. The solvent was evaporated with a gentle stream of nitrogen before derivatization. The extraction method used was that described in the Experimental Procedures section for medium and low concentrates. Six samples were derivatized for each method. The composition of the standard test mixture was chosen so as to identify any low results due to transesterification or degradation of the fatty acids. The peak areas for the fatty acid methyl esters were normalized with respect to that for the C23:0Me peak, which was used as an internal standard. The results are shown in Table 5. All the derivatization procedures gave the same areas for the saturated fatty acids, but the areas for EPA and DHA varied with the derivatization procedure, giving the lowest values with the BF_3-containing reagents.

Validation and performance. For linearity evaluation, six samples of the high concentrate with identical concentrations of internal standard, and the amount of EPA and DHA varying from 0.93–9.3 mg/mL and 0.53–5.3 mg/mL, respectively, were analyzed. The responses were linear ($\varrho = 0.9992$) and proportional over the concentration range measured (Fig. 1).

Within-day variance was determined for all three sample types by one analyst analyzing six samples from the same batch within a day for each sample type (Table 6). Within-laboratory repeatability was determined for the high concentrate from 24 analyses of one batch over a five-month period. This series included analysis on two columns and use of EPA and DHA standards from two sources (Table 7). Accuracy was determined by examining the recovery of EPA ethyl ester and DHA ethyl ester for all three sample types. Each sample type was spiked

TABLE 5

Peak Areas (mean ± SD) for the Fatty Acid Methyl Esters Normalized with Respect to the Peak Area of the Internal Standard C23:0ME

	Derivatization procedure				
FAME[a]	$NaOH/BF_3$ (n = 5)[b]	BF_3 (n = 6)	$NaOH/BCl_3$ (n = 6)	BCl_3 (n = 5)[b]	TMG[a] (n = 6)
C16:0ME	191.8 ± 1.0	192.6 ± 1.0	192.4 ± 1.1	191.2 ± 1.4	192.3 ± 1.0
C18:0ME	189.8 ± 0.5	190.0 ± 0.6	190.0 ± 0.7	189.6 ± 0.7	190.0 ± 0.5
C20:0ME	158.6 ± 0.1	158.3 ± 0.3	158.1 ± 0.4	158.3 ± 0.3	159.5 ± 1.1
C20:5ME	170.0 ± 0.8	171.8 ± 1.8	178.5 ± 1.9	179.2 ± 0.5	180.0 ± 0.8
C22:6ME	147.7 ± 0.9	149.7 ± 1.8	155.8 ± 2.7	157.0 ± 0.5	158.0 ± 0.9

[a]FAME, fatty acid methyl esters; TMG, tetramethyl guanidine.

[b]One sample destroyed due to leakage in the reaction vial.

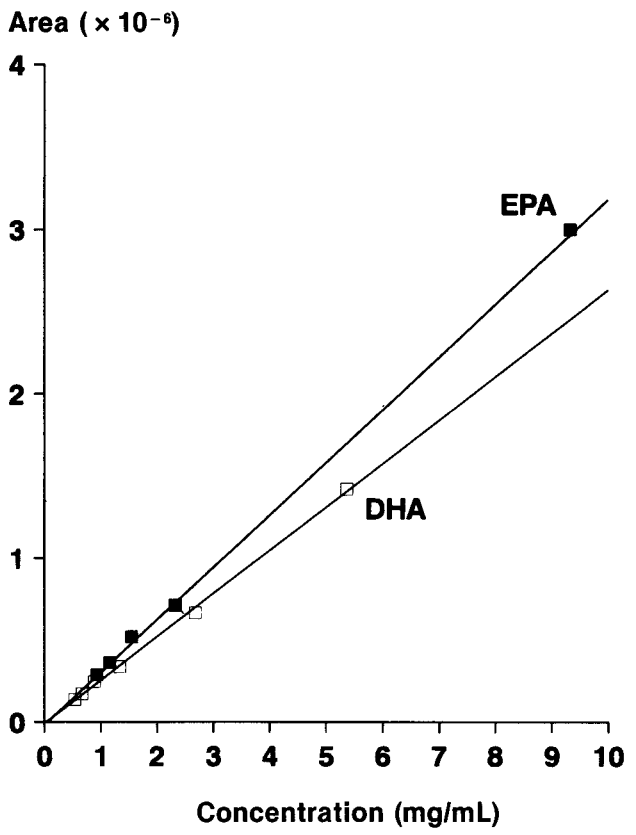

FIG. 1. Plot of area *vs.* concentration for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) ethyl esters.

with an amount of EPA ethyl esters and DHA ethyl ester equivalent to that found in the samples. Unspiked and spiked samples were analyzed and the recovery of EPA and DHA was determined. Accuracy expressed as the percentage of recovered standard is shown in Table 8.

The method gave reproducible results over a one-year period for the ethyl ester concentrate (Table 9). Determination of relative response factors gave a reproducibility of 1% when determination on two different columns and standards from two different origins were included (Table 10).

Working standard. According to European Community rules, working standards may be used for routine tests of chemical active ingredients in medicinal products (13). In our laboratory, an ethyl ester concentrate in soft gelatine capsules is analyzed and used as a working standard. In this case, a solution of approximately 175 mg working standard and 70 mg tricosanoic acid methyl ester in 10 mL *iso*-octane containing 50 mg BHT/L substitutes the standard solutions given in the Experimental Procedures section (see *High concentrates*).

DISCUSSION

The relative peak areas of the different fatty acids in a gas chromatographic analysis depends on the procedure used. Both instrument performance and the conditions for transesterification, for samples that need such treatment, may contribute to deviations from theoretical values. Table 5 shows that it is necessary to derivatize both sample and standard to correct for degradation of PUFA, particularly when the boron trifluoride reagent is used. Lack of trieicosapentaenoin and tridocosahexaenoin as commercial standards made the addition of EPA and DHA ethyl esters our choice for determining accuracy in analyzing glyceride samples. Proper standards for determining empirical relative response between the analytes and the internal standard are essential for correct results. We prefer to prepare standards by preparative HPLC and

TABLE 7

Determination of Within-Laboratory Repeatability of EPA-EE and DHA-EE in the Ethyl Ester High Concentrate (mg/g)[a]

FA-EE	n	Mean	SD	RSD (%)	Maximum	Minimum
EPA-EE	24	506.6	4.1	0.8	514.9	499.3
DHA-EE	24	313.8	2.9	0.9	317.8	307.6

[a]Abbreviations as in Table 6. FA-EE, fatty acid ethyl ester.

TABLE 6

Determination of Within-Day Repeatability for Ethyl Ester and Glyceride Samples (mg/g)

Sample no.	High concentrate (ethyl ester)		Medium concentrate (glyceride)		Low concentrate (glyceride)	
	EPA-EE[a]	DHA-EE[b]	EPA	DHA	EPA	DHA
1	512	318	252	221	162	115
2	506	316	251	221	163	115
3	506	315	252	221	165	116
4	510	316	249	222	159	112
5	502	316	251	222	148	106
6	505	314	248	219	157	111
Mean	506.8	315.8	250.5	221.0	159.0	112.5
SD	3.6	1.3	1.6	1.1	6.1	3.7
RSD (%)	0.7	0.4	0.7	0.5	3.8	3.3

[a]Eicosapentaenoic acid ethyl ester. [b]Docosahexaenoic acid ethyl ester.

TABLE 8

Determination of Recovery (%) of EPA and DHA in Glyceride and Ethyl Ester Samples[a]

Sample no.	High concentrate (ethyl ester)		Medium concentrate (glyceride)		Low concentrate (glyceride)	
	EPA-EE	DHA-EE	EPA-EE	DHA-EE	EPA-EE	DHA-EE
1	100.9	100.0	97.9	102.7	100.2	99.4
2	99.5	98.9	97.0	97.8	98.3	97.5
3	100.1	98.9	98.7	97.3	99.8	100.9
4	100.1	98.4	98.8	97.5	100.5	104.5
5	101.6	100.3	99.5	97.6	99.2	98.5
6	102.7	101.3	99.2	96.7	100.4	101.2
Mean	100.8	99.6	98.5	98.3	99.7	100.3
SD	1.2	1.1	1.0	2.3	0.9	2.6

[a]Abbreviations as in Table 6.

TABLE 9

Results from Stability Studies (soft gelatin capsules stored in high density polyethylene bottles) (mg/g)[a]

Time (mon)	Storage conditions (°C/%R.H.)	*n*	Eicosapentaenoic acid ethyl ester	Docosahexaenoic acid ethyl ester
0		3	502 ± 4	310 ± 3
3	30/50	3	499 ± 4	308 ± 2
6	30/30	3	505 ± 2	312 ± 1
6	30/50	3	505 ± 2	312 ± 1
6	30/75	3	506 ± 2	312 ± 2
9	30/50	3	502 ± 1	314 ± 1
12	30/30	3	506 ± 2	318 ± 1
12	30/50	3	506 ± 3	317 ± 1
12	30/75	3	505 ± 3	316 ± 1
12	25/50	3	508 ± 2	318 ± 1
12	25/75	3	509 ± 2	318 ± 1

[a]Mean ± SD.

TABLE 10

Determination of Empirical Response Factors for EPA-EE and DHA-EE Relative to Tricosanoic Acid Methyl Ester[a]

FA-EE	*n*	Mean	SD	RSD (%)	Maximum	Minimum
EPA-EE (day-day)[b]	6	0.9886	0.0113	1.1	1.0030	0.9733
DHA-EE (day-day)[b]	6	0.9079	0.0044	0.5	0.9136	0.9020
EPA-EE (std-std)[c]	12	0.9928	0.0111	1.1	1.0075	0.9729
DHA-EE (std-std)[c]	11	0.9180	0.0084	0.9	0.9339	0.9079
EPA-EE (inj-inj)[d]	26	0.9935	0.0117	1.2	1.0143	0.9729
DHA-EE (inj-inj)[d]	25	0.9156	0.0082	0.9	0.9347	0.9020

[a]Abbreviations as in Table 7.

[b]Results from one standard preparation analyzed six different days. Storage at −20°C between each analysis.

[c]Results from 12 different standard preparations.

[d]Results from each injection over a five-month period.

assess the purity as described in the literature (1). For routine tests, well-characterized samples containing both EPA and DHA ethyl esters, quantified by purified standards, may be used as working standards. Normal behavior in our quality control analyses is that the empirical response factors are stable over time. After long-term use and injection of dirty samples, a decrease of the empirical response factors for EPA and DHA ethyl esters relative to tricosanoic acid methyl ester are observed. Cutting off 10 cm of the column on the injector end and changing to a clean liner restored the values to a stable level.

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