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ORIGINAL ARTICLE

Determination and evaluation of EPA and DHA ethyl esters in fish oils using the TMAH transesterification method

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Abstract

A simple and dependable technique, known as THAM method, has been developed to detect and measure ethyl eicosapentaenoate (EE-EPA) and ethyl docosahexaenoate (EE-DHA) in encapsulated fish oils. This technique involves using tetramethylammonium hydroxide (TMAH) as a catalyst, followed by analysis using gas chromatography equipped with a flame ionization detector. Recoveries of EE-EPA and EE-DHA spiked between 5 mg/g and 20 mg/g were found to be between 90.8% and 95.2%, with coefficients of variation ranging from 0.2% to 2.5%, demonstrating the accuracy and precision of the technique. Additionally, its limitation of quantitation of EE-EPA and EE-DHA in fish oil samples was 0.2%. When compared with the direct injection method, the TMAH method yielded relative percent differences of no more than 3.8% in the amounts of ethyl esters of EPA and DHA in fish oil, while preventing contamination and maintaining its performance over time. Furthermore, when compared the total amounts of EPA and DHA with the boron trifluoride method, the relative percent differences were no more than 4.7% by the TMAH method. The advantages of using the TMAH method in distinguishing the ester forms of EPA and DHA and determining the total content of fatty acids in fish oils, which can provide an auxiliary check for evaluating the compliance of applications with the regulation related to the purity and form of EPA and DHA.

Keywords: Encapsulated fish oil, Ethyl docosahexaenoate, Ethyl eicosapentaenoate, GC, TMAH

1. Introduction

Fish oil is one of the main sources of omega-3 fatty acids. The benefits of the major omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in fish oil to human health have been studies a lot as to decrease the cardiovascular risk by reducing blood lipids [1–3], to alleviate the symptoms of arthritis [4], to improve depression-like behaviors [5], and to visual acuity outcomes and neurodevelopment on child growth [6]. In general, fish oil contains approximately 30% total EPA and DHA, along with other omega-3 fatty acids, in the form of triacylglycerides naturally [7,8]. This concentration can be increased by improving the processing techniques, such as chemically or enzymatically hydrolyzing the oil, combining it with urea complexation, and using molecular distillation to concentrate the ethyl esters of EPA and DHA (EE-EPA and EE-DHA) [9–11]. The concentrated EE-EPA and EE-DHA obtained through chemical modification can have a purity level higher than 85% [12,13].

Both the "Regulation for the Use Restriction of Fish Oil as a Food Ingredient" [14] and the "Specification Standards for Fish Oil Health Food" [15] specify the total daily intake of Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA). The latter standard also requires that the purity of EPA and DHA should not only be within the range of 30–50%, but also in the form of triglycerides.

There are different techniques available for analyzing fatty acids in lipids. Methyl esters are the most commonly used derivatives of fatty acids for gas chromatography. Typically, after extracting the oils

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from food, saponification and transesterification are performed, and then the resulting methyl esters are analyzed using GC-FID [16−21] or GC−MS [22,23]. However, most of these methods cannot differentiate between the original types of EPA and DHA in fish oils since all fatty acids are converted into methyl esters, unless the sources are known in advance [18].

As reported in the references, TMAH can be used as an efficient transesterification catalyst for converting triglycerides to methyl esters [16,24,25]. In the study, all the triglycerides of coconut oil were conversed to methyl esters in TMAH transesterification after 5 min [16].

Another study reported using the diluted oil samples directly (DI method) for analyzing the EE-EPA and EE-DHA [26]. Besides, there was a study comparing the TMAH and DI analytical methods for the determination of free fatty acids in dairy products [27].

This study was developed for the auxiliary evaluation the compliance of the applications for the "Specification Standards for Fish Oil Health Food" in terms of the purity and form of EPA and DHA. In the market, EPA and DHA products are available in forms of triglycerides or ethyl esters. The promulgated method, catalyzed by boron trifluoride (BF3 method) [21], provides the total amounts of EPA and DHA in the fish oil, however through the methylation results cannot distinguish the original form of the fatty acids. Therefore, the simple TMAH method is proposed as an alternative approach to identify the existence of EE-EPA and EE-DHA, and quantify the total amount of EPA and DHA in fish oil simultaneously. Besides, comparing the results obtained from the TMAH method with those from the BF3 method for the total amounts of EPA and DHA, as well as with DI method for the ethyl esters of EPA and DHA, would help assess the applicability of this method.

2. Materials and methods

2.1. Samples and standards

Twenty-seven encapsulated fish oil samples were purchased from various stores or online shops. The first 17 samples, including 11 health food fish oil samples (S7−S17), were used to develop the TMAH method. The remaining 10 samples, including 6 health food fish oil samples (S22−S27), were prepared for the comparisons between methods. The FAPAS QC material T14222QC, marked as S28, was used as a quality control sample for the total amounts of EPA and DHA. Standards used included methyl eicosapentaenoate (ME-EPA), methyl docosahexaenoate (ME-DHA), ethyl eicosapentaenoate (EE-EPA), ethyl docosahexaenoate (EE-DHA), methyl tricosanoate, and methyl tricosanoate (internal standard), all of which purchased from NU-CHEK, MN, USA. were standards grades.

2.2. Chemicals and reagents

25% Tetramethylammonium hydroxide solution, 14% boron trifluoride in methanol, were original from Sigma−Aldrich®, MO, USA. The other chemicals and reagents were analytical grade or higher.

2.3. GC-FID instrumentation and setting

A Shimadzu GC-2010 GC system equipped with a flame ionization detector (Kyoto, Japan) was used to analyze the fish oils. Sample separation was carried out using a DB-23 capillary column (Agilent Technologies) with a thickness of 0.25 μm, length of 30 m, and diameter of 0.25 mm. The oven temperature was initially set at 175 °C for 35 min, then increased at a rate of 3 °C/min to 230 °C, and held for 30 min. The injection temperature was set at 250 °C with a split ratio of 40:1, and helium was used as the carrier gas at a rate of 1.0 mL/min. The volume of injection was 1 μL, and the detection temperature was set at 270 °C.

2.4. Methods

Two parts of the experimental design were addressed. The former part involved developing a method for determining EE-EPA and EE-DHA by transesterification with a TMAH-methanol solution (TMAH method). The latter part involved comparing the TMAH method with two methods: detecting the ester forms of EPA and DHA by direct injection after dilution without transesterification (DI method), and analyzing the total amounts of EPA and DHA by transesterification with a boron trifluoride-methanol solution (BF3 method).

2.4.1. Transesterification with TMAH-methanol solution (TMAH method)

2.4.1.1. Preparation of standard solutions. Standards were prepared in hexane. Methyl tricosanoate was used as an internal standard at a concentration of 1 mg/mL. The concentrations of EPA methyl ester, EPA ethyl ester, DHA methyl ester, and DHA ethyl ester were 5 mg/mL each. Working solutions of EPA and DHA esters were prepared by diluting the stock solutions with n-hexane to a concentration

range of 10–2000 μg/mL, which included a 250 μg/mL internal standard solution, and were ready for injection.

2.4.1.2. Transesterification. 20 mg of oil sample was transferred to a 16 mL glass tube and 1 mL of internal standard solution and 3 mL of diethyl ether were added. The tube was closed with a Teflon cap and vortexed to dissolve the mixture. Next, 0.1 mL of 25% TMAH in methanol was added to the tube and the mixture was vortexed and kept at 25 °C for 5 min (with frequent shaking). Then, 3 mL of deionized water was added to the mixture and vortexed for 30 s to end the reaction. After that, 1 mL of saturated NaCl was added, the tube was fastened with a Teflon cap, gently shaken, and allowed to stand for stratification. Finally, the supernatant was ready for injection.

2.4.1.3. Calibration curve and calculation. The calibration curves for fatty acid esters were generated by plotting the peak area of each fatty acid ester (Ax) divided by the peak area of the internal standard (Ais) against the concentration of each fatty acid ester (Cx) divided by the concentration of the internal standard (Cis). The fatty acid esters were identified by comparing the retention time of peaks in the sample solutions to those in the standard solutions, which were calculated as follows:

$$C_x\,(\mu g/mL) = \frac{Ax \times Cis}{Ais}$$

C_x: concentration of fatty acid ester in sample solution (μg/mL).

C_{is}: concentration of the internal standard (μg/mL).

A_x: peak area of fatty acid ester in sample solution.

A_{is}: peak area of internal standard in sample solution.

The amounts of fatty acid esters in sample solutions were calculated as following:

$$F_E\,(mg/g) = \frac{C_X \times V \times D}{W \times 1000}$$

F_E: amount of fatty acid ester (mg/g).

V: volume (mL).

D: dilution factor.

W: sample weight (g).

1000: conversion factor of μg to mg.

The amounts of fatty acids converted from fatty acid esters were calculated as.

F_A (mg/g) = F_E/CF_x

F_A: amount of fatty acid (mg/g).

CF_x: conversion factor of fatty acid methyl ester to fatty acid is 1.04; fatty acid ethyl ester to fatty acid is 1.08.

2.4.2. Validation of TMAH method

The linearity was conducted from the calibration curves as described in 2.4.1.3 by adding each standard of the fatty acid ethyl ester and methyl ester ranging from 10 to 2000 μg/mL, with internal standard 250 μg/mL. Recoveries were evaluated by spiking standards of EPA and DHA ethyl ester into fish oil samples (S17), ranging from 5 to 20 mg/g, repeated five times for each spiking. The limitation of quantification was determined by evaluating the recoveries of the lowest spiked amount, which signal-to-noise ratio should lager than 10. A commercial grapeseed oil was used as the blank sample. A fish oil sample (S6), detected EE-EPA 8.0 mg/g and EE-DHA 6.5 mg/g, was analyzed as described in section 2.4.1.2 for the intra-day and inter-day tests.

2.5. Direct injection (DI method)

A sample solution was prepared by mixing 20 mg of oil sample with 1 mL of internal standard solution and 3 mL of hexane, ready for injection. The analytical conditions and other processes, including the preparation of standard solutions, calibration curve, and calculations, were the same as those described in sections 2.3, 2.4.1.1, and 2.4.1.3.

2.6. Transesterification with BF3 solution (BF3 method)

To prepare the sample, 20 mg of the sample was mixed with 1 mL of the internal standard solution and 1 mL of 1 N sodium hydroxide in methanol, and heated at 80 °C for 15 min for saponification. Then, 1 mL of 14% boron trifluoride in methanol was added, and the mixture was heated at 110 °C for 15 min for transesterification. After that, 1 mL of n-hexane was added and vortex-mixed for 1 min, followed by the addition of 3 mL of saturated sodium chloride solution. The resulting supernatant was taken as the sample solution [21].

The other processes, as standard solution preparation, calibration curve and calculation were all the same described in 2.4.1.1 and 2.4.1.3.

2.7. Statistical analysis

The percentage of fatty acids, the relative percent difference (RPD), the coefficient of variation (CV), were all processed by Microsoft Excel 2019.

3. Results

3.1. Gas chromatography condition optimization

All five peaks, which include three methyl esters and two ethyl esters, were successfully separated

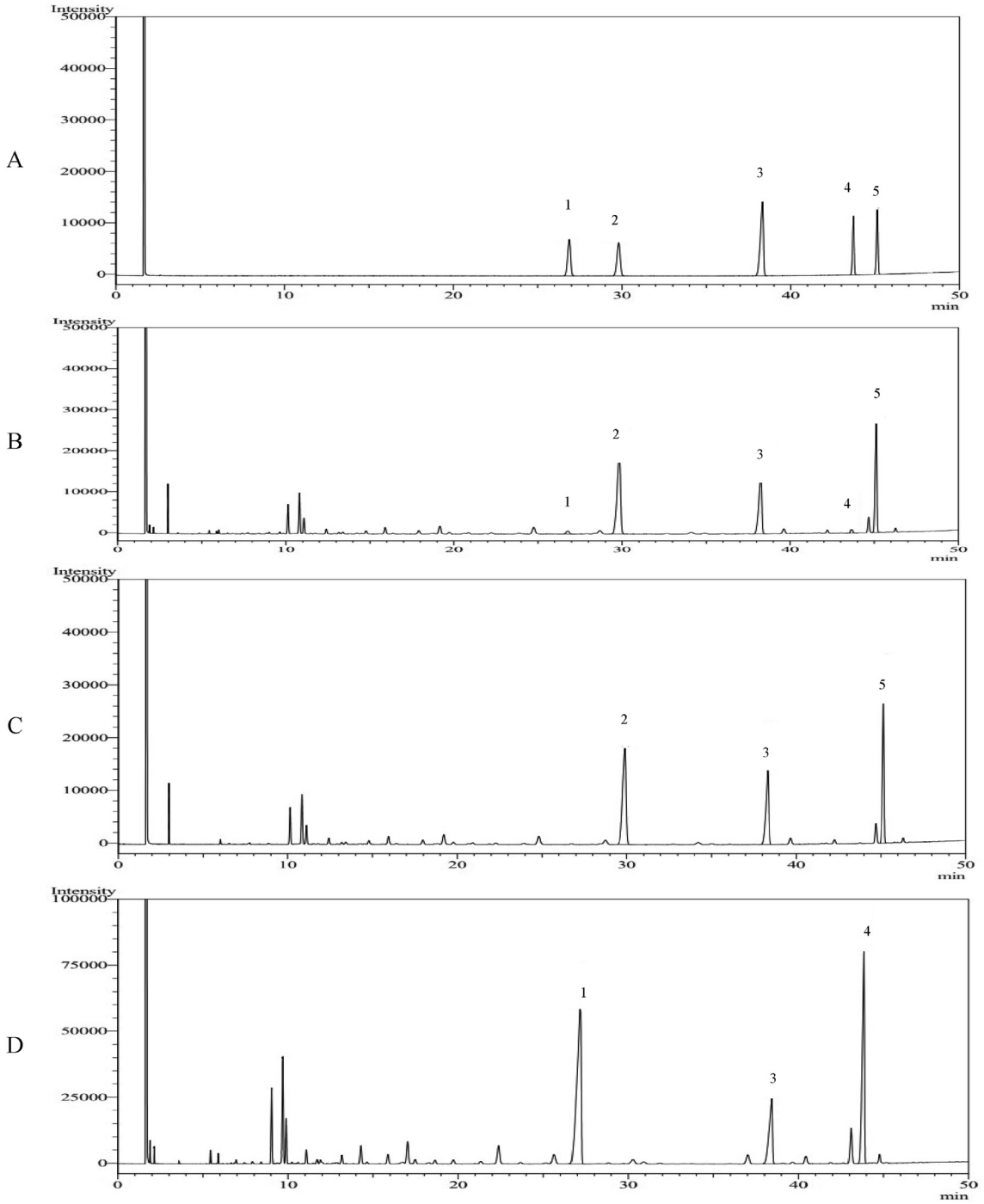

Fig. 1. Gas chromatograms of (A) standards, and S18 assayed by (B) TMAH method, (C) Direct injection method, (D) BF3 method. (Peak number represented as following: 1. methyl eicosapentaenoate, 2. ethyl eicosapentaenoate, 3. methyl tricosanoate, 4. methyl docosahexaenoate, and 5. ethyl docosahexaenoate).

well within 50 min in the following order: ME-EPA, EE-EPA, ME-C23:0, ME-DHA, and EE-DHA, as depicted in Fig. 1 (A).

3.2. Analysis of EE-EPA and EE-DHA using TMAH method

The analysis of fatty acid amounts in fish oil is typically done by converting them to fatty acid methyl esters (FAME) through the process of saponification and methylation [16−21]. The amounts of fatty acid ethyl esters (FAEE) in oils are then calculated by converting the FAME using molecular weight factors [18]. However, this method does not distinguish between the presence of EE-EPA or EE-DHA unless the source of the oil is known in advance. A report [25] suggests that it takes a certain amount of time for the ethyl esters of EPA

and DHA to fully convert to their corresponding methyl esters. To address this issue, an appropriate method for the qualification and quantification of FAEE in fish oil has been developed.

3.2.1. Reaction time of TMAH method

TMAH was utilized as a catalyst in the transesterification of free fatty acid (FFA) and triacylglycerides (TG) to fatty acid methyl ester (FAME) to determine the amounts of fatty acids in dairy products [16]. In a report on TMAH-catalyzed transesterification [25], it was found that ethyl esters of EPA and DHA were fully converted to their corresponding methyl esters using the TMAH-catalyzed method at room temperature for 40 min and 20 min, respectively.

To ensure the quality and quantity of fish oil samples, the presence of EE-EPA and EE-DHA was analyzed using the TMAH method for an appropriate reaction time. The profiles of the amounts of ethyl esters of EPA and DHA during transesterification within 10 min in four fish oil samples are shown in Fig. 2. The changes in the amounts of EE-EPA and EE-DHA were demonstrated by the ratio of the remaining amounts to the initial amounts. The ratios of the total amounts summed up by each methyl and ethyl ester over the labeled amounts were also recorded.

After transesterification for 5 min, sample S3 (which contained 294 mg/g EE-EPA and 150 mg/g EE-DHA) had 99% of EE-EPA and 97% of EE-DHA remaining. In sample S6 (which contained 8.0 mg/g EE-EPA and 6.5 mg/g EE-DHA), both 98% of ethyl esters remained. Sample S12 (which contained 2.5 mg/g) had 84% of EE-DHA remaining, and no EE-EPA or EE-DHA was detected in sample S17 (Fig. 2). The degradation of EE-EPA and EE-DHA in samples S3 and S6 was slow, while the declining tendency was more obvious in sample S12, which had a slight amount of EE-DHA. Overall, it appears that the majority of EE-EPA and EE-DHA in fish oil still remain within 5 min in TMAH transesterification.

On the other hand, the ratios of the total amounts of EPA and DHA over the amounts labeled increased and then remained almost constant after 5 min of TMAH transesterification. Thus, the TMAH method not only identifies and quantifies the existence of EE-EPA and EE-DHA in fish oils with the remaining ethyl esters of fatty acids but also determines the total amounts of fatty acids by summing up each ester.

3.2.2. Validation of TMAH method

The recoveries of EE-EPA and EE-DHA, spiked in the concentrations of 5, 10 and 20 mg/g ($n = 5$) into the fish oil sample (S17), were between 90.8—93.6% and 92.7—95.2%, respectively. The coefficients of variation were between 0.2—2.5% and 0.7—1.7%. The limit of quantification (LOQ) of EE-EPA and EE-DHA were both 2 mg/g, with signal-to-noise values of 12.5 and 17.8, respectively (Table 1). As no reference material containing ethyl ester type EPA and DHA were available for verification, the intra-day and inter-day results of analyzing sample S6 (containing ethyl esters of EPA and DHA) showed that the coefficients of variation for EE-EPA on the same day and different day were 1.9% and 4.0%; for EE-DHA were 1.4% and 3.5%, respectively. The total amounts detected over the amounts labeled for EE-EPA on the same day and different day were 98.3% and 99.9%; for EE-DHA were 104.1% and 104.8%, respectively (Table 2). The results showed that TMAH method were accurate and precise.

3.2.3. Results of investigation using TMAH method

Seventeen commercial encapsulated fish oil samples were analyzed, including 11 samples marketed as health food products. Among them, sample S3 contained 294 mg/g of EE-EPA, and sample S6 contained 8.0 mg/g of EE-EPA. The amounts of EE-DHA detected in samples S3, S6, S10, and S12 were 6.5 mg/g, 150 mg/g, 2.9 mg/g, and 2.0 mg/g, respectively. No EE-EPA or EE-DHA was detected in the remaining samples. The total amounts of methyl and ethyl esters of EPA and DHA, summed up and expressed as a percentage of the labeled amounts, are shown in Fig. 3. The values ranged from 82% to 130%, all are compliant with the "Regulations on Nutrition Labeling for Prepackaged Food Products", which range of allowable error for nutrition labeling values of nutrition labeled voluntarily was $\geq$80% of the labeled value [28]. Additionally, the other 11 health food fish oil samples (S7—S17) were all complied with their registered specifications.

3.3. Comparison of TMAH method with DI method and BF3 method

The TMAH method has several advantages, as it can distinguish between the different ester forms of EPA and DHA in fish oil and determine the total content by summing up the fatty acids. Therefore, it's worth further evaluating the correlations between the TMAH method and the widely applied BF3 method for assaying the total amounts of EPA and DHA, as well as the DI method for directly analyzing of the esters of EPA and DHA without saponification and methylation.

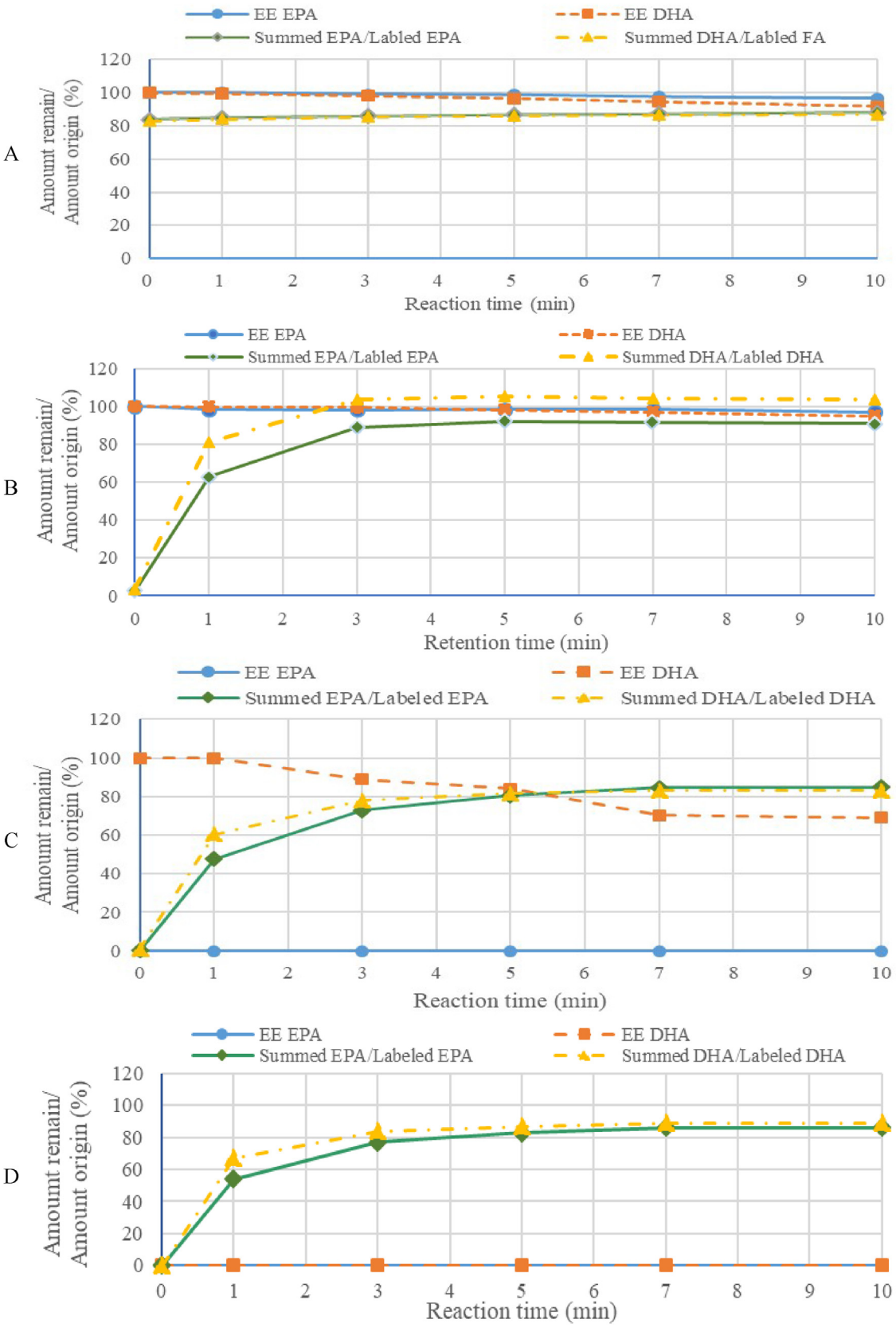

Fig. 2. The changed of EPA and DHA during transesterification within 10 min in four fish oil samples (A) S3 (contained EE EPA 294 mg/g, and EE DHA 150 mg/g), (B) S6 (contained EE EPA 8.0 mg/g, and EE DHA 6.5 mg/g), (C) S12 (contained EE DHA 2.5 mg/g, EE EPA was none detected), (D) S17 (EE EPA and EE DHA were none detected).

Table 1. The accuracy and precision of EE-EPA and EE-DHA in fish oil analyzed by using TMAH method.

Fatty acid	Amount added (mg/g)	Recovery (%)	Coefficient variation (%)	S/N
EE-EPA	2[a]	91.1	0.9	12.5
EE-DHA		95.0	4.3	17.8
EE-EPA	5	90.8	2.5	
EE-DHA		95.2	1.4	
EE-EPA	10	91.1	0.2	
EE-DHA		94.0	0.7	
EE-EPA	20	93.6	1.1	
EE-DHA		92.7	1.7	

[a] Grape seed oil was used as samples blank for LOQ test.

3.3.1. Comparison between TMAH method and DI method

Four out of the ten samples (S18–S21) were found to contain high levels of EPA and DHA, with EPA ranging from 241 to 497 mg/g and DHA ranging from 169 to 234 mg/g. The sum of EPA and DHA in these fish oil samples accounted for more than 55.8%. According to the reports, the sum of natural EPA and DHA in fish oil is typically around 30% [7,8], so the high amounts of EPA and DHA in these four samples could be due to some special processed products. Using the TMAH method, it was found that more than 94.0% of the majority of EPA

Table 2. The results of S6 analyzed by using TMAH method on the same days and different days.

Fatty acid[a]	Amount Labeled	Intra-day[b]			Inter-day[c]		
		Amount detected	Amount detected/ Amount labeled *100	CV	Amount detected	Amount detected/ Amount labeled *100	CV
	(mg/g)	(mg/g)	(%)	(%)	(mg/g)	(%)	(%)
ME EPA	300	287.7		0.3	292.2		1.8
EE EPA		7.8		1.9	8.1		4.0
(ME + EE) EPA		295.5	98.3		300.3	99.9	
ME DHA	200	202.5		0.3	203.7		1.0
EE DHA		6.1		1.4	6.3		3.5
(ME + EE) DHA		208.6	104.1		210.0	104.8	

[a] All the amounts of fatty acids were calculated as free form. The abbreviation of methyl ester expressed as ME, ethyl ester as EE, the sum of methyl ester and ethyl ester as ME + EE, eicosapentaenoic acid as EPA, docosahexaenoic acid as DHA, coefficient of variation as CV.
[b] n = 5.
[c] n = 10.

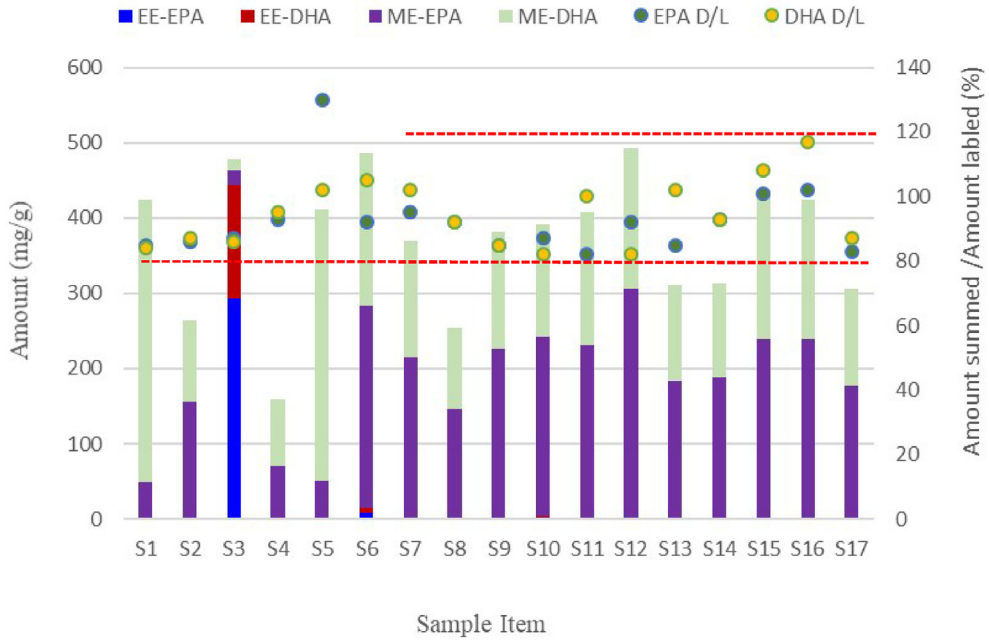

Fig. 3. The investigations of EPA and DHA in 17 samples. (S1~S6: general encapsulated fish oil, S7~S17: health food; EE-EPA, EE-DHA, ME-EPA, ME-DHA refer to methyl or ethyl esters of EPA or DHA; EPA D/L and DHA D/L refer to the percentage of amount summed over amount labled of EPA or DHA.)

Table 3. Comparisons the amounts and relative percent difference of EPA, DHA and their esters in fish oil samples using TMAH method, DI method, and BF3 method.

	Method	TMAH Method			DI Method		BF3 Method	Amount Labeled	Percentage of EE detected by TMAH method	Percentage of Total Amount Detected by TMAH Method over Amount Labeled	Relative Percent Difference of EE Amount Detected by TMAH Method and DI Method	Relative Percent Difference of Total Amount Detected by TMAH Method and BF3 Method
	Fatty Acid[a]	ME	EE	ME + EE	ME	EE	ME					
	Code & Equation	A	B	C	D	E	F	G	$\frac{B}{C}\times 100$	$\frac{C}{G}\times 100$	$\left\|\frac{(B-E)}{(B+E)/2}\right\|\times 100$	$\left\|\frac{(C-F)}{(C+F)/2}\right\|\times 100$
Sample	Unit	mg/g (%)[b]							%			
S18	EPA	13.3 (1.6)	304 (0.0)	317 (0.0)	–	310 (0.1)	315 (0.6)	350	95.9	90.6	2.0	0.6
	DHA	7.9 (9.2)	210 (0.3)	218 (3.0)	–	216 (0.0)	215 (0.9)	232	96.3	94.0	2.8	1.4
S19	EPA	15.4 (0.6)	441 (1.8)	456 (1.7)	–	450 (1.6)	451 (1.1)	497	96.7	91.8	2.0	1.1
	DHA	10.1 (1.8)	201 (1.7)	211 (1.7)	–	207 (1.6)	202 (0.9)	183	95.3	115	2.9	4.4
S20	EPA	10.6 (1.0)	395 (2.0)	406 (2.0)	–	402 (1.8)	399 (1.4)	351	97.3	116	1.8	1.7
	DHA	9.4 (7.7)	235 (2.1)	244 (1.8)	–	242 (2.0)	233 (1.6)	234	96.3	104	2.9	4.6
S21	EPA	13.7 (0.5)	290 (0.6)	304 (0.6)	–	296 (0.7)	305 (0.6)	335	95.4	90.7	2.1	0.3
	DHA	11.8 (3.7)	188 (0.9)	200 (1.0)	–	194 (0.6)	195 (0.7)	223	94.0	89.7	3.1	2.5
S22	EPA	214 (1.9)	37.8 (1.5)	252 (1.9)	–	36.4 (1.3)	264 (1.6)	241	15.0	105	3.8	4.7
	DHA	167 (1.6)	31.6 (1.7)	198 (1.6)	–	31.4 (1.6)	193 (1.6)	169	15.9	118	0.6	3.1
S23	EPA	172 (1.1)	4.5 (1.6)	177 (1.0)	–	4.5 (0.5)	175 (1.8)	293[c]	2.5	100	0.0	1.1
	DHA	113 (1.4)	3.4 (5.0)	116 (1.5)	–	3.4 (0.4)	115 (1.6)		2.9		0.0	0.9
S24	EPA	277 (0.2)	2.0 (10.2)	279 (0.2)	–	2.0 (5.0)	282 (1.6)	243	0.7	115	0.0	1.1
	DHA	187 (0.5)	–	187 (0.5)	–	–	192 (1.3)	162	–	115	–	2.6
S25	EPA	269 (1.8)	2.0 (1.0)	271 (1.9)	–	2.0 (0.4)	278 (1.2)	262	0.7	103	–	3.3
	DHA	177 (2.4)	2.8 (7.1)	180 (1.3)	–	2.6 (5.6)	188 (0.9)	160	1.6	113	7.4	4.3
S26	EPA	106 (3.2)	4.8 (4.7)	111 (3.2)	–	4.8 (0.4)	111 (1.2)	77	4.3	144	0.0	0.0
	DHA	371 (2.3)	15.7 (1.3)	387 (2.3)	–	15.1 (2.1)	403 (1.0)	349	4.1	111	3.9	4.1
S27	EPA	274 (2.4)	–	274 (2.1)	–	2.1 (0.1)	280 (1.2)	273	0.7	100	4.9	2.2
	DHA	184 (1.6)	–	184 (1.6)	–	–	191 (1.5)	162	–	114	–	3.7
S28	EPA	74.4 (2.6)	–	74.4 (2.6)	–	–	82.3 (0.7)	85.4	–	87.1	–	10.1
	DHA	93.6 (1.2)	–	93.6 (1.2)	–	–	91.9 (0.7)	99.5	–	94.1	–	1.8

[a] All the amounts of fatty acids were calculated as free form. The abbreviation of methyl ester expressed as ME, ethyl ester as EE, the sum of methyl ester and ethyl ester as ME+EE.
[b] n=2, relative percent difference of analytical results.
[c] Sum of EPA and DHA.

and DHA in these four fish oil samples (S18–S21) were in the form of ethyl esters. According to the report, oils rich in omega-3, such as ethyl esters of EPA and DHA, can be readily analyzed after dissolution [26]. Therefore, the results of ten samples (S18–S27) analyzed using the TMAH and the DI methods were compared. The distributions of EPA and DHA ethyl esters in the ten samples were divided into three sections: the high amount ones were in the range of 94.0–97.3% (S18–S21); the middle ones were at 15.0% and 15.9% (S22), and the others were between 2.0 and 4.3%. The relative percent differences (RPD) of the amounts of EE-EPA and EE-DHA in the ten fish oil samples using the TMAH method and the DI method were no more than 7.4%. The RPD of the amounts of EPA and DHA ethyl esters analyzed by the TMAH and DI methods were no more than 3.8% in the fish oil samples (S18–S22) that were detected with 31.6–441 mg/g of EE-EPA and EE-DHA.

While direct injection of EPA and DHA ethyl esters in fish oil for gas chromatography analysis would be convenient, it can cause significant damage to the chromatographic column and instrument due to the complex matrix of the fish oil sample (as reported in Ref. [27]). Therefore, the TMAH method is advantageous as it prevents contamination and maintains performance over time.

3.3.2. Comparison between TMAH method and BF3 method

The comparison between the TMAH method and the BF3 method showed that the relative percent differences of the total amounts were no more than 4.7%. The percentages of total amounts detected over the labeled amounts of EPA and DHA in the ten samples (S18–S27) using the TMAH method were in the range of 89.7–118%, except for EPA in S26, which was over its specification at 144% using both methods.

4. Discussions

The chromatograms of the samples illustrate the characteristics of each method. For instance, in S18 (which contained more than 96% ester forms of EPA and DHA), the major peaks analyzed using the TMAH and DI methods were peak 2 (EE-EPA) and peak 5 (EE-DHA), while only peak 1 (ME-EPA) and peak 4 (ME-DHA) were detected using the BF3 method (Fig. 1). The relative percent differences (RPD) of ester amounts by using TMAH and DI methods were 2.0% and 2.8%, respectively. Moreover, the RPD of the total amounts of EPA and DHA detected by TMAH and BF3 methods were 0.6% and 1.4%, respectively (Table 3). These results suggest that the TMAH method is a suitable approach for analyzing the ester forms of EPA and DHA in fish oil.

There was an interesting observation regarding S28. The percentage of total amounts of EPA and DHA detected over the labeled amounts were 96.4% and 92.4% by the BF3 method, and 87.1% and 94.1% by the TMAH method, respectively. However, no peak was detected in the S28 by the DI method, indicating that EPA and DHA in S28 were not in ester forms. Despite this, the relative percent difference (RPD) of EPA between the TMAH method and the BF3 method was 10.1% (Table 3). This phenomenon may suggest that there are other forms of EPA in addition to EE-EPA in S28, which requires further study in the near future.

The TMAH method can be used to both qualify and quantify the presence of EE-EPA and EE-DHA, as well as to determine the total amounts of EPA and DHA by summing up these two esters in fish oils. However, it is important to note that this method is time-dependent, and it is recommended to avoid processing large numbers of samples simultaneously to ensure proper time control. In addition, the direct injection method is not commonly used as it can have adverse effects on the lifespan of the column.

5. Conclusions

The TMAH method is a reliable method to determine the levels of ethyl eicosapentaenoate (EE-EPA) and ethyl docosahexaenoate (EE-DHA) in fish oil, particularly when these ethyl esters are the primary forms of EPA and DHA in the sample. In comparing the results obtained by the TMAH method and the DI method, the relative percent differences (RPD) of the amounts of these esters were found to be no more than 3.8%. Additionally, the TMAH method can prevent contamination and maintain performance over time. When compared to the BF3 method for determining the total amounts of EPA and DHA in fish oil samples, the RPD was found to be no more than 4.7%. Therefore, the TMAH method is a simple and effective way to identify and quantify the levels of EE-EPA and EE-DHA in fish oil. The results can provide feasibility assessment of using TMAH method as an auxiliary check for compliance in applications process with the "Specification Standards for Fish Oil Health Food," which regulate the purity and form of EPA and DHA.

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