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Determining Ethyl Esters in Fish Oil with Solid Phase Microextraction and GC–MS

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Abstract The long-chain polyunsaturated fatty acids (PUFA) found in fish oil, specifically eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) play an important part in human health. As a result, fish oil supplements are commonly consumed by people around the world. Supplements in the form of triacylglycerols (TAG) can be sold at a premium price, compared to those in the ethyl ester (EE) forms. Producers of TAG supplements require a simple, rapid method to determine the authenticity of their raw material. Here, we describe a method to quantify EE in fish oil using solid phase microextraction headspace analysis and GCMS. Despite the variation in linear ranges of the calibration curves with volatility of the EE, 30 individual FA were quantified including common saturated FA such as palmitic and stearic acid, as well as longer chain PUFA, such as EPA and DHA. The method was then applied to three commercial fish oils in the TAG form and two of the products were found to contain EE, with one containing EE above 1.5% w/w, indicating that contamination had occurred. With growing consumer interest in fish oil products, the method proposed here will help resolve future issues of authenticity in fish oils.

Keywords Omega-3 fatty acids · Authenticity · Ethyl esters · Triacylglycerol · Fish oil processing · Solid phase microextraction

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Introduction

The health benefits of long-chain polyunsaturated fatty acids (PUFA) have long been recognized, specifically the omega-3 fatty acids (FA) eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). They are essential for the growth and development of the brain, heart and other systems [1]. Adequate PUFA supplies are essential during retina development as well, and can be transferred from mother to infant during pregnancy and through breast milk. Deficiencies in omega-3 FA have been associated with cardiac problems, hypertension, dermal conditions, impairment of adult brain function, attention deficit disorder, diabetes, arthritis, asthma, inflammation and clinical depression [2, 3]. The best dietary source of PUFA is fatty fish; however many people to do not consume the recommended two servings of fatty fish per week [4, 5]. Fish oil can be used as a dietary supplement for those who do not consume adequate amounts of fatty fish.

Fish oil is naturally found in the form of triacylglcerols (TAG) [6], and that obtained from anchovy, sardine and herring naturally contains approximately 30% total EPA and DHA. During processing of pharmaceutical grade fish oil, TAG undergoes base-catalyzed transesterifcation with ethanol to create ethyl esters (EE) and a product commonly known as "EE oil". The EE can be fractionated, usually by molecular distillation to selectively reduce the levels of particular FA, such as saturates, and to control the amount of PUFA, ensuring that the oil contains the desired 30% PUFA [7]. An additional processing step can convert EE back into the TAG form, creating"TAG oil". Through distillation of EE and modification of PUFA content, fish oil concentrates, sometimes containing more than 65% EPA and DHA, may be created. These concentrates are often left as EE as the conversion from EE oil to TAG oil is

quite costly and for this reason, a large number of commercially available fish oil supplements are made primarily with EE. However, TAG oils are available, and fish oil refiners and producers generally specify an EE content of 3% w/w or less in their TAG products. Since natural fish oil has only trace amounts, if any, EE present, a measureable amount of EE indicates either accidental or deliberate contamination of the product. Refiners usually produce both products so accidental contamination is always a possibility. More worrisome is the potential for deliberate addition of EE to TAG oils to modify FA composition. For instance, fish oil manufacturers may dilute TAG oil concentrates that have high levels of PUFA with shorter chain EE in order to lower the EPA and DHA content, while others have been suspected of spiking TAG fish oil with EE–EPA and EE–DHA to increase their levels and reduce costs [8]. Unfortunately, EE are much less resistant to oxidation than TAG, which can lead to a lower quality product due to the production of fishy off-flavours [9]. Interestingly, some ethnic communities have been known to test fish oil deemed to be of poor quality by placing it in a polystyrene cup. If the cup dissolves, the oils would be considered to be of inferior quality. Ackman and Timmins [8] have shown that neither TAG nor free FA will dissolve polystyrene, but EE lead to rapid destruction.

In addition to the decrease in oxidative stability, there is evidence to suggest that supplements containing EE may not provide the same health benefits as TAG products. For instance, a number of studies have shown that TAG oils are better absorbed in the body than EE. Beckermann et al. [10] found that consumption of TAG fish oil resulted in 50% more plasma EPA and DHA than with EE supplements, while Lawson and Hughes [11] showed that EPA and DHA in TAG form were more efficiently absorbed by 48 and 36% respectively, when compared to EE forms. Plasma lipid concentrations of EPA and DHA were significantly higher in subjects who consumed salmon than those who consumed supplements of EE [12]. Animal studies also suggest that the ethanol molecule from the EE can be released into the liver and pancreas when EE are digested, resulting in organ damage, particularly in those with diseases that effect the liver [13–15]. Because of the negative aspects of EE, fish oil companies often promote TAG supplements as being superior and therefore charge a premium price. Thus, it becomes important to develop rapid methods to test for the presence of EE from both an authenticity and quality perspective.

Although a literature search did not return any documented methods specifically for quantifying EE in TAG oils, there are a number of chromatographic methods that may be used to determine EE in lipid mixtures. For instance, Bernhardt et al. [16] used solid phase extraction and HPLC to isolate and quantify EE from blood serum. In the same paper they also described a separation using thin layer chromatography (TLC) with petroleum ether and ethyl ether (75:5, v/v) as developing solvent. The major difficulty with these techniques is the separate steps of isolation and quantification of EE that may result in loss of EE and inaccurate recoveries. Furthermore, methods developed for biological samples are often designed to separate very different proportions and types of lipids than are found in fish oils and difficulties are often encountered when attempting to adapt these methods for analysis fish oil. Here we describe a new method to test commercial fish oil supplements for adulteration with EE using solid phase microextraction (SPME) and GCMS.

Experimental Procedures

Fish oil EE was obtained from Ocean Nutrition Canada Ltd. (Dartmouth, NS, Canada). Commercially available refined canola oil was purchased from a grocery store. Spectrophotometric grade 1,2-dichlorobenzene (99%) was used as internal standard (Aldrich, Oakville, ON, Canada).

SPME fibers with divinyl benzene/polydimethylsiloxane/Carboxen coating (50/30um), a SPME fiber holder for manual sampling, 22 ml glass vials, polytetrafluoroethylene/silicone rubber septa and phenolic screw caps were purchased from Supelco. A custom-made heating block designed to accommodate 22 ml glass vials was used to control temperature.

FA Analysis

EE in the EE oil to be used as a standard material was quantitatively converted to methyl esters (ME) using the Council for Responsible Nutrition Voluntary Monograph for Omega-3 [17] and analyzed by GC–FID. ME, rather than EE, were analyzed because accurate correction factors to account for the differential response of FA structures by FID were only available for ME. ME were separated on a DB-23 column (30 m × 0.25 mm × 0.25 μm film thickness) and helium was used as the carrier gas, at a flow rate of 1.0 ml/min. The oven temperature was initially held for 2 min at 153 °C then increased at 2.3 °C/min to 174 °C and held for 0.2 min. The temperature was then increased at a rate of 2.5 °C/min to 205 °C and held for 8.3 min. The total run time was approximately 32 min. The FID was maintained at 270 °C, and the injector (split mode 1:100, 250 °C, 4 mm liner) at 250 °C.

Calibration Curve

Varying amounts of EE oil ranging from 0.70 to 1.40 ml (62.0–1250 mg) were added to a 22 ml glass vial containing

a stir bar. Samples were then diluted to exactly 14 ml with canola oil and 1.0 µl dichlorobenzene was immediately added as an internal standard. Typical odd carbon-numbered FA was not used as internal standards because these fish oils contained traces of such structures. Dichlorobenzene was chosen instead because it had been successfully employed as an internal standard to monitor other volatile components using SPME [18]. Vials were capped with phenolic screw caps containing PTFE/silicone rubber septa and samples were stirred at 80 °C for exactly 15 min. During this equilibrium period, the SPME fiber was placed in the hot injector port of the GC to desorb any volatiles that may have accumulated between uses. After the 15 min equilibration time, the SPME fiber was inserted into the vial to a depth of 2.0 cm and exposed to the headspace for exactly 45 min. The equilibration and fiber exposure times were based on an optimized procedure developed to extract volatiles from fish oil. Extracted compounds were analyzed by GCMS in electron ionization mode. The fiber was then inserted in the injector port (splitless mode, 250 °C, 0.75 mm liner) to a depth of 5.0 cm, to the center of the injector, and remained there for 15 min. Volatile analytes were separated on a free FA phase (FFAP) column (30 m × 25 mm × 0.25 µm film coating) and helium was again used as the carrier gas at a flow rate of 1.0 ml/min. The oven temperature was held initially at 40 °C for 5 min, then increased at a rate of 10 °C/min to 220 °C and held for 10 min (total run time of 38 min). Volatiles were identified using library matches from NIST library and standards. The ratio of the area counts of each EE to the internal standard was then calculated and standard curves of ratio versus EE were constructed. Samples of commercially available fish oil supplements, purchased at retail outlets, were analyzed using the same method as for the standard curve, that is 14 ml of sample was added to a 22 ml glass vial along with 1.0 µl internal standard. Analysis was conducted in the same manner as for the standard curve.

Table 1 Concentration of individual EE (mg/g ± SD) in standard EE oil used to construct the calibration curves

FA	Concentration (mg EE/g EE oil)	FA	Concentration (mg EE/g EE oil)
14:0	3.73 ± 0.09	18:2n-4	4.42 ± 0.04
14:1n-9	0.10 ± 0.01	18:3n-6	2.08 ± 0.04
14:1n-7	0.01 ± 0.03	18:3n-4	1.68 ± 0.01
i-15:0	0.22 ± 0.03	18:3n-3	8.53 ± 0.04
ai-15:0	0.07 ± 0.01	18:3n-1	0.61 ± 0.04
15:0	0.50 ± 0.01	18:4n-3	21.7 ± 0.2
i-16:0	0.46 ± 0.01	18:4n-1	3.5 ± 0.3
16:0	29.0 ± 0.3	20:0	3.1 ± 0.1
16:1n-11	1.10 ± 0.01	20:1n-11	2.9 ± 0.7
16:1n-9	0.53 ± 0.01	20:1n-9	19 ± 1
16:1n-7	13.4 ± 0.1	20:1n-7	5.93 ± 0.06
16:1n-5	0.37 ± 0.01	20:2n-9	0.7 ± 0.2
i-17:0	0.76 ± 0.01	20:2n-6	4.4 ± 0.3
16:2n-6	0.26 ± 0.01	20:3n-6	2.96 ± 0.07
ai-17:0	0.30 ± 0.06	20:4n-6	16.31 ± 0.06
16:2n-4	1.84 ± 0.03	20:3n-3	2.03 ± 0.07
17:0	2.20 ± 0.03	20:4n-3	11.8 ± 0.1
16:3n-4	1.69 ± 0.01	20:5n-3	265 ± 1
17:1	0.88 ± 0.05	22:0	1.2 ± 0.2
16:3n-3	0.19 ± 0.04	22:1n-11	14.7 ± 0.1
16:4n-3	1.52 ± 0.04	22:1n-9	2.6 ± 0.1
16:4n-1	2.86 ± 0.01	22:1n-7	2.5 ± 0.1
18:0	37.2 ± 0.1	22:2n-6	0.8 ± 0.2
18:1n-13	1.04 ± 0.07	21:5n-3	8.2 ± 0.2
18:1n-11	0.8 ± 0.3	22:4n-6	1.3 ± 0.6
18:1n-9	75.2 ± 0.4	22:5n-6	4.3 ± 0.2
18:1n-7	37.1 ± 0.2	22:4n-3	1.04 ± 0.07
18:1n-5	1.63 ± 0.02	22:5n-3	26.7 ± 0.1
18:2Δ5,11	0.40 ± 0.07	22:6n-3	172.7 ± 0.7
18:2n-7	0.42 ± 0.02	24:1	6.87 ± 0.05
18:2n-6	12.9 ± 0.2		

Results and Discussion

FA present in the standard EE oil were typical of nutraceutical grade fish oil, containing the major constituents of EPA and DHA, as well as smaller amounts of monounsaturated, saturated and branched-chain FA. The concentrations of EE in the standard material (Table 1) as determined by GC–FID, combined with the EE concentrations from the SPME GC–MS analysis were used to create a standard curve (mg/g of EE) for each FA EE present, in order to compensate for the varying volatilities of EE with different structures. Because of the variance in molecular masses with different FA structures, not all EE present in the oils could be quantified by SPME. Some were simply not volatile enough and/or were not present at concentrations sufficient for quantification. For example, 16:3n-3 was quantified in trace amounts as ME by GC in the standard oil, but did not adsorb to the SPME fiber in large enough amounts to allow for quantification. Similarly, nervonic acid (24:1) was not volatile enough to be quantified, despite being easily detected as ME by GC. However, in many situations it is only EPA and DHA that need to be verified as TAG since they are generally considered the active ingredients in fish oil supplements and are likely to be added as EE to meet product specifications. Both of these FA were easily quantified using SPME. Alternatively, if TAG is diluted with EE in order to decrease the PUFA content, some EE likely to be used would be myristic acid EE (14:0), palmitic acid EE (16:0) and stearic acid EE (18:0). These FA were accurately

quantified by SPME with high coefficients of determination because of their relatively low molecular weights and high volatility. Lower volatility of longer chain PUFA does, however, become a problem when combined with low concentrations of those EE in the standard material, resulting in calibration curves with poorer fit. For instance, 20:1n-7 and 20:3n-6 were present in the standard at levels <10 mg/g and all had $r^2 < 0.96$; however, levels of myristic acid (14:0) and 15:0 were similarly low in the standard (concentrations of 3.73 and 0.50 mg/g, respectively) but both r^2 values were 1.00. Longer chain PUFA also have greater detection limits due to their low-volatility; for example, the lower detection limit for EPA is 5.08 mg/g, while shorter chain FA such as myristic acid can be detected at 0.04 mg/g. If one requires a lower detection limit for specific long chain FA, selection of standard material with a higher PUFA concentration will be necessary.

Of the three samples tested, two were found to contain EE. Commercial Sample A was a liquid fish oil product, purportedly containing a TAG concentrate. It was found to contain 1.6% w/w (16 mg/g) EE with the primary EE detected as EPA (20:5n-3), DHA (22:6n-3) oleic acid (18:1) (Table 2). As EPA and DHA were the major EE present in this product, it seems likely that they were added as an inexpensive way to increase the PUFA content. The presence of shorter chain EE in the product suggests that an EE concentrate was added to the product, as opposed to only EPA and DHA being added.

Commercial Sample B caused some difficulties during analysis. The product was an encapsulated fish oil concentrate product with an enteric coating and an alginate/glycerol based capsule that claimed to be the natural form of fish oil. Upon analysis it was clear that the sample contained EE as well as two large peaks, not typically found in fish oil (Fig. 1a, b). A NIST library search suggested that these peaks were glycerol and caprylic acid (8:0). Free FA are commonly determined by SPME using a variety of fibers [19–22], while glycerol determination with SPME is rarer. It has been quantified in at least one study using a similar SPME fiber coating of carboxen/polydimethylsiloxane [23] and Goicoechea et al. [24] identified it using the same fiber as this study. The capsule material seems the most obvious source of glycerol. Caprylic acid, on the other hand, can arise from TAG breakdown; however, it is unlikely that substantial amounts of a single, very short-chain FA would arise from that process, particularly from degradation of fish oil. It seems more likely that the free FA is also associated with the capsule material or the enteric coating. Unfortunately it was impossible to quantify the EE in this sample because of the rising baseline associated with the glycerol and caprylic acid peaks.

Table 2 Structures and concentrations of EE in commercial Sample A

Ethyl ester	Amount EE (mg/g)	Lower detection limit (mg/g)	Upper detection limit (mg/g)	r^2
20:5n-3	6.6 ± 0.2	5.08	25.38	0.99
22:6n-3	4.8 ± 0.2	3.28	16.40	0.98
18:1n-9 and 18:1n-7	1.1 ± 0.1	0.72	7.23	0.99
18:0	0.6 ± 0.1	0.36	3.57	0.97
18:4n-3	0.52 ± 0.06	0.42	2.09	0.99
22:1n-11	0.37 ± 0.07	0.28	1.39	0.96
20:4n-6	0.35 ± 0.09	0.16	1.56	0.93
16:0	0.34 ± 0.09	0.00	2.81	0.98
16:1n-7	0.18 ± 0.05	0.00	1.30	0.99
18:3n-3	0.15 ± 0.05	0.08	0.82	0.97
20:1n-7	0.11 ± 0.05	0.06	0.57	0.95
18:2n-4	0.10 ± 0.03	0.09	0.43	0.98
18:2n-6	0.09 ± 0.03	0.09	0.43	0.99
20:3n-6	0.08 ± 0.03	0.06	0.28	0.95
20:0	0.07 ± 0.03	0.06	0.30	0.97
22:1n-9	0.06 ± 0.03	0.05	0.24	0.96
16:4n-1	0.05 ± 0.03	0.01	0.28	0.96
18:3n-6	0.05 ± 0.02	0.04	0.20	0.98
14:0	0.04 ± 0.03	0.04	0.37	1.00
18:3n-4	0.04 ± 0.02	0.03	0.16	0.98
16:2n-4	0.04 ± 0.02	0.02	0.18	0.99
16:3n-4 and 17:1	0.04 ± 0.01	0.03	0.16	1.00
i-17:0	0.01 ± 0.01	0.01	0.07	1.00
22:1n-7	0.01 ± 0.02	0.01	0.24	0.99
16:2n-6	0.01 ± 0.00	0.01	0.03	1.00
15:0	0.01 ± 0.01	0.00	0.05	1.00
Totals	15.86 ± 1.53			

Upper and lower detection limits and r^2 values of the calibration curves are included

Commercial Sample C consisted of a capsule without enteric coating. This sample did not contain EE, glycerol or caprylic acid. The absence of these peaks in Sample C suggests that the glycerol and caprylic acid in Sample B were likely derived from the enteric coating of the capsule, rather than the capsule itself. This also indicates that the method described here may require modification to avoid the chromatography problems encountered with analysis of glycerol, caprylic acid and EE if it is to be applied to fish oil products in capsules that contain such coatings. We did not pursue this issue further, but it could likely be resolved by selecting a SPME fiber that retains EE but has little affinity for caprylic acid and glycerol [25]. In addition, we envision this method as being useful for fish oil supplement producers who wish to test the integrity of the fish oil they are using in their products. Thus the testing of raw mate-

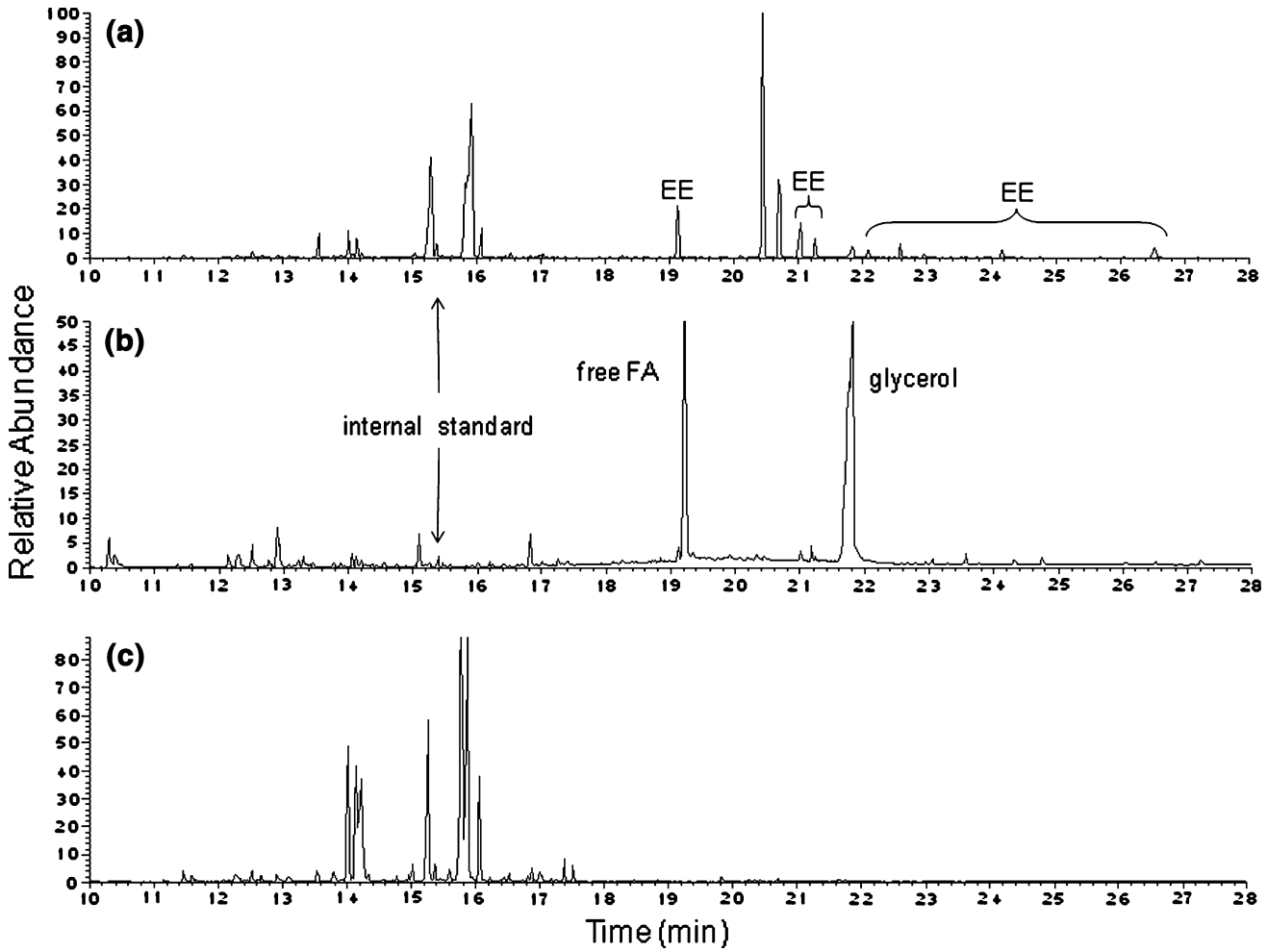

Fig. 1 Partial chromatograms of volatile compounds in TAG oils detected by SPME. **a** Commercial Sample A containing EE. The majority of compounds with large peak areas eluting before 19 min are flavours added to the product by the producer to mask fishy off-odours; **b** commercial Sample B, an encapsulated product, containing traces of EE as well as glycerol and free FA; and **c** commercial Sample C, also an encapsulated produce, without EE, glycerol or free FA. Compounds present in this sample are flavour components or products of lipid oxidation

rials purchased from third parties would be carried out before encapsulation and introduction of the enteric coating, avoiding the problem of interference by capsule materials. A particular advantage of this method is that it will simultaneously extract volatile oxidation products from oils so that it is possible to test raw materials for the presence of both EE and oxidation products at the same time.

The method described here requires very little sample preparation and is therefore, very simple to perform. Minimal sample manipulation also avoids losses of analytes that are often encountered with multi-step methods, while avoiding solvent use is particularly attractive in light of heightened awareness of solvent toxicity. Additionally this method is inexpensive and reproducible, making it ideal for testing fish oil authenticity. We found this method particularly useful in that we could monitor both EE content and volatile lipid oxidation products in a single run. We manually sampled headspace, but the method could easily be automated with the use of a SPME autosampler. In fact, the step requiring the greatest time investment is supervising the integration of peak areas and manipulating data.

Thus, this method offers a reliable alternative to HPLC and TLC for the detection of EE, with the important benefits inherent in all SPME techniques of avoiding the use of solvents, and being highly sensitive. We anticipate that this method will be particularly useful for fish oil supplement manufacturers as a means to test the quality of their source oil.

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