LETTER TO THE EDITOR

# Remarks on Official Methods Employing Boron Trifluoride in the Preparation of Methyl Esters of the Fatty Acids of Fish Oils[1]

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**ABSTRACT:** Some "official methods" for preparing methyl esters of the fatty acids from oils or fats may be referred to by users as the boron trifluoride ($BF_3$) method and invariably have two stages. The first stage, brief treatment with alkali [commonly NaOH in methanol (MeOH), sometimes $NaOCH_3$] and heat has been popularly described as a saponification step for over 30 yr. In fact, the disappearance of visible fat or oil is mostly transesterification, which can be accomplished in a few minutes under mild conditions. Free fatty acids (FFA) originally present, or produced by saponification, are not converted to methyl esters at this stage. The second stage, heating in $BF_3$–MeOH, has in practice been as short as 2 min. It can convert all FFA to methyl esters, but this step requires at least 30 min. Examples from the recent literature illustrate the necessity of extending the time for $BF_3$–MeOH transesterification of lipids or oils and methylation of FFA. No alkali transesterification is needed.
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**KEY WORDS:** Boron trifluoride–methanol, free fatty acids, methyl esters, transesterification.

The use of boron trifluoride–methanol ($BF_3$–MeOH) was originally developed by industrial chemists when gas–liquid chromatography (GLC) was in its infancy and was applied to familiar and common oils. We are now in an era when more delicate biochemical analyses, often with fatty acids of diverse structures or from very small samples, are in common use. The objective of "determining fatty acid composition" has recently been defined in *INFORM* (1). A flow schematic of the process to achieve that objective by GLC included seven steps, and each step had a factor or factors that could affect the accuracy of the results. The confidence of many North American research workers in their use of the popular $BF_3$–MeOH reagent for preparation of methyl esters may well have been shaken if they became aware of one of two papers published in sequence in the *Journal of the Japanese Oil Chemists' Society* in 1996. The first paper (2) reported an interlaboratory collaborative study and method standardization that was carried out by six laboratories in Japan. Three refined fish oils were analyzed for eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids. Methylation was carried out by transesterification with 2 M KOH in methanol. These six laboratories accessed pure EPA and DHA methyl esters, and with reference to the internal standard of tricosanoic acid (23:0), determined experimental flame-ionization detector (FID) correction factors of 1.01 for EPA and 1.06 for DHA to yield accurate results for these fatty acids, expressed in mg/g of sample. The corresponding values calculated from the theoretical FID response data of Craske and Bannon (3) are 1.02 and 1.03. In all respects, this paper (2) stands alone and satisfies one of the problems identified in the *INFORM* article, that of the availability of an appropriate reference standard. This paper (2) and its conclusions seem to be entirely acceptable. Regrettably, the alkali transesterification method cannot be used for any oil if much free fatty acid (FFA) is present, often a problem with raw fish oils. These FFA remain as FFA.

The paper immediately following in the same journal issue was published by the same senior author with a different group of coauthors (4). It tested the quantitative determination of EPA and DHA in several different fish oils, but compared the one-step KOH–methanol method of preparing methyl esters by transesterification with a two-step (alkali transesterification followed by $BF_3$–methanol) ester preparation method, the procedure included by both the Association of Official Analytical Chemists (AOAC) and the American Oil Chemists' Society (AOCS) in their recommended methods for these fatty acids in fish oils (5,6). Unfortunately, the English abstract of this paper (4) refers to "both the $BF_3$–methanol and the KOH–methanol method." This implication of $BF_3$–methanol being a separate method, even when used in combination with alkali transesterification, is a frequent pitfall in publications. For example, Yurawecz *et al.* (7) observed that $BF_3$ methylation of the fatty acids of edible oils degraded preexisting conjugated linoleic acid hydroperoxides and analogous hydroxides to give conjugated trienes. They refer expressly to the published interlaboratory trial by Joseph

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and Ackman (8) that became the basis of the AOCS official method (5). They state in their procedure description: "This method is referred to as the $BF_3$ procedure." This is almost accepted as correct common usage, in part because, unfortunately, the AOAC (6) refers to Official Method 969.33 as the "Boron Trifluoride," although the first step is described as saponification. This term follows from the procedural description in the original publication of Metcalfe *et al.* (9) on the use of $BF_3$.

The EPA content of four fish oils in mg/g sample by the KOH–methanol method alone was found by the authors (4) to be approximately 15% higher than by the "official" combined KOH–methanol and $BF_3$–methanol method; and the DHA content was also higher, by 5%, compared with the KOH–methanol method alone (4). Table 1 gives the actual published values in mg/g oil. Tuna oil C was said to be slightly oxidized (peroxide value 13.37), and tuna oil D was said to be partially hydrolyzed and to have an acid value of 6.82, possibly an important clue in explaining this mysterious result.

The problem can be set forth as follows. A much cited publication (9) referred to saponification to account for an oil going into solution. Principally, saponification, the term used in many reports and instructions, is the last thing wanted. Under anhydrous conditions, transesterification of fats, catalyzed by alkali in methanol, can be very rapid at even ambient temperature and produces methyl esters in 2–3 min (10). An in-depth study by Glass (11) used a cosolvent system with hexane to improve oil solubilization for the rapid alkali-catalyzed reaction. This recently has been clarified for the AOCS (12) with a demonstration of nearly 90% conversion of triacylglycerol to methyl esters by NaOH in methanol in 3 min of heating time. With time, or heat, the alkali will begin to saponify the esters already formed, and part of the sample or ester product converts to soaps of FFA. During the 1990 AOCS meeting in Phoenix, R.G. Einig showed (Fig. 1) that, starting with FFA, the official methods of NaOH–MeOH, followed by $BF_3$–MeOH, gave only 85–90% of methyl esters after 5 min of refluxing in the second-stage $BF_3$–MeOH solvent system. This procedure in fact required 20 min for 99% completion. In contrast, starting with a triacylglycerol, the two-step reaction was 99% complete after a total of as little as 10–12 min of refluxing in $BF_3$–MeOH (13) because most of the production of methyl esters had been achieved before that time by alkali-catalyzed transesterification alone.

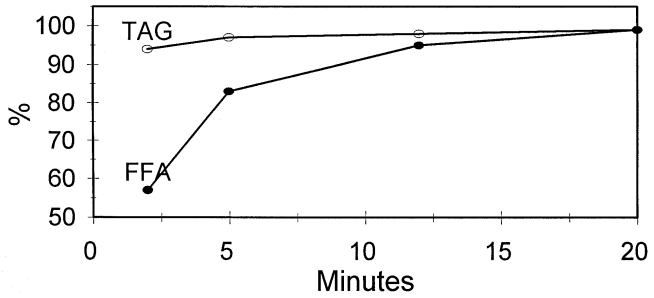


**FIG. 1.** Course of conversion with time of either free fatty acids (FFA) (●) or triacylglycerols (TAG) (○) to methyl esters by $BF_3$–MeOH. Based on 70 mg marine oil and 4.0 mL 0.5 N NaOH refluxed for 5–10 min, cooled, 5.0 mL 14% $BF_3$–MeOH added, refluxed for 2, 5, 12, and 20 min (Einig, R.G., unpublished results).

It is extremely important to point out that the masses in Table 1 are in mg/g of sample. The published results refer only to these two fatty acids, but there is an implication that they may have been damaged or lost in the two-step KOH–methanol and $BF_3$–methanol procedures. This is not true as shown by the balance of their table, reproduced herein as Table 2. The problem is that of incomplete production of methyl esters. The FID area responses and calculated weight percentages of all fatty acids in the fish oil would presumably be all equally affected, unless some were originally in the form of FFA, as in tuna oil D. These detailed data for other fatty acids are not included in the paper.

It is evident from Table 2 that the latter part (4) of the particular duo of papers, reporting only about 82% recovery of fatty acids from $BF_3$–MeOH, compared to the approximately 93% recovery from the KOH–MeOH method, illuminates this problem as too much FFA induced by the alkali step of treatment. In fact, the lowest figures for total fatty acids converted to methyl esters were reported for tuna oil D, with an acid value of 6.82 (Table 2). The plausible reason for the incomplete recovery of EPA and DHA from fish oils by the official method cited is thus that, if the authors followed the AOAC and AOCS instructions, they must have inadvertently saponified part of the sample in the first step. In the second step, the

**TABLE 1**
**Comparison of KOH–Methanol and $BF_3$–Methanol Methods in Determining EPA and DHA in Refined Fish Oils[a]**

| | EPA (mg/g) | | DHA (mg/g) | |
|---|---|---|---|---|
| | KOH | $BF_3$ | KOH | $BF_3$ |
| Tuna oil A | 70.9 ± 1.9 | 62.0 ± 1.7 | 240.4 ± 1.7 | 229.7 ± 1.7 |
| Tuna oil B | 127.1 ± 1.0 | 112.4 ± 1.3 | 201.1 ± 1.1 | 192.6 ± 1.2 |
| Sardine oil | 250.8 ± 0.9 | 214.7 ± 2.2 | 137.2 ± 1.1 | 129.0 ± 2.1 |
| Tuna oil C | 99.2 ± 1.4 | 84.3 ± 0.6 | 278.2 ± 0.7 | 224.3 ± 0.6 |
| Tuna oil D | 68.2 ± 1.9 | 58.2 ± 1.3 | 226.4 ± 0.5 | 217.7 ± 1.3 |

[a]Data from Kajishima *et al.* (4). EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; $BF_3$, boron trifluoride; KOH, potassium hydroxide.

**TABLE 2**
**Acid Values of Original Oils and Total Fatty Acids Recovered (as methyl esters) from Five Fish Oils Treated with KOH–Methanol Alone or Subsequently with a $BF_3$–Methanol Step[a]**

| | | Total fatty acids | |
|---|---|---|---|
| | Oil acid value | KOH–MeOH | $BF_3$–MeOH |
| Tuna oil A | 0.02 | 921 ± 1.7 | 799 ± 1.1 |
| Tuna oil B | 0.07 | 932 ± 1.1 | 852 ± 1.2 |
| Sardine oil | 0.08 | 946 ± 0.8 | 837 ± 2.0 |
| Tuna oil C | 0.34 | 926 ± 1.5 | 820 ± 2.3 |
| Tuna oil D | 6.82 | 886 ± 1.7 | 779 ± 1.1 |

[a]Data from Kajishima *et al.* (4). See Table 1 for abbreviations.

$BF_3$–MeOH either was too weak a solution or did not have sufficient time to convert the resulting FFA into methyl esters.

Fortuitously, a comparison through parallel applications of the combination two-step procedure of alkali–methanol and then $BF_3$–methanol treatment, with NaOH–methanol transesterification alone, is provided quite independently by data in an International Standards Organization/Technical Committee (ISO/TC) method document "ISO/CD 5509-animal and vegetable fats and oils—preparation of methyl esters of fatty acids," dated 17 Sept. 1996. Six to eight laboratories participated in a comparison of three methods for making methyl esters. The results are given only as area percentages, but Table 3 suggests no real difference among the three methods tested, including KOH–MeOH by itself and when followed by a second and esterification step in $BF_3$-MeOH.

The instructions for the 1996 ISO/CD procedure "General Method Using Boron Trifluoride" are of interest and may be quoted as follows: "Saponify [*sic*] in methanolic NaOH (5–10 min); Add $BF_3$–MeOH (12–15%) to soaps *in situ*; Boil for 3 min; For fish oils boil for 30 min (because of long-chain fatty acids); Extract methyl esters into iso-octane." The second-from-the-last instruction is especially interesting because of misunderstandings that have crept into all reports on the various methods discussed above, although the ISO instruction to extend the period of $BF_3$–methanol reaction to 30 min for fish oils may have arisen from a misunderstanding, or even a mistranslation. It would, however, have been effective in this particular set of analyses in producing a high proportion of methyl esters of fatty acids and a complete set of area percent analyses comparable to the other methods used. This document has been reissued in 1997 as "Draft International Standard ISO/DIS 5509" with the same instructions.

The remaining problem concerning the publication by Kajishima *et al*. (4) is why there should be a difference between the reduction in quantitation of EPA and DHA. The former has the first ethylenic bond in the Δ5 position, the latter in the Δ4 position. Although no comparisons of rates of esterification seem to be available, it is possible that under acidic catalyst conditions the Δ4 ethylenic bond of FFA DHA may be close enough to the carboxyl group to influence the esterification rate compared to that of FFA EPA. This view is supported by Table 3. The ISO data give a little less 16:0 and 20:5n-3 for the two-step alkali–$BF_3$ method than the one-step alkali transesterifications, but possibly more 22:6n-3. The same "saponification" problem could have existed and, if the $BF_3$–methanol step was extended to 30 min, the 22:6n-3 in free acid form could well have esterified faster than the 16:0 and 20:5n-3. The only alternative is to assume that any FFA present could have been derived from fish muscle phospholipids, and these would usually be higher in 22:6n-3 than the corresponding triacylglycerols.

**TABLE 3**
**Average Area Percentage Data from GLC of Methyl Esters of Three Major Fatty Acids of a Fish Oil Prepared by Three Different Methods[a,b]**

| Fatty acid | "General Method" NaOH plus $BF_3$–MeOH $n = 7$ | Simple KOH–MeOH transesterification $n = 8$ | Injector reaction with trimethylsulfonium hydroxide $n = 6$ |
|---|---|---|---|
| 16:0 | 18.2 ± 1.6 | 18.5 ± 1.8 | 18.8 ± 1.9 |
| 20:5n-3 | 18.2 ± 0.7 | 18.8 ± 1.1 | 17.8 ± 1.3 |
| 22:6n-3 | 10.4 ± 0.3 | 9.6 ± 4.3 | 9.9 ± 0.9 |

[a]Data from Document N615 of ISO/TC 34/SC11. GLC, gas–liquid chromatography. See Table 1 for other abbreviations.
[b]*n* = number of participating laboratories.

Method confirmation is of course highly desirable and often misunderstood. The postreaction "saponification" of methyl esters has been examined critically and shown to be sensitive to chainlength (14,15). Separation of chemical errors from instrumental errors, including injection technology (16), has been discussed in detail (17). $BF_3$–methanol was condemned for ester preparation from albacore tuna lipid on the grounds that less 18:1n-9 was obtained than by other methods (18). No mention is made of plasmalogens and by-product dimethyl acetals, but because 16:0 and even 14:0 are also relatively low, this seems to be a situation where the inclusion of these types of phospholipids should be investigated. The same paper reported a late-eluting "artifact" peak, and similar problems have suggested a modification of the AOAC official procedure by extracting nonsaponifiable materials (19). Approximately 10% of the 20:5n-3 and 4% of 22:6n-3 disappeared in the extra handling. Any esters formed and remaining after incomplete saponification would of course be removed with the unsaponifiables. In another study, microwave heating of $BF_3$–methanol alone seemed satisfactory for fish oils (20). This method also scored highly in grade among methylation techniques, and elimination of the alkali transesterification step did not seem to matter in an extensive collaborative evaluation that unfortunately did not involve fish oils (17).

A blanket condemnation of the use of $BF_3$ with fish oils seemed unwarranted because of our extensive and successful experience with $BF_3$–methanol alone, although some adverse effects from $BF_3$ esterification of sensitive fatty acids are known (21–24). Cyclopropene fatty acids rearrange in the presence of $BF_3$ (25) as do cyclopropane fatty acids (26), and squalene is destroyed by $BF_3$–MeOH (27). Hydroxylated and conjugated dienoic fatty acids are dehydrated to produce conjugated trienes (7), and care is recommended in conjugated linoleic acid (CLA) analyses (28). There have been repeated references to bad results from aged $BF_3$–MeOH solutions. At one time, a 50% solution was sold by at least one supply house. Early experience may have suffered from unnecessarily high concentrations of $BF_3$ (21–24). Frequently opening the original bottle for dilution or use no doubt allowed moisture pick-up from the atmosphere; also, fats and oils can easily contain up to 0.5% water without that being apparent. In repeated use, screw-cap centrifuge tubes (see below) can become cloudy and weakened near the neck, suggesting attack by hydrofluoric acid (HF). Possibly, HF is the actual problem when bad results are reported from the use of $BF_3$–methanol.

**TABLE 4**
**Triplicate Analyses of a Fish Oil for 20:5n-3 and 22:6n-3 Oil Transesterified with $BF_3$ Alone[a]**

| | Selected fatty acids | | Iodine value of oil | |
|---|---|---|---|---|
| | mg/g sample | w/w% by GLC | Wijs | Calculated |
| EPA | 165 | 16.5 | 193 | 192 |
| | 168 | 16.4 | 192 | 194 |
| | 167 | 16.4 | 192 | 193 |
| DHA | 127 | 12.6 | — | — |
| | 128 | 12.3 | — | — |
| | 127 | 12.3 | — | — |

[a]With area percentage corrected by flame-ionization detector response to give weight percentage of visible fatty acid esters, and by the use of 23:0 to give mg/g sample. Iodine values by the AOCS Wijs method and that calculated from the whole of the GLC w/w% analysis are also given. See Tables 1 and 3 for abbreviations.

Clearly, there should be acceptance of continuous change as and when required, even in such a simple matter as preparation of methyl esters (29,30). In my laboratory, we have for many years simply omitted the alkali transesterification step in the preparation of methyl esters of fish oil fatty acids. We only use equal volumes of $BF_3$–methanol of 7% concentration and of *n*-hexane to reduce the concentration to 3.5% of total volume, and heat for 1 h at 100°C in a screw-cap (Teflon-lined) centrifuge tube flushed with nitrogen. Table 4 shows the results of one set of triplicate analyses of a fish oil and of a comparison of the w/w% fatty acid composition by GLC to the calculated iodine values. $BF_3$–methanol alone seems to be a satisfactory basis for all of these fish oil analyses. The reasoning behind the proposal to the AOAC of the two-step procedure (8) and the times given for each of the alkali transesterification and $BF_3$–methanol treatments was to ensure rapid adoption by the AOAC of the method to have mg/g recognized as the standard listing for EPA and DHA in fish oil capsules and concentrates. Frequent abuse of GLC parameters in labeling was unfortunately common in the nutritional supplement trade at the time (31–33). Minor changes in heating times were made from existing methods, such as AOAC method 969.33, but radical changes were avoided to smooth the way for adoption by the two official bodies. In the light of the Kajishima *et al.* (2,4) studies recently published that illustrate the potential problem created by the alkali transesterification step of the official methods, including subsequent $BF_3$–MeOH treatment, it is now time to consider whether the AOAC, AOCS, and other bodies should not simply omit the alkali step in their official methods. By relying on heating oils with $BF_3$–methanol for an appropriate time alone, original FFA or those from saponification cease to be of concern and would automatically be converted to methyl esters.

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