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# A Modified Methylation Method to Determine Fatty Acid Content by Gas Chromatography

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An improved rapid method for determination of the fatty acid composition using modified methylation procedure was compared with the AOAC reference procedure based on the methylation of fatty acid with the addition of $BF_3$ catalyst before and while heating. The new method is useful for research and routine quality control and has a number of advantages over the reference procedure which are more rapid, simple and also reliable. Applicability of the modified methylation method was confirmed with three vegetable oil samples (palm oil, coconut oil and olive oil). Based on the validation method results, we obtained that a quite linear calibration curve of fatty acids was performed with $R^2$ in range of 0.9972-0.9994. The sensitivity of gas chromatography instrument was able to analyze the fatty acids up to a few ppm, the precision and accuracy were good enough with the %RSD between 1.5%-19.5% and the recovery of linolenic acid was 99.1% in the range of 80.0%-113.3%.

**Key Words :** Fatty acid methyl ester, Methylation, Boron trifluoride, Validation method

## Introduction

The fatty acid composition of vegetable oil determined by gas chromatography with capillary column has been studied for many decades and is still highly relevant for the laboratory routine analysis. Vegetable oil fatty acid analysis presents some complexity due to the wide-range of fatty acid chains and the reliability of the result. Many different methylation methods to prepare fatty acid methyl esters (FAMEs) were described in previous studies. The most widely used techniques, such as diazomethane in ether, acid-catalyzed transesterification, and base-catalyzed transesterification methods for vegetable oil separation are primarily based on solvent extraction.[1-3] The acid catalysts such as $BF_3$, HCl and $H_2SO_4$, all in methanol were commonly used to methylate fatty acid, the acid catalyst not only produced methyl esters by transesterification of triacylglycerols (TAGs) but also esterified free fatty acids in the presence of methanol. Whereas NaOH, KOH and tetramethylguadenine (TMG) commonly used as the base catalysts.[4-10] However, the esterification of free fatty acids (FFAs) was not performed by bases and synthesis of FAME also consumed much time.[11]

In the reference procedure (Association of Official Analytical Chemists),[12] lipid are extracted using a mixture of boron trifluoride and hexane after first adding an sodium methoxide. However, this extraction method are still requiring a lot of time to analysis of large numbers of fatty acid samples in quality control laboratory and must be handled cautiously because $BF_3$-methanol is harmful if inhaled or absorbed through skin. Thus there is a need for a simple, accuracy, rapid and reliable method that could be used to analyse a large number of vegetable oil in the quality control laboratory or in research field. Over the years Metcalfe *et al.* procedure has undergone modifications,[13] the modified $BF_3$-methanol procedure as proposed by Wijngaarden has performed the reflux of methanolic NaOH and fat usually takes 2-5 min.[9] However this method were not sufficiently standarized.

The aim of the research was to compare the modified methylation method with the official reference procedure to determine fatty acid composition. To test the method applicability, three vegetable oil such as palm oil, coconut oil and olive oil were examined. Moreover, to improve this new technique, the rapid, one-step and one-vial were well established, standard FAME mix and corn oil were used to evaluate this method.

## Experimental

**Sample.** Baek Sul corn oil obtained from CJ Corporation was used as the sample. Three vegetable oil such as palm oil, coconut oil and olive oil were collected from different market in Indonesia.

**Material.** Following reagents were used in this study: standard FAME mixtures and boron trifluoride (12.5% in methanol) were purchased from Sigma-Aldrich®, methanolic NaOH, saturated NaCI solution, hexane, petroleum ether were purchased from Merck.

**Fat Separation and Derivatization Methods.** The reference procedure was performed according to Association of Analytical Communities (AOAC) 969.33 procedure. Into 0.35 g oil was added 6 mL 0.5 mol $L^{-1}$ NaOH-methanol in a 250 mL round bottom flask and shaked, attached condenser and refluxed until 5-10 min at 55 °C. Added 7 mL 12.5% $BF_3$-methanol from bulb through condenser and continue

boiling for 2 min. Added 5 mL hexane through condenser and boil 1 min longer. Removed heat and added 15 mL saturated NaCl solution. To recover dry esters, transfer aqueous and hexane phases to 250 mL separatory funnel and extracted with two 50 mL petroleum ether. Washed the combined organic phases with aquabidest until free of base, evaporated the solvent using rotary evaporator. The concentrate was dissolved with 1 mL hexane prior to injection.

Modified procedure, 0.35 g corn oil was added 6 mL 0.5 mol $L^{-1}$ NaOH-methanol in a 250 mL round bottom flask and shaked, added 7 mL 12.5% $BF_3$-methanol then shaked again. The mixture was refluxed at 70 ºC for 2-3 min, added 5 mL hexane through reflux system and continue heating for an additional 2 min. During reflux, the mixture was shaked occasionally until the methylation process completed. After cooling, ± 30 mL saturated NaCl solution was added and extracted vigorously. The aqueous phase transfered into 250 mL separatory funnel and extracted with two 50 mL petroleum ether. The combined organic layer was washed with aquabidest until free of base, the solvent was evaporated using rotary evaporator. The concentrate was dissolved with 1 mL hexane prior to injection.

**Gas Chromatography.** FAMEs were analyzed on a Shimadzu gas chromatography 2010 equipped with flame ionization detector (FID). Fatty acids were separated using a INNOWax capillary column (0.25 mm i.d. 30 m in length, 0.25 μm film thickness). The carrier gas was hydrogen at flow rate of 40 mL $min^{-1}$, with air 400 mL $min^{-1}$ and make up gas of Helium at 30 mL $min^{-1}$. The column was temperature programmed at 3 ºC $min^{-1}$ to 250 ºC with initial temperature 100 ºC. The injector was set at 230 ºC with split ratio of 50:1 and the detector was set at 250 ºC.

**Validation of Analysis Method.** To ensure the analysis results, it is necessary to validate the analytical method that has been modified. Validation is an assessment to certain parameters based on laboratory experiment. The validation must give fidelity guarantee that the method meets the requirements for analytical applications, thus, having the reliability of the results. There are some parameters included in method validation that is linearity, precision, accuracy, limit of quantification (LOQ), limit of detection (LOD), sensitivity and robustness.[14,15]

The linearity illustrated the ability of analysis method to give response to the analyte concentration in sample. The linearity test was evaluated using five concentrations of FAME standard. The linearity was assessed by the linear regression equation (y = ax + b). LOD and LOQ were calculated statistically through linear regression line from calibration curve, considering that the LOD was three times the baseline noise and the LOQ was ten times the baseline noise. The precision was expressed as percent relative deviation standard (% RSD) and evaluated in terms of repeatability which was obtained by the analysis of seven replicates of the prepared samples then calculated the mean and deviation standard so as to obtained RSD. Besides using certified reference material the accuracy can also be investigated by a spike addition method.[16] Spike is addition of analyte which have been known its concentration into the sample. According to this method, the sample was spiked with a known amount of fatty acid and the percentage recovery (%R) was calculated based on the concentration of the individual sample and the spiked-sample. Analysis was carried out in seven replicates of spiked-samples.

## Results and Discussion

The compositions of fatty acids in corn oil were analyzed using AOAC reference procedure, standard FAME mix was injected at 1 μL. Qualitative data was analyzed by comparing the retention times of FAMEs of the sample with those of standard FAME mix. The relative content of individual components were calculated by area normalization method.[17,18]

$$K_i = \frac{B_i \times \Sigma C_i}{C_i \times \Sigma B_i}$$

$$\text{Fatty acid \%} = \frac{(A_i \times K_i')}{\Sigma(A_i \times K_i')} \times 100\%$$

Where $K_i$ is correlation factor of component i, $B_i$ is mass of component i in the reference mixture, $\Sigma B_i$ is mass total of the various components of reference mixture, $C_i$ is area under peak corresponding to component i, $K_i'$ is relative correlation factor, $\Sigma C_i$ is sum of the peak areas. $A_i$ is area of peak corresponding to component i. Chromatogram for standard FAME mix and oil sample (corn oil) were showed in Figure 1 and Figure 2 respectively.

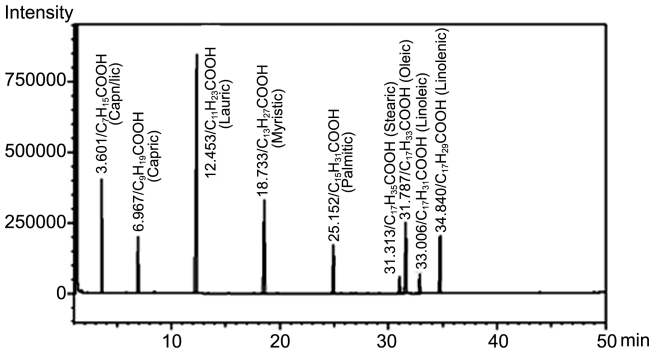


**Figure 1.** Chromatogram for standard FAME mix.

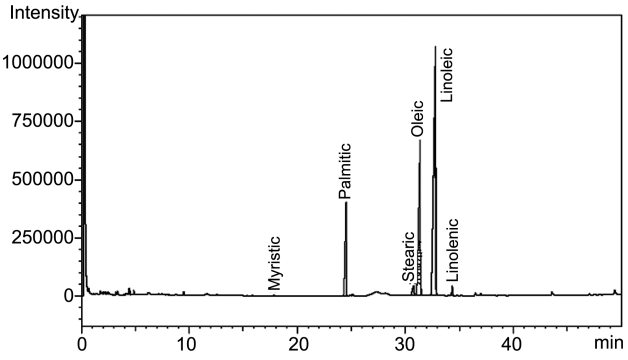


**Figure 2.** Chromatogram for corn oil.

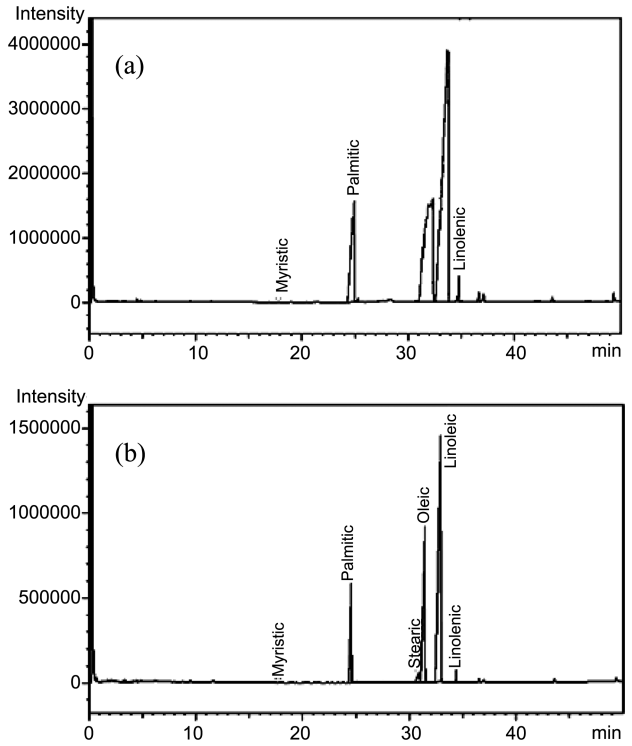


**Figure 3.** Chromatogram for methylation process at (a) 55 ºC, time 2-3 min and (b) 70 ºC, time 2-3 min.

Several values of temperature were tested to accelerate the time into 2-3 minute. The chromatogram at temperature of 55 ºC as shown in Figure 3(a) was not well separated because there was a peak widening particularly at the stearic acid and oleic acid peaks, likewise at temperature of 60 ºC, consequently the calculation of fatty acid compositions could't be done. This probably because the methylation reaction were not completed wherein not all of triglycerides or fatty acids converted into methyl esters. But after the temperature increased into 70 ºC it resulted in a well separated chromatogram Figure 3(b). Because the toxicity of $BF_3$-methanol 12.5%, which may be fatal and the vapour may be harmful and to study its effect, the $BF_3$-methanol was added in the fume hood before refluxed. The resulting chromatogram obtained as good as the chromatogram with the addition of $BF_3$-methanol in the middle of heating as in the reference procedure. This probably because the $BF_3$-methanol is the strong catalyst and extremly reactive toward many types of organic compound so the fatty acid methyl esters were more easily formed.[19] The comparison of corn oil composition with the modified and AOAC reference procedures is given in Table 1 and the comparison of methylation modified and AOAC reference procedures with respect to time is given in Table 2. The t-test was performed to assess there was no significant difference. According to t-table with the error level 0.5%, the calculated-t value was smaller than t-table (t-table: 9,925) its mean there was no significant difference between fatty acid compositions obtained upon the addition of $BF_3$-methanol before heating and while heating as in the AOAC reference procedures.

**Table 1.** Comparison of corn oil composition with the modified and AOAC reference procedures

| Fatty acid | AOAC reference procedure/% | Modified procedure/% | Calculated-t |
|---|---|---|---|
| C8:0 | nd | nd | nd |
| C10:0 | nd | nd | nd |
| C12:0 | nd | nd | nd |
| C14:0 | 0.04 | 0.04 | 0.389 |
| C16:0 | 11.86 | 11.81 | 0.291 |
| C18:0 | 1.98 | 1.92 | 1.167 |
| C18:1 | 28.01 | 27.54 | 3.140 |
| C18:2 | 57.19 | 57.77 | 8.430 |
| C18:3 | 0.92 | 0.91 | 0.471 |

The performance of the procedure was established by a validation procedure which employs the standard solutions. Linearity were observed from small concentration to large concentration, the peak area signals were plot against the corresponding concentrations in the analytical curves to obtain the linear regression models and the correlation coefficients. The parameters of the analytical curves are shown in Table 3. The values of the analytical curve lead to the conclusion that each fatty acid components gave a linear response to different concentration of fatty acids, since the correlation coefficients were closer to 1.

The LOD and LOQ for the mixture of FAMEs was calculated statistically from linear regression models, according

**Table 3.** Parameters of the analytical curve, R, LOD, LOQ and RSD (%)

| Fatty acid | Analytical curve | $R^2$ | LOD (mg $L^{-1}$) | LOQ (mg $L^{-1}$) | RSD (%) |
|---|---|---|---|---|---|
| C8:0 | Y=1524.5x-2199 | 0.9987 | 5.8 | 19.3 | nd |
| C10:0 | Y=1641.2x+2815.6 | 0.9972 | 6.1 | 20.3 | nd |
| C12:0 | Y=1630.3x-883.2 | 0.9994 | 28.0 | 93.4 | nd |
| C14:0 | Y=1622.4x-1102.6 | 0.9992 | 9.6 | 32.1 | 19.5 |
| C16:0 | Y=1587.2x+899.5 | 0.9987 | 6.4 | 21.5 | 3.1 |
| C18:0 | Y=1489.8+559 | 0.9976 | 6.4 | 21.2 | 4.7 |
| C18:1 | Y=1529.9x-1362 | 0.9982 | 11.9 | 39.8 | 2.2 |
| C18:2 | Y=1456.6x-267 | 0.9986 | 4.4 | 14.7 | 1.5 |
| C18:3 | Y=1389x+1547.8 | 0.9992 | 6.8 | 22.7 | 6.1 |

**Table 2.** Comparison of methylation AOAC reference and modified procedures with respect to time

| AOAC reference procedure | Time | Modified procedure | Time |
|---|---|---|---|
| Addition NaOH-methanol, refluxed | 5-10 min | Addition NaOH-methanol and 12.5% $BF_3$-methanol, refluxed | 2-3 min |
| Addition 12.5% $BF_3$-methanol, refluxed | 2 min | Addition hexane, refluxed | 2 min |
| Addition hexane, refluxed | 1 min | | |

**Table 4.** The fatty acid profiles of palm oil, coconut oil and olive oil

| Fatty acid | Reference palm oil content | Palm oil content/% | Reference coconut oil content | Coconut oil content/% | Reference olive oil content | Olive oil content/% |
|---|---|---|---|---|---|---|
| C8:0 | - | - | 4.6-10 | 9.15 ± 0.45 | - | - |
| C10:0 | - | - | 5-8 | 7.90 ± 0.10 | - | - |
| C12:0 | 0.1-0.5 | 0.53 ± 0.03 | 45.1-53.2 | 49.59 ± 0.31 | - | - |
| C14:0 | 0.9-1.4 | 1.29 ± 0.07 | 16.8-21 | 17.81 ± 0.03 | < 0.1 | 0.10 ± 0.01 |
| C16:0 | 38.2-42.9 | 36.85 ± 0.24 | 7.5-10.2 | 7.56 ± 0.02 | 7.5-20.0 | 10.82 ± 0.10 |
| C18:0 | 3.7-4.8 | 2.97 ± 0.06 | 2-4 | 2.19 ± 0.06 | 0.5-5.0 | 3.75 ± 0.06 |
| C18:1 | 39.8-43.9 | 41.90 ± 0.13 | 5-10 | 4.80 ± 0.07 | 56.0-83.0 | 77.79 ± 0.17 |
| C18:2 | 10.4-13.4 | 16.09 ± 0.23 | 1-2.5 | 1.00 ± 0.06 | 3.5-21.0 | 6.53 ± 0.04 |
| C18:3 | 0.1-0.6 | 0.37 ± 0.02 | nd-0.2 | - | < 1.5 | 1.00 ± 0.03 |

to calculation the LOD between 4.4-28.0 mg $L^{-1}$ and the LOQ between 14.7 mg $L^{-1}$-93.4 mg $L^{-1}$, as shown in Table 2. The precision expressed as %RSD was 1.5% to 19.5%. According to AOAC, the analysis method give the good repeatability if the %RSD less than 3% for fatty acid contents larger than 5% whereas the repeatability tend to decrease (the %RSD may be larger than 3%) if the fatty acid contents less than 5%.[12] Because the limited number of the single spike to be added into sample the accuracy (%) can only be performed for linolenic acid. The percent recovery for linolenic acid was 99.1% with the assay value range between 80.0%-113.3%, the accuration criteria fulfilled if the percent recovery in the range of 80% to 120%.[20] This indicated that the linolenic acid accuracy acceptable, but to more assure the accuracy for the other fatty acid should be checked.

**Applicability of the Method.** After the validation, the method was applied to other oil samples in order to evaluate its applicability. Three oil samples (palm oil, coconut oil, and olive oil) was analyzed using this modified procedure to know the fatty acid compositions and to verify whether this modified procedure could be applied to other oil samples besides the corn oil. The experiment was carried out in three replicates. Table 4 shows the fatty profile of the three oil samples.

## Conclusion

This modified methylation procedure can be an alternative analysis procedure to determine fatty acid profile of vegetable oil in quality control laboratory. This proposed procedure are convenient, rapid and reliable for preparation FAMEs and didn't gave a significant difference in analysis result when compared to AOAC reference procedure. The method applicability to other vegetable oil such as palm oil, coconut oil and olive oil with different fatty acid content was demonstrated in our study.

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