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 BF₃ 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 R² 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. The acid catalysts such as BF₃, HCl and H₂SO₄, 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 tetramethylguadeneim (TMG) was used as the sample. 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 NaCl 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 shaken, attached condenser and refluxed until 5-10 min at 55 °C. Added 7 mL 12.5% BF₃-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 shaken, added 7 mL 12.5% BF$_3$-methanol then shaken again. The mixture was refluxed at 70 °C for 2-3 min, added 5 mL hexane through reflux system and continue heating for an additional 2 min. During reflux, the mixture was shaken 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 phase 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 °C min$^{-1}$ to 250 °C with initial temperature 100 °C. The injector was set at 230 °C with split ratio of 50:1 and the detector was set at 250 °C.

**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.$^{17,18}$

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

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.
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 couldn’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 and 12.5% BF$_3$-methanol before heating and while heating as in the AOAC reference procedures.

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 to 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.

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.

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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 5% whereas the repeatability tend to decrease (the %RSD may be larger than 3%) if the fatty acid contents less than 5%.\(^\text{12}\) 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 accuracy criteria fulfilled if the percent recovery in the range of 80% to 120%.\(^\text{20}\) 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 give 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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### References