Determination of acid, peroxide, and saponification value in patin fish oil by FTIR spectroscopy combined with chemometrics

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Abstract

Simple, rapid, and reproducible methods for determining the acid value (AV), peroxide value (PV), and saponification value (SV) of patin fish oil (PFO) were developed using Fourier Transform Infrared (FTIR) spectroscopy combined with chemometrics of Principal Component Regression (PCR) and Partial Least Square (PLS). The relationship between actual values was determined using AOCS method and predicted value was determined with FTIR spectroscopy and chemometrics. From the validation work, the high coefficient of determination ($R^2$) reached up to > 0.99. This study concluded that by means of FTIR spectra that combined with PCR and PLS technique can be used to determine AV, PV, and SV of PFO.

1. Introduction

Patin (Pangasius micronemus) is a freshwater fish in Indonesia and have exported for years to the western market. Indonesia is the second largest of patin producer, with production reaching up to 16.1% of the total production of patin in the world (Ramadhan et al., 2016). Hashim et al. (2015) have successfully extracted fatty acid from patin and get lipid up to 31% with the level of omega-3 was 4.7%. Hence, patin can be a good source of omega-3. While in Thailand and Vietnam, patin was extracted to obtain the fish oils and the patin fish oils (PFO) have been widely traded.

In the market, that is important to know the quality of fish oil. Some parameters used to determine the quality of fish oil. To determine the quality of fish oil can be performed by determining of physico-chemical values such as acid, peroxide, and saponification value (Rohman et al., 2015). Titration method was used to determine the acid, peroxide, and saponification value, based on the chemical reaction between free fatty acids and the reagent. But, the titration method has some disadvantages such as being time-consuming and labor-intensive, requiring large amounts of organic solvents, use of the highly toxic, and carcinogenic reagent, difficulty in distinguishing the end-point with samples containing coloured substances and largely dependent on the skills of the analyst (Rohman et al., 2012; Jiang et al., 2016).

Currently, Fourier transform infrared spectroscopy (FTIR) combined with chemometrics has been widely used for determining acid, peroxide, and saponification value. FTIR has been used for determination of acid value on edible oil-based on -O-H stretching (Jiang et al., 2016), peroxide value on edible oils based on -COO-stretching (Yu et al., 2009; Hu et al., 2019), to monitoring peroxide value in oxidized emulsions (Hayati et al., 2005), and saponification value on red fruit oil using partial least square calibration (Rohman et al., 2015).

However, the application of FTIR spectroscopy for the determination of acid, peroxide, and saponification value of patin fish oil (PFO) has not been reported yet. Therefore, FTIR spectroscopy combined with principal component regression (PCR) and partial least square (PLS) was developed for the determination of acid, peroxide, and saponification value of PFO from some
2. Materials and methods

2.1 Materials
Patin from Tulungagung, East Java, Indonesia. n-hexane, chloroform, ethyl acetate, ethanol, HCl, acetic acid, sodium thiosulphate, KI, acetone, KOH were obtained from Merck (Germany), phenolphthalein indicator and starch from Progo Mulyo store in Yogyakarta.

2.2 Extraction method

Extractions of PFO were performed using maceration method with wet and dried samples and soxhlet method also with three variations of solvent are n-hexane, ethyl acetate, and chloroform. The patin flesh was cutting into small pieces then extracted with the solvent for maceration with wet samples. Patin flesh was dried in the oven at 60°C for 24 hrs before extracted using maceration with dried samples and Soxhlet method. Maceration process was performed at room temperature while the Soxhlet method at 82°C. After the extraction step, the solvent was evaporated using the rotary evaporator at 50°C to get the PFO.

2.3 Acid value determination

Determination of acid value using the AOCS official method Cd 3a–63 (AOCS, 2004). The oil sample (2 g) was mixed with 20 mL ethanol and added with 2 mL phenolphthalein in a 250 mL Erlenmeyer flask. The mixture was titrated with 0.01 M of KOH and shaken vigorously until the color was changed (from white to pink). The result was expressed as the number of milligrams of KOH required to neutralize the free fatty acid in 1 g of the sample.

2.4 Peroxide value determination

Peroxide value is a measure of peroxides contained in the oil and is determined by measuring iodine released from potassium iodide. Determination of peroxide value was performed according to AOCS official method Cd 8b-90 (AOCS, 2005). The oil sample (5±0.01 g) was dissolved in 30 mL acetic acid-chloroform (3:2) solution. Then saturated KI solution and distilled water were added and shake the flask vigorously to liberate iodine from chloroform layer. The mixture was titrated with 0.01 N sodium thiosulphate using starch solution as an indicator.

2.5 Saponification value determination

The saponification value was expressed as the number of milligrams of potassium hydroxide (KOH) required to saponify 1 g of oil. Determination of saponification value was according to AOCS method Cd 3-25 (AOCS, 1990). The 2 g of oil were dissolved with ethanol in Erlenmeyer flask. Then connected with an air condenser and boil gently for 1 hr in order that sample is completely saponified. After it cooled and added 1 mL phenolphthalein. The mixture was titrated with 0.5 N HCl until the pink colour has just disappeared.

2.6 FTIR analysis

Samples were analysed using FTIR spectrometer (Thermo Scientific Nicolet is10, Madison, WI) using Omnic software. The measurements were done in the middle infrared region of 650–4000 cm⁻¹ with 32 scans and at the resolution was 16 cm⁻¹. The background scan was performed to reduce the effect of the reference spectrum of the air. Before and after analysis sample, ATR crystal was cleaned with acetone p.a. Replications were done with scanning the same samples for three times.

2.7 Data analysis

Multivariate analysis of the acquired data was performed using principal component regression (PCR) and partial least square (PLS) with TQ Analyst software version 9 (Thermo Fisher Scientific Inc.) while the statistic analysis was done using Minitab 18. In this research, Principal component regression (PCR) and partial least square (PLS) were used to determine the physico-chemical value from FTIR spectral. PCR is one of inverse calibration technique in which the physico-chemical value from titration method (x-axis) is used as a predictor, while responses such as absorbance at several wavelengths are located on the y-axis. PCR performs multiple inverse calibrations of predictor variables against the scores (knowns as principal components) rather than original variables (Roehman and Putri, 2019). Whereas the PLS method uses a correlation between changes in spectral absorption and sample concentration then computing with other spectra that can disturb the analyte spectra (Ballabio and Todeschini, 2009). In this research using normal, first derivative and second derivative spectra to identify the targeted spectra from the compounds by increasing the spectral resolution (Putri et al., 2019).

The collected data set was divided into calibration and validation subset. Prediction models were validated with cross validation ‘leave one out’ technique. Cross validation evaluates the data by excluding selected samples in the regression model and then constructing a new model for the remaining samples. The new model was evaluated and the error values for predicted observations are computed. The new samples are then
excluded from the model set and a new model is constructed. This procedure is repeated until all samples in the PCR and PLS models have been excluded once (Rohman and Che Man, 2010). In order to compare the performances of the developed calibration models and express their predictive ability, two statistical values, namely root mean square error of calibration (RMSEC) and root mean square error of prediction (RMSEP) were calculated. Among these values, RMSEP gives the best estimation of future performance of the calibration model (Temiz et al., 2017).

3. Results and discussion

3.1 FTIR Spectra of PFO

The FTIR spectra of PFO was shown in Figure 1. Each peak corresponded to a functional group of fatty acid structure. The peak at wavenumber 721 cm⁻¹ was due to the rocking vibration of methylene (-CH₂), while the peaks at 1114 and 1234 cm⁻¹ were from -C=O vibrations. The bending vibration of methylene was observed at wavenumber 1461 cm⁻¹. Meanwhile the carbonyl (C=O) stretching vibration was observed at a wavenumber 1744 cm⁻¹. The peaks at wavenumbers 2854 and 2921 cm⁻¹ represent asymmetric and symmetric stretching vibration of methylene (-CH₂) respectively (Rohman et al., 2011; Putri et al., 2019).

3.2 Determination of AV

The acid value (AV) is an explanatory parameter for evaluation of the level of hydrolysis of oil. Free fatty acid and glycerol were the results of the hydrolysis reaction. The acid value usually can be determined by acid/base titration (Mahboubifar et al., 2016). The AV from both titration and FTIR methods were shown in Table 1. The AVs from FTIR were obtained from optimization method (Table 2). Based on the ANOVA test using Minitab 18, p-value from both methods was 0.00. So, the result from both methods not significantly different (p<0.05). The AV from the Soxhlet method were higher than in the maceration method. According to FAO about the standard for fish oils (2017), the acceptable of AV on fish oil is ≤ 3 mg KOH/g. The high of AV can be caused by heat treatment while in the extraction process. The heat treatment cause oxidation and hydrolysis on PFO to produce free fatty acid (Herchi et al., 2016).

According to Table 2, the PCR technique gave the best result than others. The wavenumber of 2000-3100 cm⁻¹ was chosen to determine AV because give the highest coefficient determination for calibration and validation, 0.9879 and 1.000 respectively with the lowest RMSEC and RMSEP were 0.210 and 0.972.

Figure 2 presents the linear regression of the PCR method expressing the relationship between AVs from the titration method (as actual value) and FTIR spectral (as calculated value). The peak at wavenumber of 2000-3100 cm⁻¹ corresponding to the stretching vibration of cis -C=H at 3006 cm⁻¹ and vibration of CH₂ stretching at the peak at 2854 and 2921 cm⁻¹ from free fatty acid (Che Man et al., 2010) (Figure 3).

3.3 Determination of PV

Peroxide value (PV) is used to measure the level of peroxide/hydroperoxide formed at the initial process of oil oxidation by measuring iodine released from potassium iodide on titration process (Nunes, 2014; Naz and Saeed, 2018). The PVs of PFO by titration and FTIR combined with PCR method was shown in Table 1. Based on the ANOVA test, the PVs from titration and FTIR method were not significantly different (p<0.05). The accuracy of iodometric titration to determine the PVs depends on some other experimental factors such as precise timing and protection of the reaction mixture from oxygen (Armenta et al., 2007). Determination of PVs by titration method could have an error caused by

Figure 1. FTIR spectra of Patin fish oil from different extraction method at mid infrared region (4000-650 cm⁻¹) scanned with resolution of 16 cm⁻¹ and number of scanning of 32.
Table 1. Determined of acid, peroxide, and saponification value by titration and FTIR methods after optimization method

| Extraction methods | Solvent  | Acid Value | Peroxide value | Saponification value |
|--------------------|----------|------------|----------------|----------------------|
| Maceration methods (wet samples) | Hexane | 5.27 | 5.42 | 9.59 | 9.48 | 135.21 | 142.48 |
| Chloroform | 2.66 | 2.71 | 7.18 | 7.16 | 287.71 | 288.79 |
| Ethyl acetate | 7.01 | 7.36 | 9.61 | 9.51 | 221.74 | 220.80 |

| Maceration methods (dried samples) | Hexane | 5.27 | 5.14 | 13.18 | 12.96 | 137.83 | 132.63 |
| Chloroform | 8.86 | 8.88 | 13.18 | 12.97 | 287.64 | 284.41 |
| Ethyl acetate | 10.57 | 10.84 | 13.16 | 13.50 | 187.84 | 189.93 |

| Soxhlet | Hexane | 15.6 | 15.67 | 8.38 | 8.52 | 144.28 | 145.44 |
| Chloroform | 8.84 | 8.75 | 17.92 | 17.96 | 268.59 | 265.31 |
| Ethyl acetate | 12.41 | 12.35 | 14.30 | 14.44 | 192.05 | 190.17 |

Table 2. Optimization of PCR and PLS methods to determine the acid value

| Multivariate Calibration | Wavenumber (cm\(^{-1}\)) | Spectra | Calibration RMSEC | R\(^2\) | Prediction RMSEP | R\(^2\) |
|--------------------------|---------------------------|---------|------------------|-------|-----------------|-------|
| PCR                      | 653-826                   | Normal  | 1.78             | 0.8653 | 2.03            | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 3.56         | 0.0395 | 3.58            | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 3.54         | 0.0888 | 3.65            | 1.000 |
|                          | 1007-1600                 | Normal  | 1.96             | 0.8344 | 1.96            | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 2.55         | 0.6969 | 3.57            | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 3.19         | 0.4421 | 4.03            | 1.000 |
|                          | 1500-2111                 | Normal  | 1.42             | 0.9171 | 2.33            | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 2.09         | 0.8088 | 0.361           | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 2.07         | 0.8125 | 0.576           | 1.000 |
|                          | 2000-3100                 | Normal  | 0.21             | 0.9983 | 0.972           | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 0.6184        | 0.985  | 1.68            | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 0.82         | 0.9731 | 1.5             | 1.000 |
|                          | 649-3005                  | Normal  | 1.12             | 0.9488 | 2.2             | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 1.5          | 0.9068 | 0.666           | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 1.59         | 0.8952 | 0.377           | 1.000 |
|                          | 653-826                   | Normal  | 3.56             | 0.0383 | 3.57            | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 3.56         | 0.0271 | 3.57            | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 3.56         | 0.0272 | 3.57            | 1.000 |
|                          | 1007-1600                 | Normal  | 3.52             | 0.1491 | 3.97            | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 3.55         | 0.0744 | 3.78            | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 3.55         | 0.0604 | 3.75            | 1.000 |
|                          | 1500-2111                 | Normal  | 1.92             | 0.8424 | 1.75            | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 3.46         | 0.2276 | 3.9             | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 3.5          | 0.1801 | 3.91            | 1.000 |
|                          | 2000-3100                 | Normal  | 1.84             | 0.8564 | 2.04            | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 1.84         | 0.2778 | 4.07            | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 3.45         | 0.2429 | 4.01            | 1.000 |
|                          | 649-3005                  | Normal  | 3.4              | 0.2979 | 3.67            | 1.000 |
|                          |                           | 1\(^{st}\) derivative | 3.48         | 0.205  | 3.91            | 1.000 |
|                          |                           | 2\(^{nd}\) derivative | 3.47         | 0.2196 | 4.09            | 1.000 |

Figure 2. Plots of acid value result obtained by titration method (actual value) and FTIR method (calculated) in calibration and validation set using PCR technique
the mistake of distinguishing on the endpoint (Jiang et al., 2016). Peroxide/hydroperoxide which is intermediate species. These intermediates are unstable species that can react very quickly that make the mistake when the determination of endpoint (Mahboubifar et al., 2016). The acceptable of PV for fish oil is ≤ 5 meq O₂/kg oil (FAO, 2017). The oxidation rate increase with the temperature results high amount of peroxide, that make PVs of PFO from the Soxhlet method were highest than others (Popa et al., 2017).

Based on the optimization method, PLS with first derivative spectra showed the best result in the prediction of the desired response (Table 3). PLS at wavenumbers of 1500-2111 cm⁻¹ were selected for determining the PVs because it gives the highest coefficient value for determination and validation. The coefficient value (R²) of determination for the calibration and validation were obtained at 0.9982. The RMSEC and RMSEP value were 0.200 and 0.564 respectively. The excellent linear relationship between the PVs measured by titration and FTIR method shown in Figure 4. Figure 5 presents the area 1500 - 2111 cm⁻¹ is attributed for -C=O ester stretching at wavenumber of 1700 cm⁻¹ (Che Man et al., 2010). According to Guillén and Cabo (1999), under the oxidative conditions, the oils have a very intense absorption due to the ester carbonyl functional group of the triglycerides causes a peak at area 1746 cm⁻¹. They also found that secondary oxidation products that cause an absorption at 1728 cm⁻¹ which overlaps with the peak of the ester functional group.

### 3.4 Determination of SV

Saponification value (SV) is an index of the average molecular mass of fatty acid in the oil sample (Zahir et al., 2017). The SV of PFO was determined by titration and FTIR combined with PCR and PLS methods were shown in Table 1. Both methods give not significantly different result (p<0.05). From the optimization method, the area at wavenumber of 1007-1600 cm⁻¹ was chosen to determine PVs Table 4. The PLS method with first derivative spectra gave the best coefficient of determination for calibration and validation, 0.9970 and 1.000 respectively with the RMSEC and RMSEP were 0.666 and 0.564 respectively. The excellent linear relationship between the PVs measured by titration and FTIR method shown in Figure 4. Figure 5 presents the area 1500 - 2111 cm⁻¹ is attributed for -C=O ester stretching at wavenumber of 1700 cm⁻¹ (Che Man et al., 2010). According to Guillén and Cabo (1999), under the oxidative conditions, the oils have a very intense absorption due to the ester carbonyl functional group of the triglycerides causes a peak at area 1746 cm⁻¹. They also found that secondary oxidation products that cause an absorption at 1728 cm⁻¹ which overlaps with the peak of the ester functional group.

### Table 3. Optimization of PCR and PLS methods to determine the peroxide value

| Multivariate Calibration | Wavenumber (cm⁻¹) | Spectra | Calibration | Prediction |
|--------------------------|-------------------|---------|-------------|------------|
|                          |                   |         | RMSEC       | R²         | RMSEP      | R²         |
| PCR                      | 653-826           | Normal  | 1.26        | 0.9256     | 1.85       | 0.8461     |
|                          |                   |         | 1.41        | 0.9062     | 2.36       | 0.6824     |
|                          |                   |         | 1.41        | 0.9056     | 2.13       | 0.7450     |
|                          |                   | 2nd derivative | 1.57        | 0.8829     | 2.09       | 0.7716     |
|                          | 1007-1600         | Normal  | 0.765       | 0.9733     | 0.599      | 0.9929     |
|                          |                   |         | 1.57        | 0.8827     | 2.29       | 0.7091     |
|                          |                   |         | 1.32        | 0.9184     | 1.60       | 0.8695     |
|                          | 1500-2111         | 1st derivative | 1.26        | 0.9263     | 1.21       | 0.9605     |
|                          |                   | 2nd derivative | 1.91        | 0.8194     | 3.22       | 0.3592     |
|                          |                   |         | 1.47        | 0.8980     | 3.03       | 0.4763     |
|                          | 2000-3100         | Normal  | 0.998       | 0.9542     | 0.843      | 0.9869     |
|                          |                   |         | 0.680       | 0.9790     | 0.330      | 0.9957     |
|                          |                   |         | 0.811       | 0.9700     | 0.642      | 0.9904     |
|                          | 649-3005          | Normal  | 2.30        | 0.7255     | 2.82       | 0.7611     |
|                          |                   |         | 2.02        | 0.7966     | 2.61       | 0.7397     |
|                          |                   |         | 2.13        | 0.7707     | 2.68       | 0.7493     |
|                          | 1007-1600         | 1st derivative | 1.81        | 0.8391     | 3.47       | 0.2069     |
|                          |                   | 2nd derivative | 1.64        | 0.8707     | 2.79       | 0.5305     |
|                          |                   |         | 1.65        | 0.8695     | 2.81       | 0.5314     |
|                          | 1500-2111         | 1st derivative | 2.10        | 0.7680     | 1.88       | 0.8719     |
|                          |                   |         | 0.800       | 0.9982     | 5.64       | 0.9982     |
|                          |                   |         | 0.683       | 0.9788     | 0.666      | 0.9871     |
|                          | 2000-3100         | Normal  | 1.80        | 0.8411     | 3.31       | 0.3054     |
|                          |                   |         | 1.77        | 0.8485     | 3.52       | 0.2450     |
|                          |                   |         | 1.81        | 0.8402     | 3.61       | 0.1963     |
|                          | 649-3005          | Normal  | 2.25        | 0.7374     | 2.77       | 0.7514     |
|                          |                   |         | 2.28        | 0.7297     | 2.82       | 0.7501     |
|                          |                   |         | 2.31        | 0.7211     | 2.86       | 0.7541     |
Figure 4. Plots of peroxide value result obtained by titration method (actual value) and FTIR method (calculated) in calibration and validation set using PLS technique.

Figure 5. FTIR spectra to determine the peroxide value of Patin fish oil (1500-2111 cm$^{-1}$).

Figure 6. Plots of saponification value result obtained by titration method (actual value) and FTIR method (calculated).

Figure 7. FTIR spectra to determine the saponification value of Patin fish oil (1007-1600 cm$^{-1}$).
conducted by Rohman et al. (2015) that use absorbance at wavelength of 1400-1600 cm\(^{-1}\) with PLS technique to determine the SVs. The linear regression as shown in Figure 6. First derivative spectra were displayed in Figure 7.

4. Conclusion

In this work, the AV, PV, and SV of PFO were successfully determined via FTIR spectroscopy combined with chemometrics using PCR and PLS method. The rapid determination of AV, PV, and SV by FTIR spectroscopy is, therefore, suitable and practical option for process control. Another advantage of FTIR spectroscopy method is that it is environmentally friendly as no chemical is needed except acetone for cleaning the ATR crystal. By utilizing this method, the chemical cost is negligible as compared to the AOCS standard method.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

The author thanks to The Ministry of Research, Technology, and Higher Education of the Republic of Indonesia for its scholarship so that this research can be done.

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