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The Employment of ATR-FTIR Spectroscopy and Chemometrics for Authentication of Bawal (*Colossoma macromopum*) Fish Oil from Palm Oil

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Abstract: Bawal fish is a product with high nutritional content, and the development of fish oil has made it easier for consumers to consume. High-quality fish oil can be a target for adulteration, such as with vegetable oils like palm oil. This study aims to develop FTIR spectroscopy for authentication of bawal fish oil (BFO) from palm oil (PO). Bawal fish oil and palm oil were prepared in binary mixtures with concentrations from 0-100%, resulting in 21 mixtures. The oil was directly measured by attenuated total reflectance (ATR) spectral measurement in the mid-infrared region (4000-600 cm^{-1}). The results were combined with linear discriminant analysis (LDA) and multivariate calibration, such as PLSR or PCR. The results showed that LDA could make clear discrimination between bawal fish oil, palm oil, and their mixtures without any misclassification observed in 4000-600 cm^{-1} region. Multivariate calibration with PLSR using the first derivative spectrum provided the best model for the relationship between actual and predicted FTIR values. At these conditions, the R2 values for the calibration and validation models obtained were 0.9966 and 0.9969, with RMSEC and RMSEP values of 0.0249 and 0.0248, respectively. Therefore, FTIR spectroscopy combined with LDA and PLSR is an effective method for authenticating bawal fish oil from palm oil.

Keywords: Bawal fish oil, palm oil, oils adulteration, chemometrics, ATR- FTIR spectroscopy

1. INTRODUCTION

Fish is one of the foods that have high nutritional content. Currently, its utilization is widely used in the form of fish oil. Fish oil has a higher price compared to fish, making it easier to be adulterated. Authentication of food supplements needs to be a significant concern due to high demand and lack of regulation [1]. Freshwater bawal fish (Colossoma macropomum) or also known as tambaqui fish is a native fish originating from the Amazon valley [2]. This fish is easily cultivated

due to its ease of breeding, fast growth, productivity, and market acceptance. The nutritional content of bawal fish is quite varied, ranging from 1-2% mineral, 15-24% protein, and 0.1-22% fat which is dominated by 60% unsaturated fatty acids with 45% monounsaturated fatty acids (MUFA) and 15% polyunsaturated fatty acids (PUFA) [3]. Currently, fish oils have emerged as promising functional foods due to its function to treat several diseases. Some analytical methods of fish oil authentication have been developed, such as chromatography method. Liquid chromatography-mass spectrometry (LCMS) and gas chromatography-mass spectrometry (GCMS) have high sensitivity and high selectivity analytical method [4,5]. Comprehensive two-dimensional tandem gas chromatography-mass spectrometry (GC x GC-MS) combined with chemometrics is able to provide a fingerprint profile of fatty acid composition and has been successfully used for the authentication of fish oil products in Brazil [1]. However, these methods require several steps of sample preparation such as the need for derivatization, skilled analysis, can damage the sample, takes longer time, and requires a lot of chemical solvents [6]. An alternative method that has been developed is infrared spectroscopy. This method is used because it has advantages such as non-destructive to the sample, fast, easy to use, simple, and does not require time-consuming sample preparation [7]. This method is also considered as a green method of analysis because it does not use hazardous solvents and there are no chemical residues or waste that can pollute the environment [8].

The application of spectroscopy in food identification is increasingly developing with the aim of controlling its quality and quantity. The spectroscopy region that is widely applied is the Mid Infrared Spectroscopy or MIRS region, which is an electromagnetic region of 4000-600 cm^{-1}. The principle of this method is the difference in the absorption characteristics produced by a material with certain chemical bonds when illuminated with a spectrum of light waves. In connecting the data set obtained from FTIR spectra with the condition of the material or sample, statistical methods, namely chemometrics, are used for authentication [9]. There have been many studies involving oil in the analysis using spectroscopy with LDA, PCR or PLS chemometrics , such as successfully identified patin fish oil from palm oil, patin fish oil from corn oil, tuna oil from pork fat, pork oil in cow's milk, tuna fish oil from palm oil and corn oil [10, 11, 12]; [6]; [13]. The application of infrared spectroscopy for the authentication of fish oil is developing and providing good results. Therefore, the aim of this study was to develop the process of authentication bawal fish oil using ATR-FTIR combined with chemometrics with linear discrimination analysis (LDA) and multivariate calibration (PLS and PCR) from palm oil.

2. MATERIALS AND METHODS

2.1. Materials

The bawal fish samples were obtained from fish farms in the Yogyakarta region, Indonesia. The researcher utilized the dry rendering method to extract the full body of bawal fish. The sample cleaning and cutting 4-5 cm are performed, followed by heating using a cabinet drying method for 24 hours at 50°C. Subsequently, the sample is subjected to pressing with a pressure of 150 kN for 5 minutes. Palm oil was used as the adulterant agent and was obtained from a supermarket in Yogyakarta, Indonesia. Palm oil was chosen because it is cheaper and more readily available in the market as food grade.

2.2. Preparation of samples

The bawal fish oil (BFO) was obtained using the dry rendering extraction method at a temperature of 50°C for 24 hours. Subsequently, a set of calibration mixtures was prepared using 21 samples. The oil of bawal fish was mixed with palm oil in binary mixtures with concentrations ranging from 0-100%. The composition of the binary mixture used can be seen in Table 1.

Table 1. The composition of Bawal Fish Oil (PFO) in biner mixtures with Palm oil

Sample	Bawal Fish Oil (BFO) (%)	Palm Oil (PO) (%)
1	100	0
2	95	5
3	90	10
4	85	15
5	80	20
6	75	25
7	70	30
8	65	35
9	60	40
10	55	45
11	50	50
12	45	55
13	40	60
14	35	65
15	30	70
16	25	75
17	20	80
18	15	85
19	10	90
20	5	95
21	0	100

2.3. ATR-FTIR Spectroscopy Measurement

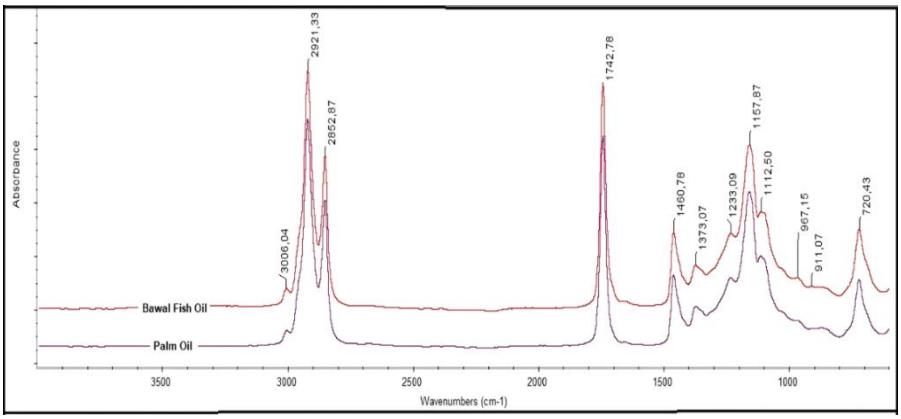
The BFO treated with bentonite as well as those mixed with Palm oil (PO) were measured using a FTIR spectrophotometer (FTIR Nicolet iS20) with a DTGS (deuterated triglycine sulfate) detector connected to the OMNIC® and Windows® software. The sample reading technique used was attenuated total reflectance (ATR). All spectra were measured in the region of 4000-600 cm-1 by placing the sample directly on the ATR crystal. The resolution used was 8 cm^{-1} with 32 scans. All spectra were recorded in absorbance mode to facilitate quantitative analysis according to the Lambert-Beer law. Each sample was read three times. Before measuring each sample, a background spectrum was recorded using an air spectrum. After measuring each sample, the ATR crystal was cleaned using acetone. The data obtained will be analyzed using the TQ-Analyst software.

2.4. Chemometrics analysis

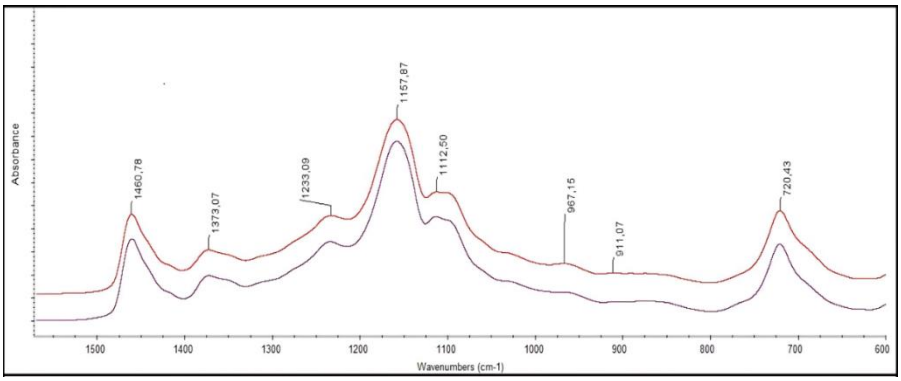
The TQ Analyst software version 9 (Thermo Fisher Scientific, Inc.) was used for chemometric analysis. Two chemometric techniques were used in this study is pattern recognition and multivariate calibration. For pattern recognition analysis, a supervised technique called LDA was used. The LDA model evaluated the discrimination between the BFO treated with bentonite and the adulterated ones according to the concentrations that were prepared using a Cooman score plot. Multivariate calibration analysis was performed using PLSR and PCR models. These models were evaluated using the root mean square error of calibration (RMSEC), root mean square of prediction (RMSEP), and coefficient of determination (R2).

3. RESULTS AND DISCUSSION

In this study, mid-infrared spectroscopy (4000-600 cm^{-1}) combined with chemometrics was used to determine the authentication of bawal fish oil (BFO) from palm oil (PO). The ATR-FTIR reading results will provide absorbance data at each wavenumber from 4000-600 cm^{-1}. The comparison spectra of BFO and PO can be seen in Figure 1 (a). It can be seen that the FTIR spectra of BFO and PO have similar spectra, but if observed in the fingerprint region (1500-600 cm^{-1}) in Figure 1 (b), there are some peaks that indicate differences in peak intensities. These differences can be used as the basis for chemometric analysis. Both spectra show typical Triglyceride (TG) spectra, as animal and vegetable oils mostly consist of TG [14].



(a)



(b)

Figure 1. ATR-FTIR spectra of Bawal fish oil and Palm Oil at (a) mid infrared region (4000-600 cm^{-1}) and finger print region (1500-600 cm^{-1})

Table 2. FTIR absorption of common bands detected in bawal fish oil

Wavenumber (cm^{-1})	Functional grup	Vibration
3006.04	Alkena	=C-H stretching
2921.33	Alkana	Methylene asymmetric C-H stretching
2852.87	Alkana	Methylene symmetric C-H stretching
1742.78	Alkana	C=O stretching
1460.78	Alkana	Methylene scissoring CH2
1373.07	Alkana	Methyl siymmetrcial C-H bending
1233.09	Alcohol and phenols	C-O stretching
1157.87	Alcohol and phenols	C-O stretching
1112.50	Esters	Aliphatic C-O stretching
967.15	Alkena	=C-H out-of-plane bending
911.07	Alkena	=C-H out-of-plane bending
720.43	Alkana	Methylene rocking

Table 2 contains information about several functional groups that can be identified. FTIR spectroscopy is a suitable method because it has fingerprinting in its analysis, so that the similarity of characteristics between samples can be distinguished in the fingerprint region, making it highly sensitive [18]. The ATR technique is used in sample reading. The principle of ATR utilizes the phenomenon of total internal reflection. The sample of oil liquid will be dropped on an ATR crystal, which is insoluble in water or organic solvents and has a high refractive index. In ATR spectroscopy technique, a crystal with excellent infrared transmitting properties and high refractive index is placed close to the sample and used as an internal reflection element (IRE) [19]. A beam of radiation that enters the crystal will undergo total internal reflection when the angle of incidence at the interface between the sample and the crystal is greater than the critical angle, where the critical angle is a function of the refractive index of both surfaces. A small portion of the wavelength penetrates outside the reflecting surface, and when a material that selectively absorbs radiation is in close contact with the reflecting surface, the beam loses energy at the wavelength absorbed by the material. The weakened radiation generated is measured and plotted as a function of wavelength by a spectrometer, and it produces spectral absorption characteristics of the sample. The ATR technique in FTIR spectroscopy allows for the measurement of samples in liquid or solid form without complex sample preparation. Therefore, this technique is often used in the analysis of oils and fats. ATR also has the advantage of minimizing interference by water or other impurities because only the surface of the sample is involved in the measurement [15]. In Figure 2, it can be seen that all 21 mixture FTIR spectra at mid infrared region (4000-600 cm^{-1}).

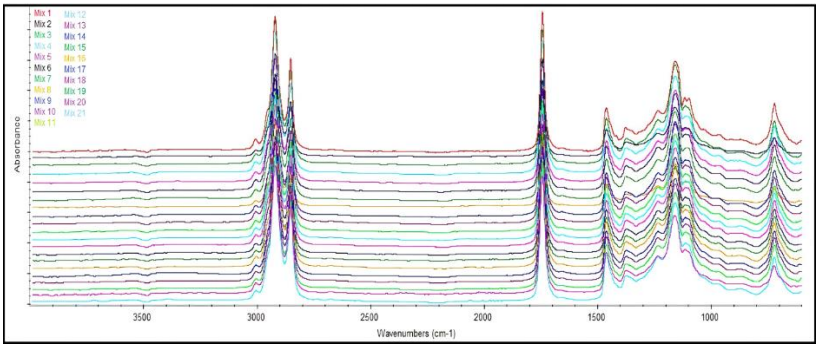


Figure 2. ATR-FTIR spectra of Bawal Fish Oil (BFO) and Palm Oil (PO) mixture at mid infrared region (4000-600 cm^{-1}) with number of scanning of 32 and at resolution of 8 cm^{-1}

The authentication analysis of fish oil using chemometrics was carried out using linear discriminant analysis (LDA) to see the classification between pure fish oil and adulterated oil. In LDA analysis, the samples were divided into three classes, bawal fish oil treated with bentonite, adulterated BFO with palm oil, and palm oil. In FTIR reading, the wavenumber was used as a variable and the absorbance data obtained from 4000-600 cm^{-1} were used as variables, which were then converted into Mahalanobis distance to classify bawal fish oil and its mixture. LDA applied to predict class membership of samples (individuals) from quantitative profiles made by several measured [20]. From Figure 3, it can be clearly seen that the classification is well-formed. This indicates that LDA successfully distinguished authentic bawal fish oil from palm oil as adulterant.

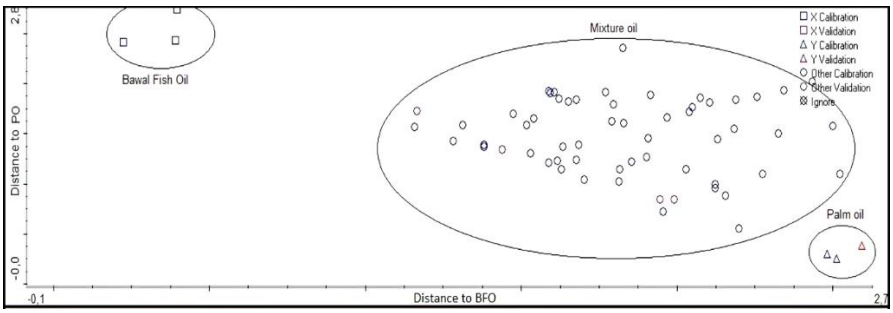


Figure 3. Linear discriminant analysis (LDA) Coomans plot for the discrimination between BFO adulterated with PO using the absorbance values of ATR-FTIR spectra at (3500-600 cm^{-1})

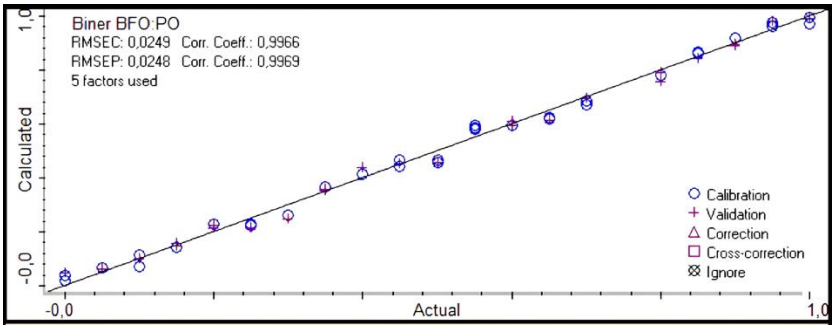
PLSR and PCR are multivariate calibration methods used for quantification of fish oil adulteration. In both methods, the concentration of the analyte (y-axis) is modeled with the principal components, which are a linear combination of absorbance values (x-axis) [21]. The combination of FTIR spectroscopy with multivariate calibration is suitable for quantitative analysis of oil samples, as it follows the principles of the Beer-Lambert law where the absorbance of specific functional groups is proportional to the analyte concentration [22]. The obtained FTIR spectra are preprocessed using spectral preprocessing, such as first and second derivative transformations. The aim of the derivative transformations is to increase the sensitivity of the analysis by eliminating interfering spectra [23]. The first derivative is used to enhance spectral resolution and simplify the baseline, while the second derivative is used to remove broad band absorption. The resulting normal and transformed spectra are then analyzed using PLSR and PCR to obtain the best prediction for PO as an adulterant agent in BFO. Statistical parameters used include the coefficient of determination (R2) to determine the

accuracy between actual and predicted values, and the root mean square of calibration (RMSEC) and root mean square error of prediction (RMSEP) to evaluate the precision of the samples (Table 3).

The selection of spectral was based on its ability to provide a high R2 value and low RMSEC and RMSEP values [24]. Based on the normal and derivative spectra, PLSR used the first derivative spectrum in the wavenumber range of 3500-600 cm^{-1} to provide the best model for the relationship between the actual and predicted values of BFO and PO. In the adulteration of BFO with PO, the R^2 values for the calibration and validation models were 0.9966 and 0.9969, with RMSEC and RMSEP values of 0.0249 and 0.0248. These results demonstrate that the use of FTIR combined with LDA and PLSR can be an effective method for authenticating BFO from PO as an adulterant agent. This is indicated by the accurate and precise results, as evidenced by the high R^2 value and low RMSEP and RMSEC values. Figure 4a shows the relationship between the actual and predicted values of BFO and PO, while figure 4b shows the errors that occurred during modeling. It can be seen that the errors that occurred during modeling were randomly distributed around zero difference. This indicates that there were no systematic errors and the developed model can be relied upon to predict BFO contaminated with PO.

Table 3. The wavenumber region for authentication of BFO in binary mixture with PO.

Multivariate calibration	Wavenumber (cm-1)	Spectra	Calibration		Prediction	
			RMSEC	R^2	RMSEP	R^2
PLS	**3500-600**	Normal	0.0272	0.9959	0.0300	0.9954
		1st Derivative	**0.0249**	**0.9966**	**0.0248**	**0.9969**
		2nd Derivative	0.0352	0.9933	0.0413	0.9920
	3000-600	Normal	0.0364	0.9926	0.0298	0.9960
		1st Derivative	0.0672	0.9744	0.0257	0.9970
		2nd Derivative	0.0609	0.9790	0.0361	0.9936
	1800-600	Normal	0.0732	0.9757	0.0425	0.9975
		1st Derivative	0.0678	0.9736	0.0361	0.9940
		2nd Derivative	0.0664	0.9747	0.0405	0.9922
	1500-600	Normal	0.0622	0.9846	0.0520	0.9843
		1st Derivative	0.0582	0.9806	0.0470	0.9909
		2nd Derivative	0.0541	0.9833	0.0589	0.9850
PCR	3500-600	Normal	0.0863	0.9576	0.0372	0.9934
		1st Derivative	0.0699	0.9725	0.0386	0.9930
		2nd Derivative	0.0682	0.9738	0.0476	0.9891
	3000-600	Normal	0.0543	0.9834	0.0409	0.9924
		1st Derivative	0.0681	0.9737	0.0386	0.9926
		2nd Derivative	0.0663	0.9751	0.0497	0.9889
	1800-600	Normal	0.0861	0.9571	0.0338	0.9946
		1st Derivative	0.0687	0.9729	0.0464	0.9899
		2nd Derivative	0.0628	0.9774	0.0462	0.9916
	1500-600	Normal	0.0836	0.9596	0.0664	0.9793
		1st Derivative	0.0855	0.9576	0.0382	0.9928
		2nd Derivative	0.0723	0.9700	0.0446	0.9927



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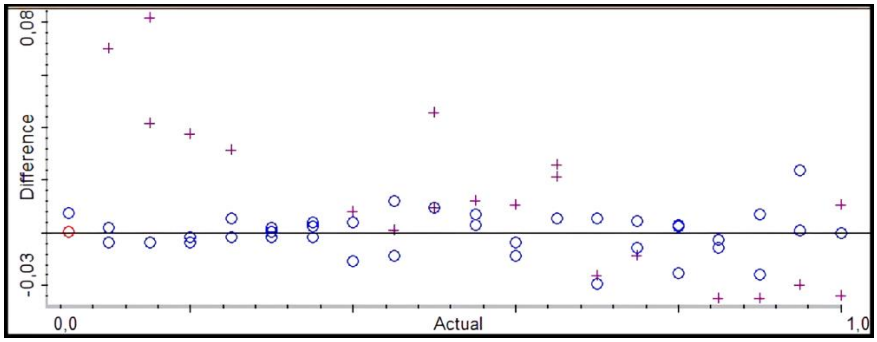

(b)

Figure 4. The correlation between the actual value of PO with FTIR predicted values facilitated by partial least square calibrations 1st derivative (a) along with residual analysis (b)

FTIR-ATR is able to predict the fatty acid profile of fish oil with other components based on the vibrations of its functional groups [25]. Linear discriminant analysis (LDA) based on FTIR data is able to be the initial step in detecting potential fish oil adulteration. Once potential adulteration has been identified, the next step can be multivariate calibration in the form of PLSR or PCR to quantify it. Again, FTIR is a highly potential method to be developed in fish oil adulteration models.

4. CONCLUSION

Combining ATR-FTIR with chemometric analysis such as LDA, PLS or PCR can classify and quantify adulteration in bawal fish oil. The LDA analysis was able to classify bawal fish oil, palm oil, and their mixtures perfectly. In addition, using multivariate calibration such as PLS or PCR can quantify the adulteration model of bawal fish oil with palm oil, which can be seen from the obtained values of RMSEC, RMSEP, and R2. Therefore, the development of ATR-FTIR spectroscopy methods is very promising for the adulteration detection of other fish oils because FTIR spectroscopy method provide a fast, non-destructive, and environmentally friendly method for the authentication of bawal fish oil and adulterants from other fish oil.

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