Application of FTIR Spectroscopy and Chemometrics for the Prediction of Radical Scavenging Activities of Fish oils

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ABSTRACT

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Patin (Pangasius micronemus), Gabus (Channa striata) and Bandeng (Chanos chanos) are the freshwater fishes that are widely cultivated in Indonesia. Fish oils are believed to have biological activities including antioxidant activities. The objectives of this study were (1) to determine radical scavenging activity (RSA) of fish oils using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radicals, (2) to classify fish oils from different species and extraction methods using chemometrics of principal component analysis (PCA) and cluster analysis (CA) and (3) to predict the antiradical activities of fish oils from different species and extraction methods using FTIR spectroscopy combined with principal component regression (PCR) and partial least square regression (PLSR). The results exhibited that RSA of Patin fish oil extracted from flesh using maceration technique gave the highest RSA. PCA and CA were successfully applied for the classification of fish oils from different species and extraction based on PC1 and PC2 score plots. Based on dendrogram, the fish oils could be classified into 4 groups. The absorbance values of second derivative FTIR spectra at wavenumber region of 1440 – 1741 cm⁻¹ provided the highest correlation between actual values of RSA and FTIR predicted values with coefficient of determination (R²) of 0.9794, root mean square error of calibration (RMSEC) of 0.737 and root mean square error of prediction (RMSEP) of 0.927. From this study, FTIR spectroscopy combined with chemometrics can be used for classification of fish oils and for predicting the antioxidant activities of fish oils from different species and extraction method.

Key words: fish oil, antioxidant activity, FTIR spectroscopy, chemometrics, DPPH radical scavenging

INTRODUCTION

As maritime country, Indonesia has numerous fish species. It is estimated that there are 4000 species of fishes found (Muchlisin and Azizah, 2009). Patin (Pangasius micronemus), Gabus (Channa striata) and Bandeng (Chanos chanos) are the freshwater fishes widely cultivated in Indonesia. Fish oil is a good source of essential fatty acid having antioxidant activities, therefore, fish oils could be considered as functional oils to be supplemented in any types of food and pharmaceutical products (Erdoğan et al., 2004; Zzaman et al., 2014; Latip et al., 2014). The recent studies supported that fish oils are essential for improvement the human health caused by the presence of some bioactive compounds in the fish tissue extracted in fish oils (Kuvendziev et al., 2018).

The problems related to oxidative deterioration of lipid-based foods and...
pharmaceuticals is of great economic importance. During oxidation reactions, the mono- and polyunsaturated fatty acids present in edible fats and oils could produce some undesirable odors contributing to off-flavor. The oxidation can also decrease the nutritional values and quality of food and pharmaceutical products, therefore the stable antioxidants are needed (Farvin et al., 2014). Antioxidant can be defined as any substances or materials capable of preventing the oxidation of molecules (Rao, 2016; Archibong et al., 2018). Antioxidants contained in fish oil are influenced by the extraction method used to extract fish oils from different part of fish species. Hydraulic pressing, Soxhlet extraction based on heat extraction, solvent extraction using non-polar solvents (Garcia-Moreno et al., 2014; De Oliveira et al., 2016) and newer extraction methods such as ultrasound-assisted extraction, super critical fluid as well as some green extraction-based on enzymatic methods (Zhang et al., 2021) are reported to extract fish oils (Bonilla-Mendez and Hoyos-Concha, 2018). Some extraction conditions also affected the extraction efficiency, as a consequence, it is necessary to select the appropriate extraction method in order to get high antioxidant activities in fish oil (Chaves et al., 2020).

Some methods have been applied for evaluating antioxidant activities of fish oils in vitro including radical scavenging methods using 2,2’-diphenyl-1-picrylhydrazyl (DPPH) and 2,2’-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), lipid peroxidation inhibition (Akmal and Roh, 2017; Putri et al., 2021), and metal-chelating efficacy (Bag and Chattopadhyay, 2018). These methods are typically required some organic solvents which are hazardous to human health and environment and also involved sample preparation, as a consequence, some instrumental responses are developed for rapid evaluation of antioxidants. One of potential spectroscopic techniques are infrared spectroscopy caused by large information contained in FTIR spectra which are fingerprint in nature (Rohman, 2012).

The use of Fourier transformed-infrared (FTIR) spectroscopy in combination with chemometrics has emerged as powerful technique for the prediction of biological activities (Wu et al., 2012). Among chemometrics techniques, the multivariate calibrations are commonly used to establish the prediction models of biological activities including antioxidants by making the correlation between actual values of antioxidant activities as determined by standard methods and FTIR predicted values (Lu and Rasco, 2012). The combination of FTIR spectroscopy and multivariate calibration has been successfully applied for prediction of the total antioxidant capacity in four onion varieties and shallot (Lu et al., 2011), red wine (Versari et al., 2010) and chocolate products (Hu et al., 2016). Using literature study, there are no publications regarding the use of FTIR spectroscopy in combination with chemometrics for prediction of antioxidant activities of fish oils from different species. The current study aimed to use FTIR spectra combined with chemometrics to classify fish oils and to predict the antioxidant activities from fish oil.

MATERIALS AND METHODS

Materials

Patin fishes were obtained from several locations in Yogyakarta, East Java Central Java, South Jakarta, and South Kalimantan, Indonesia. Bandeng fishes (milkfish fish) were collected from traditional fish market in Juwana Pati Central Java, while Gabus fishes were obtained from fish farmer at Yogyakarta. The chemicals of 2,2’-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma (Aldrich, USA). The other reagents and solvents used for analysis were of analytical grade and obtained from E. Merck (Darmstadt, Germany).

Sample preparation

Fish oils were extracted from body parts of flesh, head and offal (Patin), head (Bandeng), as well as offal, head and bone (Gabus). All body parts of fish samples were cut into small pieces and then dried in cabinet dryer about 24 hours, except flesh of Patin which is dried about 2 days at temperature of 50°C.

Fish oil extractions

All samples were extracted using press method except Patin flesh which is extracted using maceration, Soxhlet, and Ultrasound Assisted Extraction (UAE) using organic solvents. During maceration, dry and wet fish samples were extracted using n-hexane, ethyl acetate, and chloroform for 24 h. Soxhlet extraction was applied on dry and wet fish samples using n-hexane and chloroform as extracting solvents for 4 hours. Furthermore, UAE method was carried out using ultrasonic probe UP 200S (Hielscher Ultrasound Technology, Berlin, Germany) with solvent composition of 42%: 58% (n-hexane-isopropanol), an amplitude of 41%, a solvent-to-sample ratio of 20:1, a cycle of 0.8 s⁻¹, temperature at 59°C and
extraction time of 25 minutes. The solvent was then removed using rotary evaporator at 40℃. The direct pressing method for fish oil extraction was carried out according to Bako et al. (2017) with slight modification using direct pressing with 100 kN force for 2 minutes. The oil was separated from sediment by centrifugation at 5000 rpm for 10 minutes. All fish oils obtained were then subjected to antioxidant evaluation using DPPH radical scavenging assay and FTIR spectral measurement.

**DPPH radical scavenging activity assay**

DPPH radical scavenging assay was determined according to Akanbi and Barrow (2018) with some modification. A-50μL of each fish oil samples were added with 1mL 2,2-diphenyl-1-picryl-hydrazyl (DPPH) 0.4mM and diluted with ethanol to 5.0mL. The solution mixture was allowed to stand at room temperature in the dark during 30min. The absorbance of evaluated solutions was measured using spectrophotometer (Hitachi, U-2900, Japan) at 515nm and corrected with blank solutions containing solvent and the studied samples. The absorbance of control solutions containing DPPH• solution was also measured. DPPH radical scavenging activity was calculated as:

\[
\%\text{RSA} = \left( \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100\%
\]

**FTIR spectra measurement**

All fish oil samples were analyzed using FTIR spectrophotometer (Thermo Scientific Nicolet iS10, Madison, WI) equipped with software of OMNIC included in FTIR instrument. The spectra were scanned at mid-infrared region of 4000-650cm⁻¹, scanning number of 32 and the resolution 8cm⁻¹. All FTIR spectra were corrected against FTIR spectrum of air as background. All samples were measured in three replicates. After each scanning, ATR crystal was cleaned with n-hexane twice and acetone once. The spectra were recorded as absorbance values at each data point and used for making the correlation models between antiradical activity and FTIR spectra assisted with multivariate calibrations.

**Chemometrics Analysis**

Chemometrics of principal component analysis (PCA) and cluster analysis for classification of fish oils was performed using software of Minitab version 19 (Minitab Inc., USA), while multivariate calibration of partial least square (PLS) and principle component regression (PCR) used for the prediction of radical scavenging activity was carried out using TQ analyst software (Thermo Fisher Scientific Inc, Madison). Leave-one-out cross-validation was performed to verify PLS and PCR calibration models. The values of root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP) and coefficient of determination (R²) were used as criteria for evaluating the validity of developed models.

**RESULT AND DISCUSSION**

The radical scavenging activities (RSA) toward DPPH radicals of fish oils extracted from different parts (flesh, head, offal, head and bone) of fish species (Patin, Bandeng and Gabus) using different extraction techniques (Table 1). The highest RSA in Patin fish oil was observed from flesh part extracted using maceration extraction technique accounting of 23.57±0.14%. Bandeng fish oils extracted from offal part using pressing method had RSA value of 10.71±0.18%, while Gabus fish oil extracted using pressing method had RSA of 16.26±0.7%. In general, Patin fish oil had RSA value > Gabus Fish oil > Bandeng fish oil. In Patin, fish oils extracted by chloroform in dried sample using maceration extraction technique had higher antiradical activity than other technique. From this results, it is clear that extraction techniques affected RSA values of fish oils.

In order to classify fish oil samples based on antiradical activities, two unsupervised pattern recognitions, namely principal component analysis (PCA) and cluster analysis (CA) were used. PCA is known as exploratory data analysis which is used for reducing the number of variables if the correlation among variables existed. PCA is an approach to reduce a large of datasets without eliminating the important information in datasets. One of PCA outputs is called principle components (PCs) in which two or more samples having the same PC values are considered as the same samples (Irnawati et al., 2021). As variable, the absorbance values of FTIR spectra of evaluated samples at wavenumber regions of 4500-650cm⁻¹ were used during PCA. The score plot of PCA from samples based on species and extraction method (Figure 1). The score plot performs the projection of the evaluated samples, as described by the first and second principle component (PC1 and PC2). The closer the distance to one another, the more similar characteristic of them.
Figure 1. The PCA score plot of fish oil samples from different species and extraction methods. 1 = PFO by Maceration (n-hexane, wet samples); 2 = PFO by Maceration (ethyl acetate, wet samples); 3 = PFO by Maceration (chloroform, wet samples); 4 = PFO by Maceration (n-hexane, dried samples); 5 = PFO by Maceration (ethyl acetate, dried samples); 6 = PFO by Maceration (chloroform, dried samples); 7 = PFO by Soxhlet (n-hexane, dried samples); 8 = PFO by Soxhlet (chloroform, dried samples); 9 = PFO by UAE (n-hexane:isopropanol; dried samples); 10 = PHO by Pressing (dried samples); 11 = POO by Pressing (dried samples); 12 = BHO by Pressing (dried samples); 13 = GHO by Pressing (dried samples); 14 = GOO by Pressing (dried samples). PFO = Patin flesh oil; PHO = Patin Head Oil; BHO = Bandeng Head oil; GHO = Gabus Head Oil; GOO = Gabus Offal oil.

Table I. Antioxidant activity of fish oil from different species and extraction methods

| Sample code | Studied oils | Part of fish | Extraction method | RSA actual value | RSA prediction value |
|-------------|--------------|--------------|-------------------|------------------|---------------------|
| 1           | PFO          | Flesh        | Maceration (wet samples, n-hexane) | 19.11±0.00 | 20.30±0.15 |
| 2           | PFO          | Flesh        | Maceration (wet samples, ethyl acetate) | 13.67±0.00 | 13.68±0.03 |
| 3           | PFO          | Flesh        | Maceration (wet samples, chloroform) | 21.90±0.14 | 21.15±0.44 |
| 4           | PFO          | Flesh        | Maceration (dried samples, n-hexane) | 20.50±0.14 | 21.65±0.44 |
| 5           | PFO          | Flesh        | Maceration (dried samples, ethyl acetate) | 14.04±0.08 | 14.27±0.29 |
| 6           | PFO          | Flesh        | Maceration (dried samples, chloroform) | 23.57±0.14 | 22.43±0.04 |
| 7           | PFO          | Flesh        | Soxhlet (dried samples, n-hexane) | 19.20±0.21 | 18.83±0.04 |
| 8           | PFO          | Flesh        | Soxhlet (dried samples, chloroform) | 19.20±0.16 | 20.91±0.14 |
| 9           | PFO          | Flesh        | UAE (dried samples, n-hexane:isopropanol) | 22.78±0.08 | 22.22±0.59 |
| 10          | PHO          | Head         | Pressing (dried samples) | 10.71±0.18 | 11.00±0.15 |
| 11          | POO          | Offal        | Pressing (dried samples) | 10.51±0.30 | 10.27±0.17 |
| 12          | BHO          | Head         | Pressing (dried samples) | 10.71±0.18 | 10.80±0.32 |
| 13          | GHO          | Head-bone    | Pressing (dried samples) | 16.06±0.00 | 16.31±0.32 |
| 14          | GOO          | Offal        | Pressing (dried samples) | 16.26±0.7 | 15.94±0.45 |

*PFO: Patin Flesh Oils; PHO = Patin Head Oils; POO =Pation Offal Oils; BHO: Bandeng Head Oils; GHO = Gabus Head Oils; GOO = Gabus Offal Oils; and RSA = Radical Scavenging Activity.*
PFO extracted by maceration using ethyl acetate (wet and dried samples) and PFO extracted by UAE having similar characteristic since they are close to each other in score plot. Based on score plot values, PC1-PC5 could extract more than 95% of information from original data.

Cluster analysis (CA) is a process of identifying the grouping of evaluated samples based on the similarity measure which is represented as a dendogram (Omran et al., 2007). CA is based on Euclidian distance (Ed) in which two or more samples with Ed equal to zero can be considered as the same samples (Miller and Miller, 2005). In this study, clustering algorithm can be used as a simple tool for categorizing fish oil samples based on antioxidant scavenging activity from different species and extraction methods, and the dendogram obtained was depicted (Figure 2).

The firstly joined samples are Patin head oil (PHO) extracted by pressing and Bandeng head oil (BHO) extracted by pressing followed by Patin flesh oil (PFO) Soxhlet (n-hexane) and PFO Soxhlet (chloroform) and so on until all samples are grouped into one group. Cluster analysis suggests that fish oil samples based on variable of antioxidant activities could be divided into four groups. Group 1 consisted of PFO by maceration, PFO by soxhlet (n-hexane), PFO by soxhlet (chloroform), and PFO by maceration (chloroform, dried samples). Group 2 consisted PFO by maceration (chloroform, dried samples), PFO by maceration (chloroform, dried samples), and PFO by UAE. Group 3 consisted PFO by maceration (ethyl acetate, wet samples), PFO by maceration (ethyl acetate, dried samples), GHO by pressing, and Gabus Offal oil (GOO) extracted by pressing method. Group 4 consisted Patin head oil (PHO) extracted by pressing, Bandeng head oil (BHO) by pressing, and Patin offal oil (POO) by extracted by pressing method.

In order to predict the radical scavenging activity (RSA) of fish oil samples, two multivariate calibrations namely principle component regression (PCR) and partial least square (PLS) were applied. The variation in FTIR spectra of fish oil samples can be exploited to build the calibration model for the prediction of RSA.
The wavenumber regions and FTIR spectral treatment were optimized to obtain the best prediction model in terms of providing highest $R^2$ values and lowest RMSEC and RMSEP values. Figure 3 showed attenuated total reflectance-FTIR spectra along with functional groups present in studied fish oils responsible for the absorption of infrared radiation. Some optimizations of wavenumber regions and spectral treatment along with multivariate calibration techniques were carried out, and the statistical performance (Table II). The optimal prediction condition was obtained using second derivative FTIR spectra at wavenumbers region 1440–1741 cm$^{-1}$ assisted by PCR with $R^2$-calibration value of 0.9854, $R^2$-prediction value of 0.9781, RMSEC value of 0.737 and RMSEP value of 0.927. Figure 4 revealed the correlation between actual value of RSA (x-axis) and FTIR predicted value (y-axis) assisted with PCR. High $R^2$ value and low errors (RMSEC and RMSEP) indicated that FTIR spectroscopy in combination with PCR could be an alternative method for predicting RSA accurately and precisely.
Figure 3. Attenuated total reflectance-FTIR spectra of fish oil samples from different species and extraction methods, scanned at mid infrared region (4000-650 cm\(^{-1}\)).

Figure 4. PLS calibration model for correlation between actual and predicted value of radical scavenging activities of fish oil samples. The x-axis showed the actual radical scavenging activity, the y-axis showed the calculated radical scavenging activity based on FTIR spectra using the optimum condition.
CONCLUSION
FTIR spectroscopy in combination with PCA and CA has been successfully applied for classification of fish oils originating from fish species of Patin, Gabus and Bandeng. Moreover, FTIR spectroscopy at wavenumber 1440-1741 cm\(^{-1}\) combined with PCR could be an alternative method for predicting RSA with high accuracy and low error based on high \(R^2\) and low RMSEC and RMSEP values.

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