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Research Article

Optimization of microwave-assisted fish oil extraction from Patin (*Pangasius micronemus*) using Response Surface Methodology-Box Behnken Design (RSM-BBD)

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ABSTRACT

Patin (*Pangasius micronemus*) fish oil is rich in omega-3 fatty acids such as α-linolenic acid and eicosapentaenoic. To obtain the benefits of omega-3, it is necessary to develop an efficient extraction method for patin oil (PO). This study aimed to develop the microwave-assisted extraction (MAE) method for fish oil from patin. Three independent variables with three levels were priorly evaluated using Box–Behnken design, including temperature (30, 60, 90°C), solvent composition (30, 60, 90% of ethyl acetate in methanol), and solvent to sample ratio (10:1, 15:1, 20:1). The significant variables were then optimized by response surface methodology (RSM). A second-order quadratic model of RSM suggested an extraction at 60°C with 56% ethyl acetate in methanol as an extraction solvent and a solvent-to-sample ratio of 20:1. A kinetics study under the optimum MAE conditions approved the complete recovery (38.84%) starting at 15 min of extraction time. The high precision of the MAE process was confirmed by the coefficient of variation less than 3%. Additionally, the microwave-produced fish oil was characterized by gas chromatography-mass spectrometry (GC-MS) to contain α-linolenic acid and eicosapentaenoic as omega-3. Henceforth, it has been demonstrated that microwave-assisted fish oil extraction developed in this study is efficient for high-quality PO production.

Keywords:
Patin oil, Box-Behnken design, Response surface methodology, Omega-3, GC-MS

1. INTRODUCTION

Fish oils are the primary food source of omega-3 fatty acids. Some species of Pangasius have been studied to contain omega-3, i.e., *Pangasius micronemus* (patin fish), *Pangasius nasutus*, and *Pangasius bocourti*[1-2]. Among these species, patin contains the highest level of omega-3 fatty acids[1]. The main omega-3 fatty acids in patin fish include α-linolenic acid and eicosapentaenoic acid[3]. These fatty acids positively affect human health, such as preventing cognitive decline, providing anti-inflammatory and antioxidant effects[4-5]. Additionally, omega-3 fatty acids also decrease the risk of coronary heart disease and may help protect against the development of dementia[6-7]. Henceforth, consuming fish oil may provide the aforementioned health effects.

Extraction is the necessary attempt to obtain the benefits of fish oil from patin. Conventionally, the oil was extracted using Soxhlet, maceration[8], Bligh and Dyer method[2], and Folch method[1]. However, these methods are

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inefficient due to time-consuming up to 48 hours (Folch method) with recovery 26-31%[1]. Newer extraction techniques, such as microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), accelerated-solvent extraction (ASE), and supercritical fluid extraction (SFE), are considered methods for enhancement of the extraction efficiency. However, the utmost advantage is the possibility to conduct multiple extractions provided by MAE. Hence, this extraction technique is considerably faster than the conventional one[9]. Priorly, MAE has been successfully used to extract oil from eel (*Monopterus albus*), Mackarel, catfish, and *Tilapia*[10-13]. In this study, MAE was developed for patin oil (PO) extraction.

The basic principle of MAE is the unique heating of the moisture by microwave energy transmission inside the cells. This condition leads the water compound on the cell membrane to evaporate, resulting in high pressure on the cell membrane that increases the biological matrix's porosity and enhances the release of analyte to the extracting solvent. Consequently, the MAE performs rapidly with low solvent consumption while achieves high recovery and reproducibility[9-11,14-15]. Nonetheless, the extraction process was influenced by several factors, such as temperature, extraction time, power, solvent type, solvent-to-sample ratio, and solvent composition[10-17]. Previous studies revealed that when temperature, extraction time, and power were increased, the yield of an extract increases[10,12]. The increase in yield might be due to increase in the penetrating power of solvent towards sample matrix when the irradiation time was prolonged while the later reduction in extraction yield might be as a result of over-exposure or overheating of the sample matrix leading to thermal degradation of effective chemical constituents in the sample[10]. Provided that the interactions between extraction factors may significantly influence the recovery, a chemometric approach helps develop a new extraction method. Furthermore, in contrast with other factorial designs, Box-Behnken design (BBD) requires fewer experiments to provide sufficient information for statistically acceptable results, and the experiments are performed avoiding extreme conditions[18-20].

Therefore, in this study, a BBD in conjunction with response surface methodology (RSM) was applied to optimize the extraction factors. The optimized method was subsequently used to produce PO, and the resulting oil was analyzed for the fatty acids composition by gas chromatography-mass spectrometry (GC-MS).

2. MATERIALS AND METHODS

2.1. Materials

Patin was obtained from a freshwater fish farm in Ngunut, Tulungagung, East Java, Indonesia (Latitude: of 8°6'37.32"S; Longitude: 111°59'16.87"E). The chemicals used in the study were of analytical purity and obtained from the suppliers described below: n-hexane ≥97.0% HPLC grade (Riedel-de Haen, Germany), ethyl acetate (Labkem, Barcelona, Spain), Potassium hydroxide (KOH) and HPLC-grade methanol (MeOH) from Merck (Darmstadt, Germany). Potassium methoxide solution 2 M was prepared by dissolving 11.2 g KOH in 100 mL MeOH. Supelco 37 Component FAME Mix from Merck (Darmstadt, Germany) was used as a standard reference of fatty acids. Whatman Filter paper No. 42 (Sigmaaldrich, Singapore).

2.2. Sample preparation

First, the whole fish was cleaned, and flesh was separated manually. Subsequently, the flesh was cut into small pieces (approximately 100 mg/piece) and dried in the oven at 60°C for 24 h. The dried flesh was then finely ground with an electric blender (Philips blender Power Titanium 1250 W, Valencia, Spain). Before the extraction process, the ground samples were stored at -4°C to keep the sample integration.

2.3. Extraction of patin oil

The Microwave-assisted Extractions (MAE) were carried out using a MARS 6 240/50 (One Touch Technology, CEM Corporation, Matthews, NC, USA). The fish sample of 1 g was accurately weighed and placed in an extraction tube made from modified polytetrafluoroethylene (PTFE-TFM). Then, a mixture of ethyl acetate and methanol was added according to the pre-determined solvent composition and solvent-to-sample ratio in the design of the experiment. The MAE conditions set for the extractions were: temperature (30, 60, 90°C), solvent composition (30, 60, 90% ethyl acetate in methanol), and solvent to sample ratio (10:1, 15:1, 20:1 mL solvent/g sample). After the extraction had been finished, the tubes were cooled down inside the MAE for 10 min until they reached ambient temperature. Then, the solid material was removed by filter paper, while the solvent was eliminated from the extract using a vacuum rotary evaporator (Lab1st, USA) at 40°C. The resulting oil was weighted to calculate the extraction yield and kept in the refrigerator at 4°C until GC-MS analysis.

2.4. Box-Behnken design (BBD) and response surface methodology (RSM)

A Box-Behnken design was employed to assess the influencing MAE factors. The studied factors were temperature (A, °C), solvent composition (B, % ethyl acetate in methanol), and solvent to sample ratio (C, mL of solvent/g of sample) at three levels (coded as -1, 0, and 1). The range of these factors is listed in (Table 1), while the whole design consists of 15 experiments performed in random order are presented in (Table 2). The extraction

Table 1. The studied factors in Box-Behnken design.

Code	Factors	-1	0	+1	Units
A	Temperature	30	60	90	°C
B	Solvent composition	30	60	90	% (v/v)
C	Solvent to sample ratio	10:1	15:1	20:1	mL/g

Table 2. Box-Behnken design matrix with coded variables, actual, and predicted yield of Patin Oil.

Run	Factors			Response (% yield)	
	Temperature (°C)	Solvent composition (%)	Solvent to sample ratio	Observed	Predicted
1	0	0	0	34.25	35.38
2	-1	0	1	37.28	35.75
3	-1	1	0	34.16	35.25
4	0	1	-1	30.06	31.71
5	0	1	1	30.89	31.33
6	1	0	1	31.35	34.09
7	-1	0	-1	23.48	20.74
8	0	-1	1	35.46	33.82
9	1	1	0	30.36	27.19
10	0	0	0	34.74	35.38
11	1	0	-1	30.43	31.96
12	0	0	0	37.14	35.38
13	1	-1	0	34.66	33.57
14	0	-1	-1	16.73	16.29
15	-1	-1	0	12.76	15.94

yield (%) was used as the responses, calculated as bellow:

$$\text{Yield}\,(\%) = \frac{W_2}{W_1} \text{ x } 100 \qquad (1)$$

where W_2 is the mass of PO extracted from the sample (g), and W_1 is the mass of the dried samples (g).

The resulting BBD data was examined by the analysis of variance (ANOVA) to determine the significance of the effect of each independent factor and their interactions. The main and interaction effects that significantly influence the MAE process were then included in a polynomial model of RSM that can be expressed as follows :

$$Y = f(A,B,C)+e \qquad (2)$$

where *Y* is the extraction yield (%) as the response of the BBD, while A, B, and C are the studied factors (temperature, solvent composition, and solvent to sample ratio, respectively) and e is a random error. Since the final purpose was to optimize the response Y, it was necessary to find the best estimation for the correlation between factors and the response surface. Generally, a second-order model is applied in RSM:

$$Y = \beta_0+\sum_{1=1}^{k} \beta_i X_i+\sum_{1=1}^{k} \beta_{ii} X_i^2 + \sum_{1=1}^{k-1} \sum_{i=1}^{k} \beta_{ij} X_i X_j+\varepsilon \qquad (3)$$

Y is the dependent factor; Xi are k represents the independent factors that may affect MAE efficiency;

β_0, β_{ii} (i = 1,2,…,k), β_{ij} (i = 1, 2,…, k; j = 1, 2,…,k) are regression coefficients, and ε is a random error.

The construction of the BBD matrix and the modeling of the RSM were performed employing STATGRAPHICS Centurion 18 (Statpoint Technologies, Inc., USA). The analysis of variance (ANOVA, $p<0.05$) was utilized to define the significance of each factor of interest. Provided that ANOVA suggests a significant difference, a Least Significant Difference (LSD, $p<0.05$) test was applied to estimate the differences between mean values. The Analysis ToolPak of Excel (Microsoft Office) was used to calculate the data from a non-factorial experiment.

2.5. Evaluation of the method precision

The precision of the extraction methods was evaluated by studying the repeatability (intra-day) and intermediate precision (inter-day). Repeatability was assessed by nine independent extractions under the same conditions on the same day, while nine additional extractions evaluated intermediate precision on three consecutive days (3 extraction processes on each day). The precision value was expressed as the coefficient of variation (CV) of the extraction yield (%).

2.6. Fatty acid analysis

Fatty acid must be derivatized to methyl ester form before injected into GC-MS. The derivatization procedure followed the previous reported method with modification[8]. Potassium methoxide was used instead of sodium methoxide. The fish oil sample was accurately weighed (100 mg) in a 10 mL centrifuge tube using an analytical balance. Then, 1.2 mL hexane was stirred using a homogenizer for 1 min until completely dissolved. Next, a mixture of potassium hydroxide in methanol (2 M, 0.25 mL) was added and stirred for 5 min. The mixture was then centrifuged at 3,000 r.p.m. for 5 min. Finally, 1-2 mL of supernatant were transferred into a 2 mL GC vial and covered with silicone Teflon cap and septum.

2.7. GC-MS condition

The fatty acid methyl esters (FAMEs) were analyzed by the tandem technique of gas chromatography-mass spectrometry (GC-MS) model TQ8040 (Shimadzu, Kyoto, Japan). A capillary column with 60 m length, 0.25 mm internal diameter, and 0.25 µm film thickness, Supra-WAX-280 (Teknokroma, Barcelona, Spain), was used to separate FAMEs. The injector was used in split mode, and MS ionization mode was electron ionization (EI). Helium was used as the carrier gas, and the temperature injection was 150°C. The GC temperature program operation was initially 50°C for a 2 min, then raised with 5°C min^{-1} rate to 220°C with and hold for 15 min, then raised at a rate of 40°C/min to 250°C and hold for 2 min, and finally raised again to 270°C with a rate of 40°C/min then hold for 2 min. Data were acquired and analyzed with the help of GC-MS solution software. The FAMEs were identified employing NIST11 library and comparing the peak retention time in GC with a reference FAME mixture. The results were expressed in relative amounts to the total fatty acids (%) of duplicate sampling.

3. Results and discussion

3.1. Assessment of the effects of extraction factors

A Box-Behnken design was carried out to develop a microwave-assisted extraction (MAE) of Patin oil (PO). The extraction factors that may affect the efficiency of the MAE consisted of temperature (A), solvent composition (B), and solvent to sample ratio (C). The levels of each factor (A: 30, 60, 90°C; B: 30, 60, 90% ethyl acetate in methanol; and C: 10:1, 15:1, 20:1 mL solvent/g sample) were chosen based on literary studies on the yield of fish oil extraction[10,21-22]. The effects of the factors and the possible interactions among them were evaluated by analysis of variance (ANOVA). A standardized Pareto chart, which allows for knowledge of the influencing factors and their order of influence from a graphical point of view, is presented in (Figure 1).

A bar that crosses a vertical line in the Pareto chart (Figure 1) corresponds to a factor or interaction of factors that significantly affect the response (% extraction yield). In this case, six effects have *p*-values below 0.05,

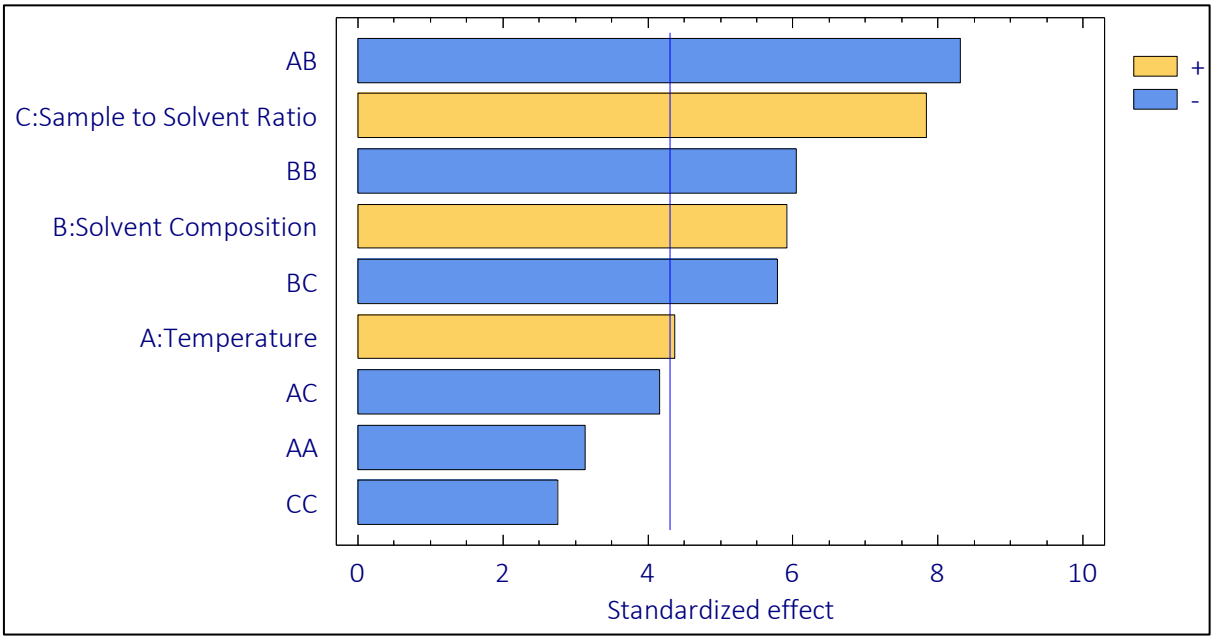

Figure 1. Pareto chart for the standardized main, interaction, and quadratic effects of MAE factors on the extraction yield.

indicating that the effects were significantly influenced the extraction yield at the 95.0% confidence level. The influencing effects consisted of main effects (temperature, solvent to sample ratio, and solvent composition), interaction effects (temperature×solvent composition and solvent composition×solvent to sample ratio), and quadratic effect (temperature×temperature). All the main effects showed a positive influence of the extraction factor on the response, which means a high extraction yield was achieved by increasing the level of these studied factors. On the contrary, the interaction and quadratic effect showed a negative direction toward the extraction yield.

The temperature of MAE is being controlled by the occurrence of a unique microwave energy transmission that heats the matrix simultaneously[23]. Since the extraction temperature had a significant positive effect on the extraction, higher temperatures could increase the extraction yield. This fact may result from the increases of solvent viscosity in higher temperatures that enhance the solvent diffusivity into the matrix. Moreover, the increase in temperature facilitates the solubility of the analyte in the solvent resulting in a higher collection of the extract can be achieved. However, after the optimum extraction temperature was achieved, further increases of the

temperature lowering the extraction yield[10]. An extremely high temperature could result in a low extraction yield caused by the analyte decomposition[24-25].

Apart from increasing the extraction temperature, a higher extraction yield could also be obtained by increasing the solvent to sample ratio. Previous study about oil extraction from *Moringa peregrina*, also confirmed that the solvent to sample ratio has a significant positive effect on the extraction yield. The study reported an increasing the concentration gradient would increase the mass transfer rate from the solid matrix to the solvent[26]. However, once the oil is completely extracted, a further increase of the solvent-to-sample ratio could not provide additional extracted oil but lower the extraction efficiency.

The effect of solvent composition also affected the extraction yield. Another research reported disclosed similar results confirming that the combination of ethyl acetate and methanol were compatible with microwave energy. The conversion efficiency of the absorbed microwave energy into heat is indicated by the dielectric constant (ε) of the extraction solvent. The higher the ε value of the solvent, the higher the temperature inside the MAE system. Methanol has a higher dielectric constant (ε = 32.70) than ethyl acetate (ε = 6.02); thus, it could absorb much of the microwave energy and transform it into heat and favorable to the extraction rate[16]. However, thermolabile compounds require lower temperatures inside the extraction system provided by ethyl acetate to protect the compounds. Henceforth, optimization of the studied dual solvent is necessary to define the suitable composition to provide the highest extraction yield.

3.2. Optimization of the extraction factors

After evaluating the effect of extraction factors and their interactions, the significant ones were included in the MAE optimization. The corresponding regression coefficient of factors and their interactions that significantly influenced the MAE yield were substituted into the following second-order polynomial model equation:

$$Y = 35.38+2.39A+3.23B+4.29C-6.42AB -4.87B^2-4.48BC \quad (4)$$

where Y is the MAE yield, and X is the studied factors (A, temperature; B, solvent composition; C, solvent to sample ratio).

The second-order polynomial model was validated by the high value of the coefficient of determination (R^2, 92.77%), representing the confidence that the regression model fitted the observed data[27]. Additionally, the model validation was performed by a lack-of-fit test comparing the variability of the current model residuals to the variability between observations at replicate settings for the factors. A nonsignificant lack-of-fit (p>0.05) is a desirable statistical parameter to prove the fitting of the model to the responses[27]. In this study, the lack-of-fit test result showed a p-value of 0.1325, which means that the models fitted well. The goodness of fit between the observed and predicted data by the proposed model is plotted in (Figure 2).

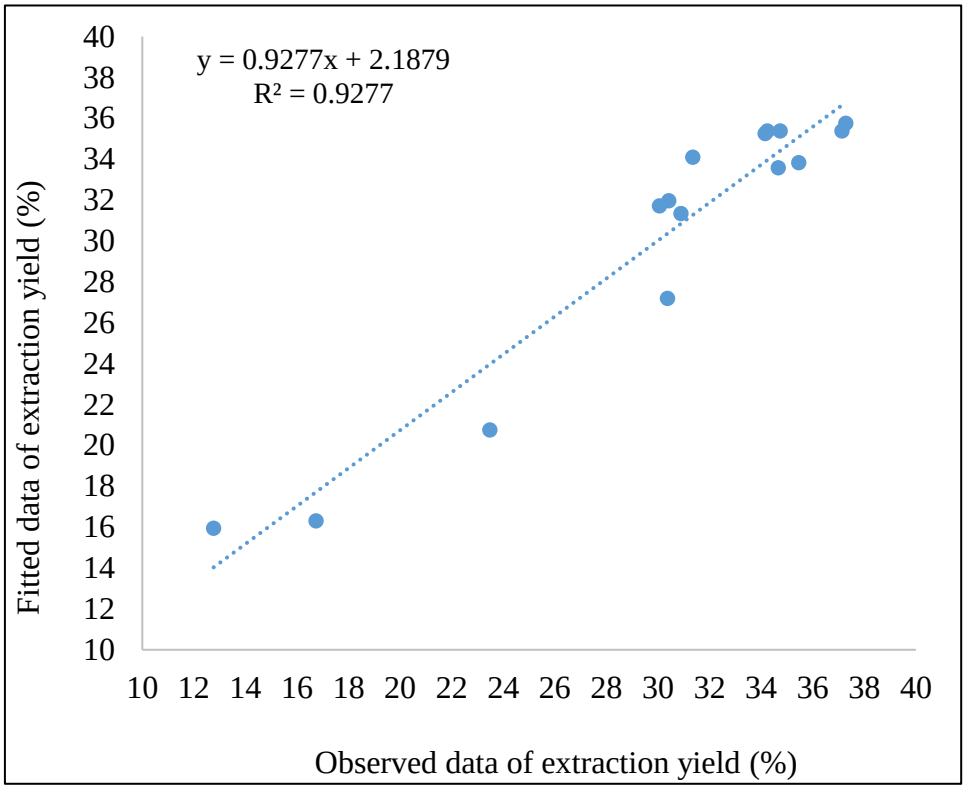

Figure 2. A scatter plot for the observed (X-axis) versus predicted (Y-axis) data by the regression model.

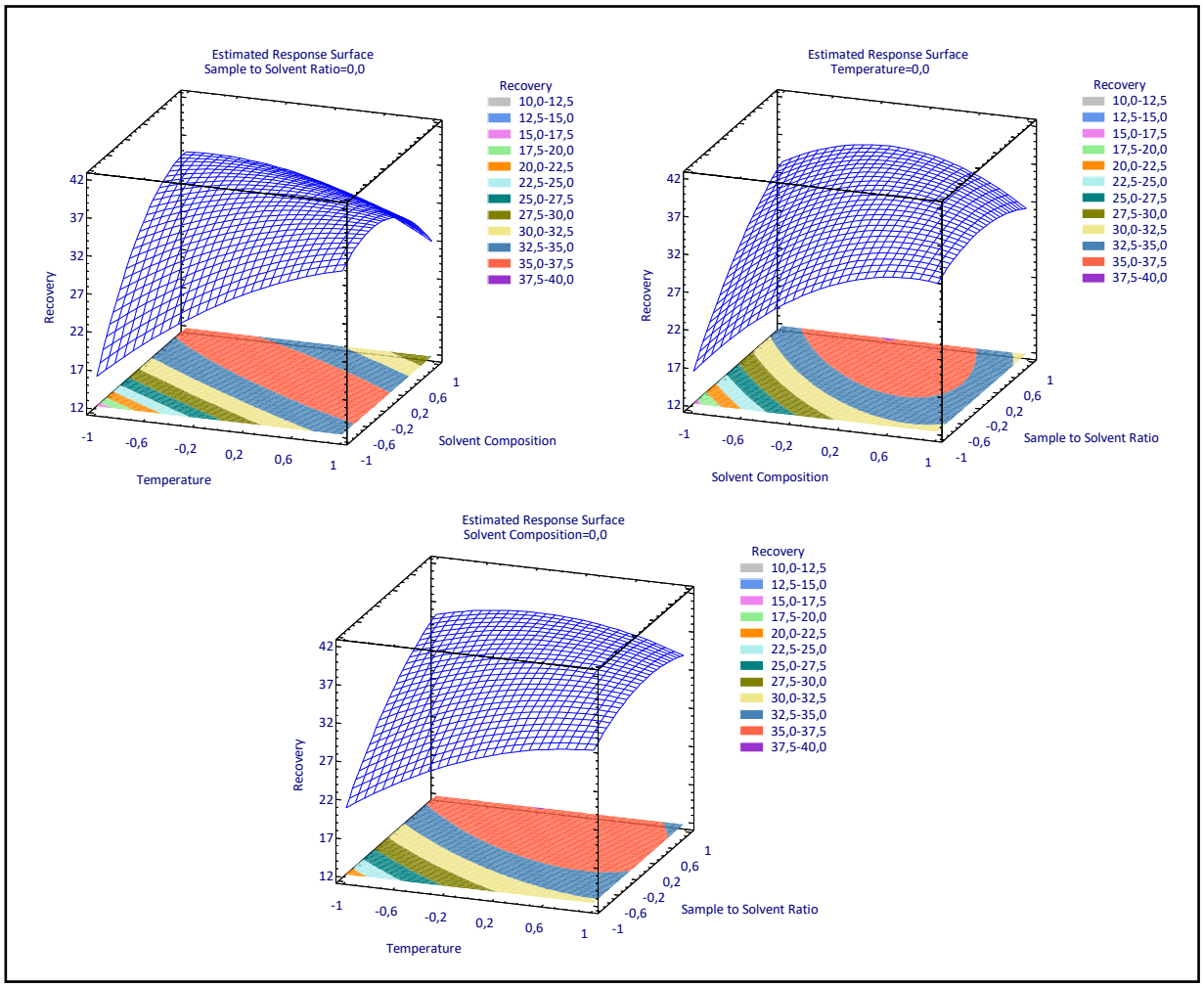

Figure 3. Response surface plot for the studied Microwave-Assisted Extraction factors.

3.3. Response optimization

The influential independent factors are essential to achieve the best extraction yield when optimizing the method. Based on the predicted model, three-dimensional surface plots were constructed (Figure 3) to predict the relationships between influencing factors and the response. Response surface methodology (RSM) plotted the prediction surface of extraction yield based on the variations of the studied factors. The RSM suggested the optimum extraction yield (38.40%) was located at coordinates -0.013, -0.120, and +1.00 for temperature (A), solvent composition (B), and solvent to sample ratio (C), respectively. Based on this optimum condition, the high PO extract was achieved by applying an extraction temperature of 55°C, a solvent composition of 56%, and a solvent-to-sample ratio of 20:1. The obtained yield was higher than Soxhlet method (18%) in another study, with the condition at the temperature of 70°C for 2 hours[28].

3.4. Extraction time

On the basis of the optimum MAE condition previously suggested by RSM, a study of the extraction kinetics was performed at 5 to 30 min. Figure 4 discloses the plot of extraction yield over the extraction time. The ANOVA was employed to calculate the significance of the extraction time (p>0.05). As the calculated F value (4.32) was higher than the F-critical (3.11), the extraction time significantly affected the PO recovered by MAE. Subsequently, a post-hoc ANOVA detected an optimal extraction time at 15 min as any longer extraction time did not provide a higher extraction yield. Hence, complete oil recovery from Patin was achieved starting at 15 min by MAE.

3.5. Precision of the MAE method

A number of alternative extraction methods, including MAE, are now applied in the food industry[29]. Apart from producing a high recovery of the extraction yield, a precise method is also necessary to achieve an efficient industrial MAE. The assessment of the method precision was done by performing repeatability (intra-day extractions) and intermediate precision (inter-day extractions). The result was shown in Table 3. The acceptable values validated the developed method for repeatability (CV, 1.85%) and intermediate precision (CV, 1.97%). Both

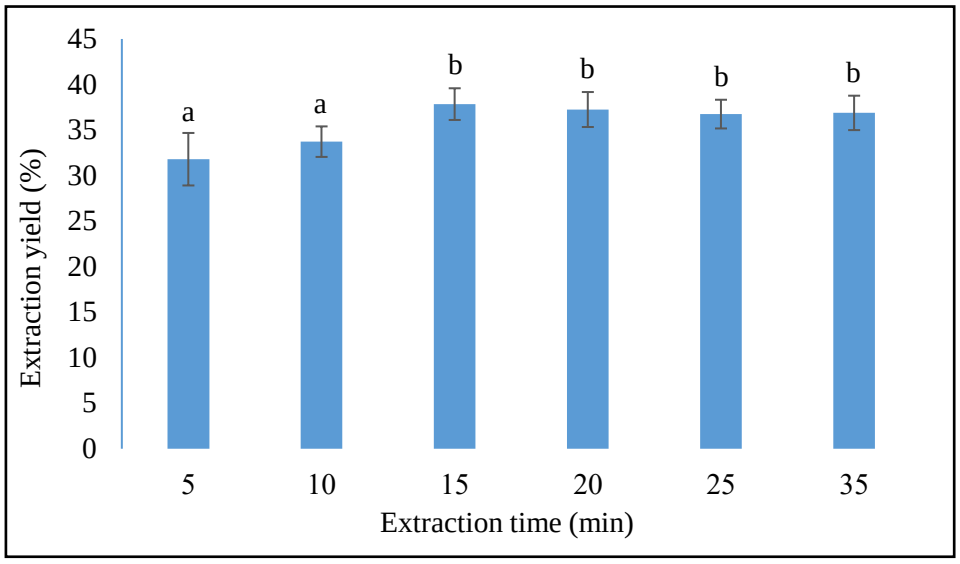

Figure 4. Patin oil recovery using different extraction times. Different letters on the bar indicate significant differences (p<0.05) among extraction yield (%) recovered under different extraction times.

Table 3. The result of Repeatability and Intermediate precision of the MAE method.

Samples	Repeatability	Intermediate precision		
	Day-1	Day-1	Day-2	Day-3
1	37.70	38.77	37.77	38.83
2	38.75	36.69	38.90	38.23
3	37.64	38.90	38.01	38.93
4	36.85			
5	38.65			
6	38.74			
7	38.81			
8	39.00			
9	38.93			
Mean	38.34		38.34	
SD	0.71		0.76	
%CV	1.85		1.97	

experiments confirmed the method precision (% CV) less than 2%, making this proposed MAE reliable for potential application in the food industry.

3.6. Fatty acid composition

The oil extracted from patin by the developed MAE method was analyzed by GC-MS for the fatty acids composition. The GC-MS chromatogram of Patin oil was shown in Figure 5. The identification and relative quantification of occurring fatty acids in PO is reported in (Table 4). The reported values for the amount of identified fatty acids were relative % to the total fatty acids in the matrix. The PO was described naturally as low in saturated fatty acids (SFA, 27.51%) and high in monounsaturated fatty acids (MUFA, 47.24%). Specifically, PO was characterized by oleic (39.15%) and linolenic (19.44%) acid as the major fatty acids in the matrix. This finding was similar to the amount reported by Hashim, et. al., (2015) that also found MUFA in PO ranging from 35 to 41%. The high level of unsaturated fatty acids contained by the microwave-produced PO makes the production of this fish oil is compatible with the cholesterol-lowering purpose[1].

Another interesting fact that resulted from the fatty acids composition of PO was the content of α-linolenic (1.41%) and eicosapentaenoic (0.50%) acid as the omega-3. Additionally, PO also contained omega-6, linoleic acid (19.44%), γ-linolenic (0.38%), and arachidonic (1.02%) acid. Meanwhile, the amount of oleic acid (omega-9) was higher than other fatty acids. The ratio between omega-3, omega-6, and omega-9 in PO was 1:11:21. Previous research disclosed similar findings on the level of oleic acid (29-33%) in PO by the Folch method[1]. Therefore, the developed MAE resulting in comparable PO with the oil extracted using the conventional method.

4. CONCLUSION

In conjunction with response surface methodology, Box-Behnken design successfully optimized the microwave-assisted extraction (MAE) for oil extraction from Patin. The proposed MAE method provided high recovery (extraction yield of 38.40%) and precision (coefficient

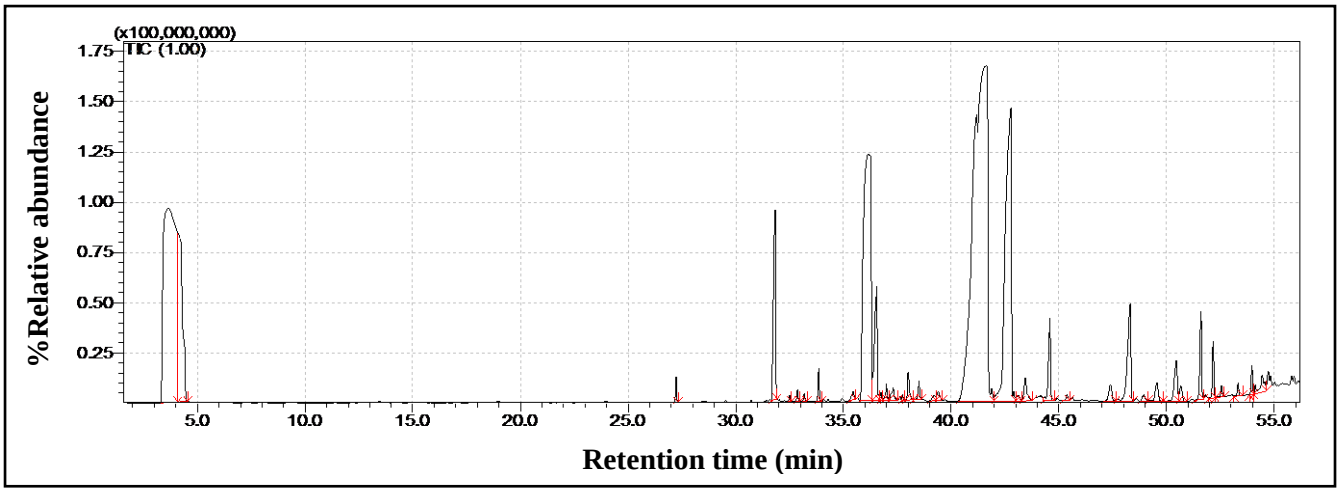

Figure 5. GC-MS Chromatogram of Patin oil.

Table 4. The Fatty acid composition of Patin Oil (n=3).

Retention time (min)	Fatty Acid	Relative to the total fatty acids (%)
	Saturated fatty acid (SFA)	
27.229	Lauric acid (C12:0)	0.39 ± 0.01
33.173	Myristic acid (C14:0)	9.49 ± 0.20
33.851	Pentadecanoic acid (C15:0)	0.52 ± 0.02
38.007	Heptadecanoic acid (C17:0)	0.52 ± 0.00
41.632	Stearic acid (C18:0)	16.35 ± 0.05
47.339	18-methyl-nonadecanoic acid (C19:0)	0.24 ± 0.01
	Total SFA	27.51
	Monounsaturated fatty acid (MUFA)	
36.472	Palmitoleic acid (C16:1)	3.59 ± 0.09
38.482	Cis-10-Heptadecanoic acid (C17:1)	0.24 ± 0.01
41.468	Oleic acid (C18:1n9)	39.15 ± 1.27
42.615	Vaccenic acid (C18:1n7)	2.58 ± 0.16
48.228	Cis-11-Eicosanoic acid (C20:1)	1.68 ± 0.06
	Total MUFA	47.24
	Polyunsaturated fatty acid (PUFA)	
42.513	Linoleic acid (C18:2n6)	19.44 ± 0.28
43.360	γ-linolenic acid (C18:3n6)	0.38 ± 0.03
47.343	α-linolenic acid (C18:3n3)	1.41 ± 0.05
49.498	8.11-Eicosadienoic acid (C20:2)	0.27 ± 0.01
50.382	Cis-11.14-Eicosadienoic acid (C20:2)	0.63 ± 0.02
50.640	Cis-5.8.11-Eicosatrienoic acid (C20:3)	0.22 ± 0.01
52.544	10.13.16-docosatrienoic acid (C22:3)	1.38 ± 0.12
53.307	Arachidonic acid (C20:4n-6)	1.02 ± 0.22
53.961	Eicosapentaenoic acid (C20:5n-3)	0.50 ± 0.01
	Total PUFA	25.25

of variation less than 3%) by applying the optimum MAE temperature (60°C), extraction solvent composition (56% ethyl acetate in methanol), solvent-to-sample ratio (20:1), and extraction time (15 min). The microwave-produced patin oil was characterized by omega-3 (α-linolenic and eicosapentaenoic acid), omega-6 (linoleic, γ-linolenic, and arachidonic acid), and omega-9 (oleic acid) with the ratio of 1:11:21. The high level of unsaturated fatty acids (47.24% MUFA and 25.25% PUFA) contained by PO, in addition to the high recovery and precision of the extraction method, make the developed MAE potential for industrial application.

5. ACKNOWLEDGEMENT

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Conflict of interest

None to declare.

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Author contribution
Concept: ARP, WS, MP, CCF, AR, SS; Supervisor: WS, AR, SS, MP; Collecting Data: ARP; Analysis Data: ARP, WS; Writing: ARP, WS, AR.

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