

Authentication analysis of milkfish oil treated with activated charcoal and bentonite using Fourier Transform Infrared Spectroscopy and Multivariate Data Analysis

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Article history:

Received: 4 January 2022

Received in revised form: 10 February 2022

Accepted: 29 March 2022

Available Online: 10

September 2023

Keywords:

Milkfish oil,
Authentication,
Multivariate calibration,
FTIR spectra,
Linear discriminant analysis

DOI:

[https://doi.org/10.26656/fr.2017.7\(5\).1012](https://doi.org/10.26656/fr.2017.7(5).1012)

Abstract

Adulteration issues are important not only for fats and oils consumers but also for producers and regulatory bodies, therefore authentication analytical methods are continuously developed. The objective of this study was to highlight the employment of attenuated total Fourier transform infrared (FTIR) spectroscopy and chemometrics for the authentication of milkfish oil (MFO) from pumpkin seed oil (PSO). In this study, ATR-FTIR spectra of MFO, PSO and its binary mixtures were scanned, and the information obtained was used for quantitative analysis of adulterants and classification between authentic MFO and MFO adulterated with PSO. Two multivariate calibrations of partial least square calibration (PLSR) and principal component regression (PCR) were compared for their performance during quantitative analysis and linear discriminant analysis was applied for discrimination between authentic MFO and MFO adulterated with PSO. The results revealed that PLSR using 2nd derivative spectra at wavenumbers of 1500-1000 cm⁻¹ was preferred for simultaneous quantitative analysis of MFO-treated with activated charcoal, while PCR using 2nd derivative spectra at wavenumbers of 1500-1000 cm⁻¹ was suitable for analysis of MFO-treated with bentonite providing the acceptable values of R², root mean square error of calibration (RMSEC) and root mean square error of prediction (RMSEP). Linear discriminant Analysis using normal FTIR spectra at the whole wavenumber region was successful for the discrimination of MFO treated with activated charcoal and bentonite with accuracy levels of 100%. It can be concluded that the chemometrics approach for ATR-FTIR spectra could be a simple and effective tool for the authentication of MFO.

1. Introduction

The supplementation of marine-based fish oil in food and pharmaceutical products is popular to get the beneficial effects of n-3 long chain polyunsaturated fatty acids (n3PUFA) eicosapentanoic acid (EPA, 20:5 n-3) and docosahexanoic acid (DHA, 22:6 n-3) (Malinowski *et al.*, 2019). The consumption of marine-derived fish oils was associated with cardiovascular, chronic inflammatory, neurodegenerative disorders, and non-alcoholic fatty liver disease after the publication in 1980 by Bang *et al.* who reported that there is a correlation between diets rich in marine oils with the rarity of ischemic heart diseases in Greenland Inuit Eskimo (Bang *et al.*, 1980). Milkfish (*Chanos chanos* Forsk.) is one of

the cultivated fish in Indonesia and it is used as a protein source needed for human development. It contains protein essential amino acids particularly lysine and polyunsaturated fatty acid (PUFA). The fatty acid component in fish oil is very easily oxidized, the oxidation process that occurs causes fatty acids to detach from bonding with glycerol resulting in an increase in the amount of free fatty acids. Increasing the amount of free fatty acids has the potential to reduce the quality and damage of oil (Dari *et al.*, 2018). The stage that can be done to prevent this is oil purification with an adsorbent, adsorbent can absorb impurities from components, pigments, and free fatty acids in the oil. Adsorbents that can be used in the purification process are activated

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charcoal, bentonite, attapulgite and chitosan (Rosmalina *et al.*, 2021). Based on research on Patin fish oil (PFO) conducted by (Diharmi *et al.*, 2019), oil refining using bentonite and activated charcoal showed a decrease in Free fatty acid levels resulting from $0.65 \pm 0.01\%$ to $0.06 \pm 0.01\%$ met the International Fish Oil Standard (IFOS, 2011). Milkfish oil (MFO) MFO is considered a functional food oil because it contains PUFAs which are beneficial to human health, therefore MFO is subjected to adulteration with low price oils such as vegetable oils (Christy *et al.*, 2004). Unethical producers can fake MFO with other vegetable oils for economic gain, but this practice can reduce the quality of MFO.

Several analytical methods have been proposed and validated which are intended for the authentication analytical purposes of high price oils including high-performance liquid chromatography (HPLC) by analysis of triacylglycerols (Ruiz-Samblás *et al.*, 2013), gas chromatography in combination with detectors of flame ionization detector (FID) or mass spectrometer (MS) through analysis of fatty acid composition (Jurado-Campos *et al.*, 2021), NMR spectroscopy through analysis of metabolite fingerprinting (Fadzillah *et al.*, 2017), differential scanning calorimetry through analysis of thermal profiles (Angiuli *et al.*, 2009), electronic nose (Marina *et al.*, 2010), and real-time polymerase chain reaction through analysis of DNA fingerprinting (Zhang *et al.*, 2012). However, some of these methods involved sophisticated instruments (chromatography, real-time PCR, NMR spectroscopy) and lack specificity and reproducibility (DSC, electronic nose), therefore, simple and reliable methods based on vibrational spectroscopy have been reported in fats and oils authentication.

Fourier transform infrared (FTIR) spectroscopy, as one of the vibrational spectroscopy based on the interaction of electromagnetic radiation in the infrared region with samples to yield vibrational transitions, has emerged as a powerful analytical technique for authentication of high price edible fats and oils due to its property as fingerprinting analytical technique (Rohman, 2017). Using certain sampling accessory like attenuated total reflectance in which the samples can be placed directly above ATR crystal in combination with multivariate data analysis, FTIR spectra has been successfully applied for authentication analysis of edible fats and oils either in binary, ternary or quaternary models such as olive oil (Rohman and Man, 2012), cod liver oil (Rohman and Che Man, 2011b), Patin fish oil (Putri *et al.*, 2020), butter (Fadzillah *et al.*, 2013) and others. However, the application of FTIR spectroscopy in combination with chemometrics for the authentication of Milkfish oil is very limited. Therefore, in this study, FTIR spectroscopy was employed for adulteration

analysis of MFO from pumpkin seed oils having close similarity with FTIR spectra of MFO based on linear discriminant analysis.

2. Materials and methods

2.1 Materials

Milkfish (Ikan Bandeng in Indonesia) was purchased from the central fish market in Central Java (in Juana Pati), Indonesia. Pumpkin seed oil was purchased from the local market around Yogyakarta, Indonesia. The authenticity of the used PSO was verified by determining fatty acid composition using gas chromatography with a flame ionization detector (Rohman and Che Man, 2011a).

2.2 Preparation of milkfish oil

Milkfish oil (MFO) was prepared by extracting the head of a milkfish by placing both fish parts in an aluminium tray according to Mustafidah *et al.* (2021). Milkfish samples were dried using a cabinet dryer at 50°C for about 24 hrs. MFO was extracted using direct pressing with 100 kN force for 2 min. The samples were subjected to centrifugation at $5000 \times g$ for 10 mins to separate sediment. Furthermore, MFO obtained was treated with bentonite and activated charcoal as bleaching agents according to (Polii, 2016) and (Kurniawan and Saputra, 2011).

2.3 Preparation of binary mixtures of milkfish and pumpkin seed oil

MFO and pumpkin seed oil (PSO) were prepared in a binary mixture with the composition as detailed in Table 1. The binary mixture used a different concentration range to facilitate the calibration and validation models spanning certain ranges. All mixtures were analyzed using an FTIR spectrophotometer and the prediction of analyte levels was performed using multivariate.

2.4 Scanning using FTIR spectrophotometer

All samples used for modelling (quantification using multivariate calibration and discrimination using LDA) were subjected to scanning with an FTIR spectrophotometer (Thermo Scientific Nicolet iS10, Madison WI) applying attenuated total reflectance (ATR) accessory. The Omnic® with Windows® (XP/Vista) Software was used to process FTIR spectra. The measurements of ATR-FTIR spectra were performed in a region of $4000\text{--}650\text{ cm}^{-1}$ using 32 scanings and a resolution of 8 cm^{-1} . Before the measurement of each sample, a background spectrum of air was measured using the same condition as that of the sample. All samples were measured in three replicates using

Table 1. The composition of Milkfish oil (MFO) and pumpkin seed oil (PSO) used during the modelling (calibration and validation) of MFO prediction assisted with multivariate calibration.

Samples	Milkfish oil %	Pumpkin seed oil %
1	72	28
2	80	20
3	84	16
4	62	38
5	39	61
6	71	29
7	31	69
8	73	27
9	48	52
10	11	89
11	37	63
12	46	54
13	26	74
14	99	1
15	62	38
16	53	47
17	98	2
18	95	5
19	53	47
20	86	14
21	10	90
22	97	3
23	12	88
24	66	34
25	44	56
26	100	0
27	0	100

absorbance mode. During the analysis of samples, ATR crystal was cleaned with *n*-hexane and acetone after FTIR spectral measurement.

2.5 Linear Discriminant Analysis of milkfish oil and milkfish oil added with pumpkin seed oil

LDA was used for supervised pattern recognition between authentic MFO and MFO added with PSO. The samples consisting of pure MFO and MFO mixed with PSO at different concentration ranges were prepared.

The Coomans plot was constructed for discrimination between authentic MFO and adulterated MFO with PSO, and the accuracy levels of discrimination were evaluated. The discrimination between authentic MFO-treated with PSO using the absorbance values of ATR-FTIR spectra at the whole IR region ($4000\text{-}650\text{ cm}^{-1}$).

2.6 Data analysis

Data analysis using chemometrics was carried out using the software of TQ Analyst. The chemometrics evaluation included the evaluation of performance characteristics of two multivariate calibrations of partial least square calibration (PLSR) and principal component regression (PCR) by evaluating of root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP) and coefficient of determination (R^2). For LDA, the accuracy levels were used as criteria during the classification between authentic MFO and MFO adulterated with PSO (Ikhsan *et al.*, 2021).

3. Results and discussion

In this study, ATR-FTIR spectra were employed for analysis of the adulteration practice of milkfish oil (PSO) with pumpkin seed oil (PSO). The selection of PSO as an adulterant model was based on the close similarity between MFO and PSO in score plots in principal component analysis, as reported in our previous study (Mustafidah *et al.*, 2021). Figure 1 exhibits FTIR spectra of MFO extracted from the flesh and head parts in which each peak represented functional groups present in the studied oils. Each peak and shoulder represented functional groups in triacylglycerols (TAG). The main components present in edible fats and oils are TAG, therefore, the functional groups in TAG dominate FTIR spectra. The presence of *cis* =CH coming from unsaturated fatty acids such as oleic and linoleic acid was confirmed by the peak at 3700 cm^{-1} . Peaks at 2921 and 2852 cm^{-1} were due to stretching vibrations of methyl (-CH₃) and methylene (-CH₂) groups from the fatty acid chain in TAG. Both functional groups are also confirmed by peaks at 1461 cm^{-1} (bending vibration of -

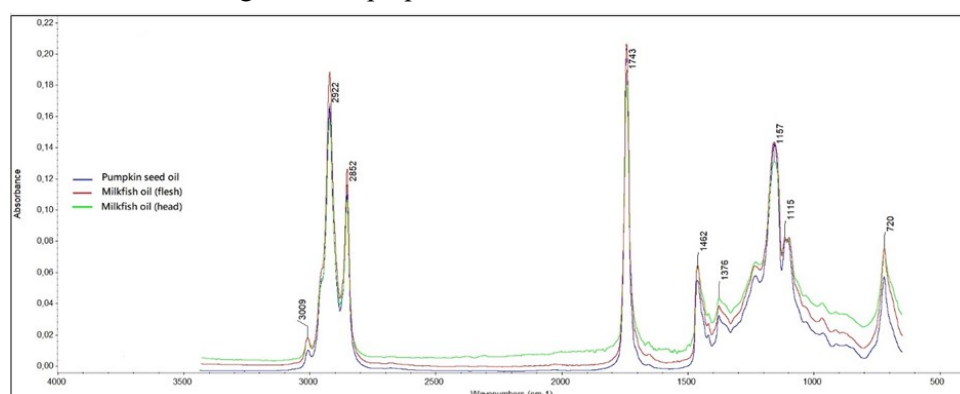


Figure 1. Attenuated total reflectance-FTIR spectra of pumpkin seed oil (blue), milkfish oil from flesh part (red) and milkfish oil from head (green) at mid-infrared region ($4000\text{-}650\text{ cm}^{-1}$) at the resolution of 8 cm^{-1} .

CH₂) and 1376 cm⁻¹ (bending vibration of -CH₃). The presence of carbonyl (C=O) was confirmed by the peak at wavenumbers of 1743 cm⁻¹. The presence of C-O in TAG was confirmed by peaks at 1157 and 1098 cm⁻¹ (Lerma-García *et al.*, 2010; Rohman and Man, 2012). All FTIR spectra of these oils are similar, however, using detailed investigation, these oils could be differentiated in terms of peak intensities, especially in fingerprint regions, because FTIR spectra are fingerprinting analytical techniques in nature.

Authentication analysis of MFO-treated with activated carbon from PSO was carried out by simultaneous quantitative analysis of PSO and MFO which was aided by two multivariate calibrations (PLSR and PCR) at certain wavenumbers or its combination. Table 2 exhibits the statistical performance of PLSR and PCR for the relationship between actual values of PSO and MFO in terms of R²-calibration, R²-prediction, RMSEC and RMSEP. The values of R² indicated the

closeness between actual values of analyte(s) (PSO and MFO) and FTIR predicted values, therefore, R² values could be used as accurate indicators of the developed method. The higher R²-values, the more accurate of methods. In addition, RMSEC and RMSEP indicated the repeatability of the developed method to provide similar results, therefore, the lower the RMSEC and RMSEP values, the more precise the method. Based on these criteria, multivariate calibrations of PLSR, the wavenumbers regions and FTIR spectral treatments (normal, 1st and 2nd) were optimized. Normal and derivative FTIR spectra at certain wavenumbers regions combined with multivariate calibrations were compared in order to get the best model for the prediction of MFO as an adulterant. Derivatization of FTIR spectra could improve the resolution of adjacent peaks which may affect the better performance of calibration models, however, the higher order of spectra derivative could decrease the model sensitivity. The selection of the FTIR

Table 2. The statistical performances of ATR-FTIR spectra at certain wavenumbers in combination with multivariate calibrations for calibration and validation modelling of simultaneous analysis of milkfish oil (MFO) treated with activated charcoal and pumpkin seed oil (PSO).

Wavenumbers (cm ⁻¹)	Multivariate calibration	Spectra	Calibration		Prediction	
			RMSEC	R ²	RMSEP	R ²
1500-1000	PLS	Normal	0.1110	0.9196	0.1270	0.8993
		1st Derivative	0.0588	0.9781	0.0616	0.9778
		2nd Derivative	0.0062	0.9998	0.0437	0.9916
	PCR	Normal	0.0351	0.9923	0.0454	0.9876
		1st Derivative	0.0414	0.9892	0.0452	0.9898
		2nd Derivative	0.0409	0.9895	0.0467	0.9892
3000-700	PLS	Normal	0.1010	0.9338	0.1170	0.9167
		1st Derivative	0.0391	0.9904	0.0529	0.9853
		2nd Derivative	0.0785	0.9607	0.0812	0.9586
	PCR	Normal	0.0424	0.9887	0.0559	0.9838
		1st Derivative	0.0364	0.9917	0.0520	0.9868
		2nd Derivative	0.0397	0.9901	0.0523	0.9855
1500-800	PLS	Normal	0.1540	0.8393	0.1680	0.8145
		1st Derivative	0.0587	0.9782	0.0610	0.9781
		2nd Derivative	0.0920	0.9456	0.0921	0.9455
	PCR	Normal	0.0392	0.9903	0.0613	0.9829
		1st Derivative	0.0449	0.9873	0.0486	0.9876
		2nd Derivative	0.0467	0.9863	0.0487	0.9870
1500-700	PLS	Normal	0.1070	0.9253	0.1150	0.9161
		1st Derivative	0.0884	0.9499	0.0924	0.9458
		2nd Derivative	0.0037	0.9999	0.0504	0.9896
	PCR	Normal	0.038	0.9909	0.0549	0.9861
		1st Derivative	0.0227	0.9968	0.0471	0.9968
		2nd Derivative	0.0271	0.9954	0.0466	0.9888
3000-1000	PLS	Normal	0.1090	0.9233	0.1330	0.8937
		1st Derivative	0.0266	0.9956	0.0562	0.9857
		2nd Derivative	0.0789	0.9603	0.0812	0.9586
	PCR	Normal	0.0411	0.9894	0.0563	0.9839
		1st Derivative	0.0367	0.9915	0.0521	0.9870
		2nd Derivative	0.0397	0.9901	0.0523	0.9859

Note: The selected condition for quantitative analysis is in bold.

spectral condition was based on its capability to provide high R^2 and low values of RMSEC and RMSEP. PLSR using absorbance values of 2nd derivative FTIR spectra at selected fingerprint wavenumbers 1500-1000 cm^{-1} was selected to simultaneously predict MFO and PSO. Using these conditions, the R^2 values in calibration and prediction models for simultaneous analysis of MFO and PSO were 0.9998 and 0.9916 with RMSEC and RMSEP of 0.0062 and 0.0437, respectively.

Figure 2 exhibits the scattering plot for the relationship between actual values of MFO-treated with activated carbon [A] and PSO [B] with FTIR predicted values. The residual analysis was also evaluated to evaluate the difference between actual and FTIR-predicted values. The residual values fall around zero (above and below zero) indicating that systematic error does not exist. While random error which is frequently occurring during analysis could be negligible as indicated by low values of RMSEC and RMSEP during modelling.

The performance of multivariate calibrations for modelling (calibration and validation) for simultaneous analysis of milkfish oil (MFO) treated with bentonite and pumpkin seed oil (PSO) applying ATR-FTIR spectra at certain wavenumbers in combination with multivariate calibrations was compiled in Table 3. PCR using absorbance values of 1st derivative FTIR spectra was selected for prediction of bentonite-treated MFO and PSO simultaneously providing R^2 calibration of 0.9780, R^2 validation of 0.9573 with RMSEC and RMSEP values of 0.0617 and 0.0859, respectively. The scattering plot for the relationship between actual values of MFO-

treated with bentonite [A] and PSO [B] with FTIR predicted values can be seen in Figure 3. Again, systematic errors were not observed during residual analysis. From these results, FTIR spectroscopy and multivariate calibrations were accurate (high R^2) and precise (low RMSEC and RMSEP) (Miller and Miller, 2010) for quantitative analysis of MFO and PSO intended for authentication analysis.

The chemometrics of linear discriminant analysis (LDA), a kind of supervised pattern recognition technique, was applied to discriminate between authentic MFO-treated with activated charcoal and bentonite and MFO adulterated with PSO using PSO the absorbance values of ATR-FTIR spectra at whole IR region. Basically, LDA is a reduction technique of original variables (original data matrix) into lower-dimensional space. To carry out the LDA process, there are three steps to be performed. The first step is to calculate the separation capability between different classes of objects, namely the mean distances between the authentic MFO and MFO adulterated with PSO. This distance is called a between-class variance. In the second step, the mean distances in each class of objects were calculated and this distance is known as a within-class variance. The third step is to build the lower-dimensional space maximizing the distance of between-class variance and minimizing the distance of within-class variance and a parameter known as linear discriminant function (LDF) was calculated. LDF reflects the difference between groups as much as possible (Tharwat *et al.*, 2017). The objects in the same group will have similar values of LDF and objects in different groups will have very

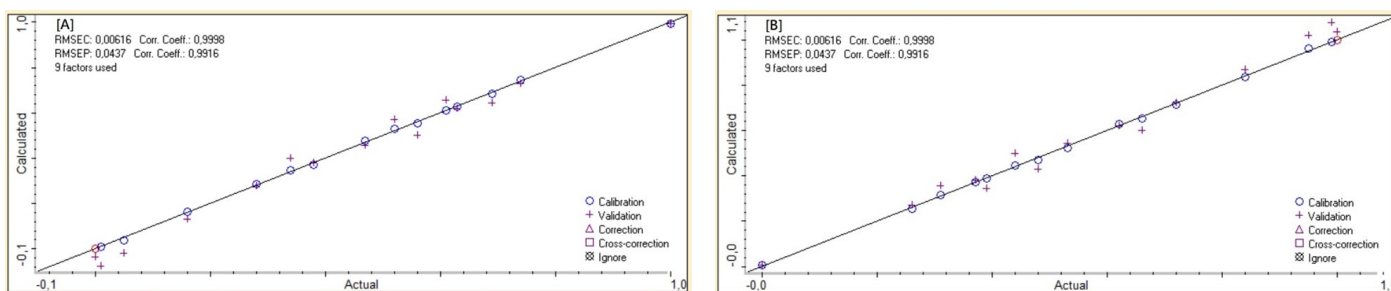


Figure 2. The scattering plot of partial least square regression (PLSR) for the relationship between actual values of MFO-treated with activated carbon [A] and PSO [B] with FTIR predicted values using 2nd derivative ATR-FTIR spectra at wavenumbers of 1500-1000 cm^{-1} .

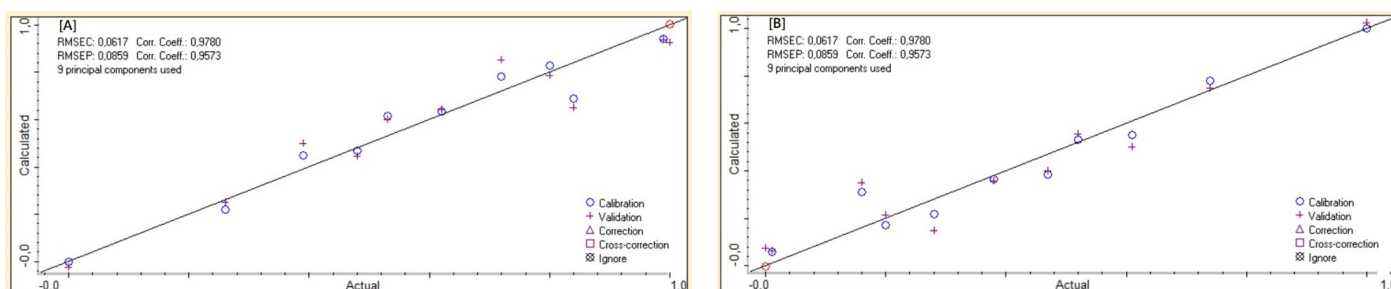


Figure 3. The scattering plot of principle component regression (PCR) for the relationship between actual values of MFO-treated with bentonite [A] and PSO [B] with FTIR predicted values using 1st derivative ATR-FTIR spectra at wavenumbers of 1500-1000 cm^{-1} .

Table 3. The statistical performances of ATR-FTIR spectra at certain wavenumbers in combination with multivariate calibrations for calibration and validation modelling of simultaneous analysis of milkfish oil (MFO) treated with bentonite and pumpkin seed oil (PSO).

Wavenumbers (cm ⁻¹)	Multivariate calibration	Spectra	Calibration		Prediction	
			RMSEC	R ²	RMSEP	R ²
1500-1000	PLS	Normal	0.0853	0.9576	0.0860	0.9569
		1st Derivative	0.1010	0.9402	0.1030	0.9381
		2nd Derivative	0.0932	0.9491	0.0928	0.9496
	PCR	Normal	0.0625	0.9774	0.1250	0.9274
		1st Derivative	0.0617	0.9780	0.0859	0.9573
		2nd Derivative	0.0478	0.9869	0.0873	0.9570
3000-700	PLS	Normal	0.1030	0.9381	0.1050	0.9355
		1st Derivative	0.0955	0.9465	0.0975	0.9442
		2nd Derivative	0.0956	0.9464	0.0951	0.9481
	PCR	Normal	0.0658	0.9749	0.0978	0.9462
		1st Derivative	0.0582	0.9805	0.0923	0.9580
		2nd Derivative	0.0444	0.9887	0.0848	0.9602
1500-800	PLS	Normal	0.0977	0.9439	0.0975	0.9443
		1st Derivative	0.1010	0.9401	0.1030	0.9379
		2nd Derivative	0.0930	0.9493	0.0929	0.9496
	PCR	Normal	0.0532	0.9837	0.0828	0.9636
		1st Derivative	0.0637	0.9766	0.0799	0.9637
		2nd Derivative	0.0116	0.9992	0.0987	0.9554
1500-700	PLS	Normal	0.1040	0.9356	0.1040	0.9357
		1st Derivative	0.1010	0.9399	0.1020	0.9384
		2nd Derivative	0.0926	0.9498	0.0919	0.9506
	PCR	Normal	0.0329	0.9938	0.0887	0.9595
		1st Derivative	0.0432	0.9893	0.1020	0.9462
		2nd Derivative	0.0187	0.998	0.0955	0.9536
3000-1000	PLS	Normal	0.0943	0.9479	0.0974	0.9443
		1st Derivative	0.0954	0.9466	0.0976	0.9442
		2nd Derivative	0.0957	0.9462	0.0953	0.9479
	PCR	Normal	0.0458	0.9880	0.1040	0.9573
		1st Derivative	0.0617	0.9780	0.0885	0.9597
		2nd Derivative	0.0571	0.9812	0.0801	0.9635

Note: The selected condition for quantitative analysis is in bold.

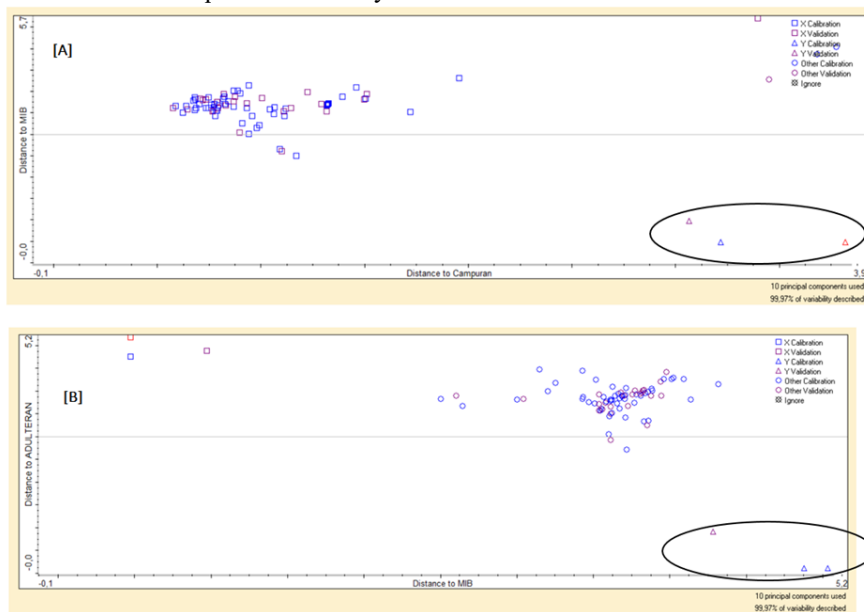


Figure 4. Linear Discrimination Analysis (LDA) for the discrimination between authentic MFO-treated with activated charcoal [A] and bentonite [B] and MFO adulterated with PSO using the absorbance values of ATR-FTIR spectra at the whole IR region (4000-650 cm⁻¹).

different values of LDF. Therefore, LDF provides a means of discriminating between the two groups (the authentic MFO and MFO adulterated with PSO). Based on Coomans plot (Figure 4), the authentic MFO and MFO mixed with PSO could be clearly separated with accuracy levels of 100%, meaning that LDA is successful to be applied for the classification of authentic and adulterated groups of MFO.

4. Conclusion

The employment of ATR-FTIR spectra and chemometrics of multivariate calibrations (PLSR and PCR) is successful to be applied for simultaneous quantitative analysis of MFO treated with activated charcoal and bentonite with acceptable accuracy and precision as indicated by high R^2 and low values of RMSEC and RMSEP. Linear discriminant Analysis using normal FTIR spectra at the whole wavenumbers region was successful for the discrimination of MFO treated with activated charcoal and bentonite with accuracy levels of 100%. The developed method is fast and reliable with minimum sample preparation and minimum use of solvents and reagents; therefore, this method can be considered a green analytical technique.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors acknowledge Universitas Gadjah Mada for providing the facilities needed to perform this study.

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