



# The employment of FTIR spectroscopy and chemometrics for the classification and prediction of antioxidant activities of pumpkin seed oils from different origins

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## ABSTRACT

Vegetable oils, including pumpkin seed oil (PSO), contained triacylglycerols and some minor components including phenolics and tocopherols which are believed to contribute to antioxidant activities. The objective of this research was (1) to determine the antioxidant activities of PSOs from different origins using 2,2'-diphenyl-1-picrylhydrazil (DPPH) radical scavenging, (2) to classify PSOs from different origins using principal component analysis (PCA) and cluster analysis (CA), and (3) to predict the antiradical activities of PSO based on Fourier transform infrared (FTIR) spectra using multivariate calibration of partial least square regression (PLSR). The antioxidant activities of PSOs were evaluated based on the capability of PSOs to reduce the absorbance values of DPPH. Classification of PSOs from different origins was evaluated using PCA and CA using variables of absorbance values of FTIR spectra, while prediction of antiradical activities using FTIR spectra was facilitated by PLSR. The results showed that radical scavenging activity (RSA) of PSO from Gunung Kidul and Tawangmangu had the highest RSA accounting for 98.71%  $\pm$  0.02% and 83.57%  $\pm$  0.13%, respectively. PCA could classify PSO from different origins using the variable of FTIR spectra absorbances based on PC1 and PC2 score plots. CA using the same variables could group PSO samples into five groups based on the dendrogram obtained. PLSR using the first derivative FTIR spectra at 3,500–650  $\text{cm}^{-1}$  could predict RSA of PSO with  $R^2$  values of 0.9996 and 0.9418 in calibration and validation models, while the root mean square error of calibration and the root mean square error of prediction were of 0.425% and 4.93%, respectively. It can be concluded that FTIR spectra combined with chemometrics are effective means for the classification of PSO from different origins and for the prediction of antioxidant activities of PSO with accurate and precise results.

## INTRODUCTION

Currently, edible vegetable oils are taken into account as major economic resources in certain nations. Vegetable oils are typically applied in some industries such as food, pharmaceuticals, cosmetics, and oleochemicals. In view of the nutritional aspects,

vegetable oils also contain some bioactive compounds like essential fatty acids, fat-soluble vitamins, and phenolic compounds needed in human metabolism and energy sources (Zhang *et al.*, 2012). Pumpkin seed oil (PSO), extracted from pumpkin (*Cucurbita maxima*) seeds, has been known as a functional oil due to its active components with certain biological activities (Rohman and Irnawati, 2020). PSO is reported to contain phenolic compounds, tocopherols, and carotenoids. These compounds are well known as a source of natural antioxidants (Al-Farsi *et al.*, 2005). Several researches have reported the antioxidant activity of PSO using *in vitro* methods including 2,2'-diphenyl-1-picrylhydrazil (DPPH) radical scavenging assay and  $\beta$ -carotene/linoleic acid bleaching test and correlated these antioxidant activities with phenolics compounds (Jiao *et al.*, 2014; Kulaitienė *et al.*, 2018; Rezig *et al.*,

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2018; Siano *et al.*, 2016). However, the determination of antioxidant activities using physicochemical tests involved reagents and several analytical steps; as a consequence, some fast techniques like the Fourier transform infrared (FTIR) spectroscopy method are continuously developed for measurement antioxidant activities.

Antioxidants are typically defined as any substance or any compound either synthetic or natural having the capability to significantly delay or prevent the oxidation reactions of the substrate when present at low levels compared with those of an oxidizable substrate (Rohman *et al.*, 2020a). Due to some restrictions regarding the use of synthetic antioxidants, the natural antioxidants coming from plants are explored and developed, and among these are antioxidants from vegetable oils (Widodo *et al.*, 2019). Some evaluations on antioxidant activities *in vitro* have existed in the scientific literature including 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay, Trolox equivalent antioxidant capacity method known as 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation decolorization assay, lipid peroxidation inhibition assay, beta-carotene bleaching method, metal chelating assay Ferric thiocyanate method, and cupric ion reducing antioxidant capacity method (Alam *et al.*, 2013). DPPH radical scavenging assay is the most popular method applied for screening the antioxidant activities of edible fats and oils (Marina *et al.*, 2009).

With the development of chemometrics or multivariate data analysis, some chemists tried to correlate the antioxidant with spectroscopic profiles for specific reasons including classification of edible oils or prediction of antioxidant activities using absorbance values of certain spectroscopic methods. Rohman *et al.* (2020b) have conducted a review on the application of FTIR spectra combined with chemometrics for authentication analysis of fats and oils. However, the application of FTIR spectroscopy combined with chemometrics for the prediction of antioxidant activities is very limited. Casoni *et al.* (2019) have successfully classified edible vegetable oils based on their UV-Vis spectra and the profiles of DPPH radical scavenging combined with chemometrics of pattern recognition of principal component analysis (PCA) and fuzzy-PCA. The combination of proton (1H)-NMR and proton (31P)-NMR spectroscopy and chemometrics of discriminant analysis were successful for the classification of 13 types of edible vegetable oils (Vigli *et al.*, 2003). The other spectroscopic techniques combined with chemometrics applied in the classification of oils in different states are FTIR spectroscopy (Lu and Rasco, 2012; Valasi *et al.*, 2020) and fluorescence spectroscopy (Cao *et al.*, 2017). However, using literature investigation, there is no reported publication on the application of FTIR spectra for the classification of PSO. Therefore, the objectives of this study were (1) to classify PSO from different origins based on FTIR spectra and chemometrics of PCA and cluster analysis (CA) and (2) to predict DPPH radical scavenging activities based on FTIR spectra combined with the multivariate calibration of partial least square.

## MATERIALS AND METHODS

### Materials

Pumpkin seeds were obtained from several locations around Yogyakarta and Central Java, Indonesia. The pumpkin fruit was authenticated in the Laboratory of Pharmacognosy, Department of Pharmaceutical Biology, Faculty of Pharmacy,

Universitas Gadjah Mada, under the supervision of Dr. Djoko Santosa. DPPH was purchased from Sigma (Aldrich, St. Louis, MO). The other reagents and solvents used for analysis were of analytical grade obtained from E. Merck (Darmstadt, Germany).

### Microwave pretreatment

Microwave pretreatment of pumpkin seeds was carried out according to the method described by Azadmard-Damirchi *et al.* (2010). Pumpkin seed samples (100 g) were placed in an even layer in Pyrex Petri dishes inside the microwave (Hitachi, model MR-5750). Samples were microwave treated at a power of 50% for 240 seconds.

### Oil extraction by pressing

PSO was extracted from the microwave-treated pumpkin seed samples by mechanical hot pressing (Maksindo MKS-J03) according to the method described by Dang and Bui (2019). The oil was later separated from the sediment by centrifugation at 2,500 rpm (Thermo Scientific) for 10 minutes and stored in a freezer at  $-20^{\circ}\text{C}$  for subsequent analysis.

### DPPH radical scavenging activity (RSA) assay

DPPH radical scavenging assay was carried out according to Casoni *et al.* (2019). PSO samples were diluted with ethanol in a 10 ml volumetric flask. For each of the analyzed PSO samples, total radical scavenging capacity was determined by measuring the absorbance of DPPH solution 0.4 mM at 515 nm (Abs control) and the absorbance of DPPH solution added with the evaluated PSO samples after a reaction time of 30 minutes (Abs sample). All absorbance measurements were corrected with the absorbance of blank containing solvent and the studied PSO samples. For the calculation of RSA percentage (%), the following formula was used:

Percentage (%) of DPPH radical scavenging activity =

$$\frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100\%$$

### FTIR spectra measurement

The FTIR spectra of studied PSO samples were carried out using the conditions as previously reported by Irnawati *et al.* (2020). The spectra were scanned using an FTIR spectrophotometer (Thermo Scientific Nicolet iS10, Madison, WI), controlled with the operating Omnic software. The measurements were carried out in a mid-infrared region of  $4,000\text{--}650\text{ cm}^{-1}$  with a scanning number of 32 and a resolution of  $8\text{ cm}^{-1}$ . The used sampling accessory was horizontal attenuated total reflectance composed of ZnSe crystal. All FTIR spectra were corrected against the FTIR spectrum of air as background. After every scan, a new reference air background spectrum was taken. These spectra recorded as absorbance values at each data point in triplicate were used for making the correlation between antiradical activity and FTIR spectra.

### Chemometrics analysis

The absorbance values of FTIR spectra were used as variables during the classification of PSO samples from different regions with the aid of chemometrics of PCA and CA. Furthermore, the prediction of DPPH RSA using a variable of absorbance values

of FTIR spectra was assisted with the multivariate calibration of partial least square. All chemometric analyses [PCA, CA, and partial least square (PLS)] were carried out using Minitab® version 17 (Minitab Inc., State College, PA).

## RESULTS AND DISCUSSION

In this study, the antioxidant activities of PSO from different regions around Yogyakarta and Central Java, Indonesia, were evaluated by DPPH radical scavenging activities. This technique was based on the capability of chemical compounds present in PSO to reduce DPPH radicals into nonradicals (Rohman *et al.*, 2006; Widyastuti *et al.*, 2021). Table 1 shows the percentage

**Table 1.** Radical scavenging activities of pumpkin seed oils (PSOs) from different origins at concentration levels of 100 µl/5 ml.

Origins	Radical scavenging activity (%)
Blora	44.20 ± 0.07
Boyolali	40.88 ± 0.26
Cepu	50.67 ± 0.75
Godean	42.82 ± 0.22
Gunung Kidul	98.71 ± 0.02
Gondomanan	48.04 ± 0.82
Grabag	73.70 ± 0.27
Kopeng	58.95 ± 0.07
Kudus	53.04 ± 0.22
Muntilan	64.77 ± 0.20
Palagan	61.23 ± 0.33
Parakan	54.51 ± 0.27
Pati	60.67 ± 0.00
Purwodadi	49.20 ± 0.86
Tawangmangu	83.57 ± 0.13
Weleri	67.62 ± 0.20

of DPPH radical scavenging activities of PSO with a concentration of PSOs of 20 µl/ml. The RSA of PSOs from Gunung Kidul and Tawangmangu had the highest RSA among others, accounting for 98.71% ± 0.02% and 83.57% ± 0.13%, respectively.

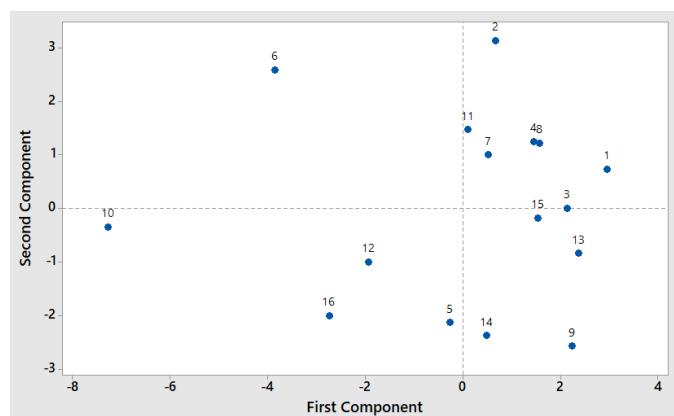
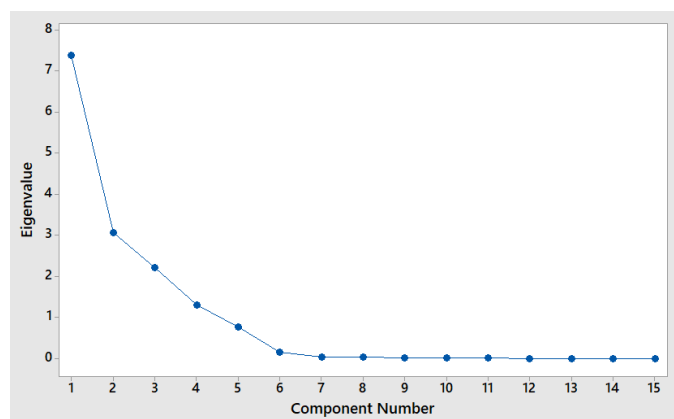
In order to classify PSOs from different origins, two chemometric classifications, namely, PCA and CA, were used. PCA and CA are unsupervised pattern recognition in chemometrics analysis (Che Man *et al.*, 2011). PCA is a feature for the reduction of the amount of data when there is a correlation present (Miller and Miller, 2010). In this study, PCA was accomplished using FTIR-ATR spectra absorbance of 16 PSO samples at 14 wavenumber and radical scavenging activities, as shown in Table 2. Each peak or shoulder is associated with structural or functional group information in PSO (Saucedo-Hernández *et al.*, 2011). The functional group and vibration mode for the absorption of peak and shoulder in PSO are presented in Table 3. The functional groups represented in FTIR spectra are correlating with chemical compounds present in PSO. Edible fats and oils including PSO are mainly composed of triacylglycerols (TAG) in approximately 95%–98%; as a consequence, the functional groups present in TAG dominate (Rohman *et al.*, 2020c). The functional groups of methyl, methylene, ether, carbonyl, and backbone of fatty acids are represented in FTIR spectra. In addition, the functional groups which are representative for the minor components like functional group of –OH phenolics disappear due to the low levels; therefore, they are not detected in FTIR spectra. Figure 1 shows the score plot of PCA of 16 PSOs from different origins representing the projection of samples defined by the first principal component (PC1) and the second principal component (PC2). An eigenvalue of about 99.3% was achieved using six PCs (Fig. 2). The variance (69.7%) is being described by PC1 and PC2. PC1 accounted for 49.2% of the variation, while PC2 described 20.4% of the variation. Figure 3 shows the loading plot for the determination of wavenumbers contributing to the separation of the PSO from different origins. Based on the loading plot, the wavenumbers at

**Table 2.** Peak absorbances for each wavenumbers and radical scavenging activity of PSO from different origins.

No.	Origins	Absorbance values at different wavenumbers (cm <sup>-1</sup> )													
		721	850	996	1,118	1,160	1,237	1,376	1,460	1,654	1,744	2,853	2,922	2,953	3,007
1	Blora	0.0993	0.0335	0.0405	0.0999	0.1651	0.0762	0.0471	0.0800	0.0104	0.2540	0.1659	0.2387	0.0783	0.0246
2	Boyolali	0.0975	0.0293	0.0386	0.0978	0.1622	0.0739	0.0459	0.0783	0.0135	0.2493	0.1609	0.2319	0.0770	0.0249
3	Gondomanan	0.1001	0.0334	0.0407	0.0985	0.1646	0.0759	0.0469	0.0788	0.0100	0.2541	0.1623	0.2339	0.0784	0.0256
4	Cepu	0.0975	0.0334	0.0407	0.0988	0.1625	0.0759	0.0465	0.0789	0.0100	0.2504	0.1625	0.2343	0.0775	0.0246
5	Godean	0.0987	0.0349	0.0491	0.1150	0.1641	0.0740	0.0456	0.0759	0.0117	0.2420	0.1538	0.2225	0.0764	0.0261
6	Gunung Kidul	0.0969	0.0312	0.0402	0.0974	0.1589	0.0721	0.0443	0.0753	0.0083	0.2429	0.1523	0.2183	0.0734	0.0241
7	Kopeng	0.0979	0.0335	0.0413	0.0994	0.1623	0.0750	0.0464	0.0786	0.0093	0.2470	0.1608	0.2307	0.0766	0.0245
8	Muntilan	0.0984	0.0333	0.0408	0.0982	0.1636	0.0753	0.0466	0.0793	0.0089	0.2517	0.1649	0.2372	0.0776	0.0244
9	Palagan	0.1030	0.0350	0.0432	0.0976	0.1660	0.0766	0.0474	0.0788	0.0098	0.2550	0.1590	0.2290	0.0783	0.0268
10	Parakan	0.1008	0.0342	0.0414	0.0930	0.1550	0.0716	0.0439	0.0726	0.0093	0.2315	0.1372	0.1946	0.0674	0.0250
11	Pati	0.0975	0.0334	0.0417	0.0994	0.1626	0.0747	0.0457	0.0777	0.0088	0.2488	0.1602	0.2305	0.0763	0.0237
12	Weleri	0.1020	0.0347	0.0424	0.0956	0.1620	0.0742	0.0460	0.0764	0.0094	0.2450	0.1510	0.2150	0.0723	0.0247
13	Kudus	0.1010	0.0347	0.0427	0.0987	0.1650	0.0761	0.0473	0.0796	0.0100	0.2540	0.1620	0.2330	0.0778	0.0251
14	Grabag	0.1030	0.0349	0.0426	0.0965	0.1650	0.0756	0.0469	0.0777	0.0098	0.2510	0.1550	0.2230	0.0760	0.0266
15	Purwodadi	0.1000	0.0348	0.0429	0.0986	0.1640	0.0756	0.0470	0.0791	0.0096	0.2510	0.1620	0.2310	0.0762	0.0242
16	Tawangmangu	0.1031	0.0336	0.0412	0.0944	0.1615	0.0748	0.0459	0.0748	0.0095	0.2439	0.1451	0.2088	0.0734	0.0268

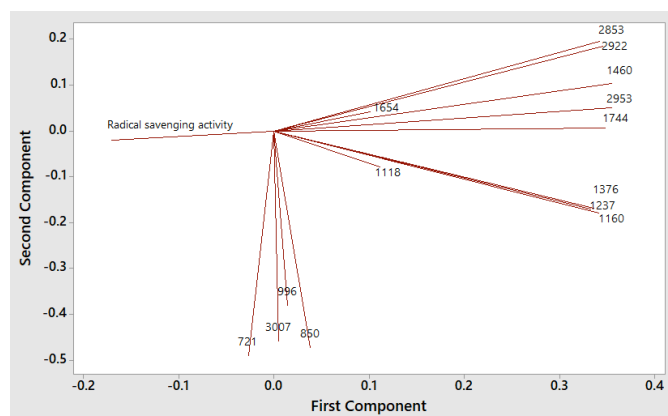
**Table 3.** Functional groups responsible for absorption of peaks and shoulders in PSO.

Wavenumber region (cm <sup>-1</sup> )	Functional groups	Vibration modes
3,007	<i>cis</i> -C=CH	Stretching vibration
2,953	Methylene (-CH <sub>2</sub> )	Asymmetric stretching vibration
2,922 and 2,852	Methylene (-CH <sub>2</sub> )	Asymmetric and symmetric Stretching vibrations
1,744	Carbonyl (C=O)	Stretching vibration
1,654	C=C	Stretching vibration
1,460	Methylene (-CH <sub>2</sub> )	Bending vibration (scissoring)
1,376	Methyl (-CH <sub>3</sub> )	Symmetric bending vibration
1,234, 1,160	Methylene (-CH <sub>2</sub> )	Bending vibration
1,118, 1,097	C-O	Stretching vibrations
996	-HC=CH-( <i>trans</i> )	Bending out of plane
850	=CH <sub>2</sub>	Wagging
721	-(CH <sub>2</sub> ) <sub>n</sub>	Rocking

Adapted from Saucedo-Herna *et al.* (2011).**Figure 1.** The PCA score plot of pumpkin seed oils (PSOs) from different origins: (1) Blora; (2) Boyolali; (3) Gondomanan; (4) Ceppu; (5) Godean; (6) Gunung Kidul; (7) Kopeng; (8) Muntilan; (9) Palagan; (10) Parakan; (11) Pati; (12) Weleri; (13) Kudus; (14) Grabag; (15) Purwodadi; (16) Tawangmangu.**Figure 2.** Eigenvalue of PCA of PSO from different origins.

2,953, 1,460, and 1,160 cm<sup>-1</sup> made a large contribution to the PCA model.

CA is a hierarchical method in which successive partition of the data set results in a cluster represented as a

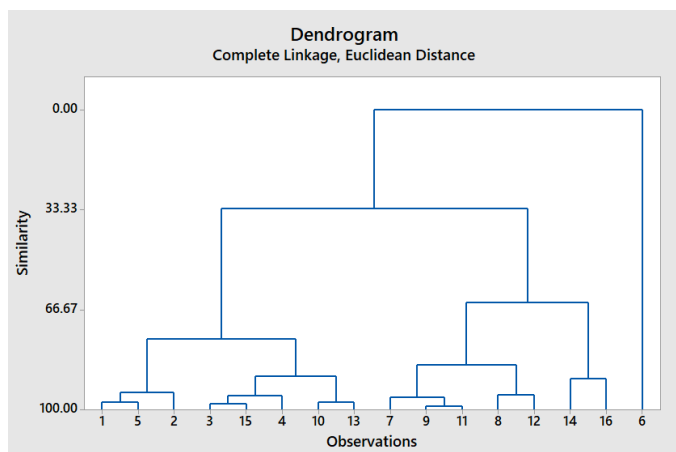
**Figure 3.** The loading plot of PCA models of PSO from different origins.

dendrogram or tree (Djuris *et al.*, 2013). In addition, CA can provide numerical values of similarity among the objects evaluated and therefore obtained more objective information (Che Man *et al.*, 2011). We divided a group of PSOs from different origins into classes so that similar characteristics of PSO are in the same class. The classification stages of PSO can be seen in Table 4. It can be stated that the first joined are PSOs from Palagan and Pati, followed by PSOs from Gondomanan and Purwodadi with distance levels being 0.5607 and 0.1643, respectively, and so on until all PSOs from different origins are grouped into one class. Figure 4 showed a dendrogram using the single linkage method. This analysis suggests that the evaluated PSOs from different origins fall into five groups. Group 1 contained PSO from Palagan, Pati, Kopeng, Muntilan, and Weleri; PSOs from Gondomanan, Purwodadi, Ceppu, Parakan, and Kudus formed group 2; PSOs from Blora, Godean, and Boyolali formed group 3; PSOs from Grabag and Tawangmangu formed group 4. Meanwhile, PSO from Gunung Kidul formed a separate group (group 5).

The multivariate calibration of PLSR was used for the prediction of antioxidant activities through DPPH radical scavenging activities using absorbance values of FTIR spectra as variables during modeling. The variation in FTIR spectra of

**Table 4.** The classification stages of PSO from different origins.

Step	Number of clusters	Similarity level	Distance level	Clusters joined	New cluster	Number of obs. in new cluster
1	15	99.0304	0.5607	9	11	9
2	14	97.9866	1.1643	3	15	3
3	13	97.6132	1.3802	1	5	1
4	12	97.4629	1.4671	10	13	10
5	11	96.0477	2.2855	7	9	7
6	10	95.4511	2.6304	3	4	3
7	9	95.0781	2.8462	8	12	8
8	8	94.2580	3.3204	1	2	1
9	7	89.7091	5.9509	14	16	14
10	6	88.8139	6.4685	3	10	3
11	5	85.0111	8.6676	7	8	7
12	4	76.4353	13.6267	1	3	1
13	3	64.2058	20.6986	7	14	7
14	2	32.9605	38.7667	1	7	1
15	1	0.0000	57.8267	1	6	1

**Figure 4.** The dendrogram of PSOs from different origins: (1) Blori; (2) Boyolali; (3) Gondomanan; (4) Ceppu; (5) Godean; (6) Gunung Kidul; (7) Kopeng; (8) Muntilan; (9) Palagan; (10) Parakan; (11) Pati; (12) Weleri; (13) Kudus; (14) Grabag; (15) Purwodadi; (16) Tawangmangu.

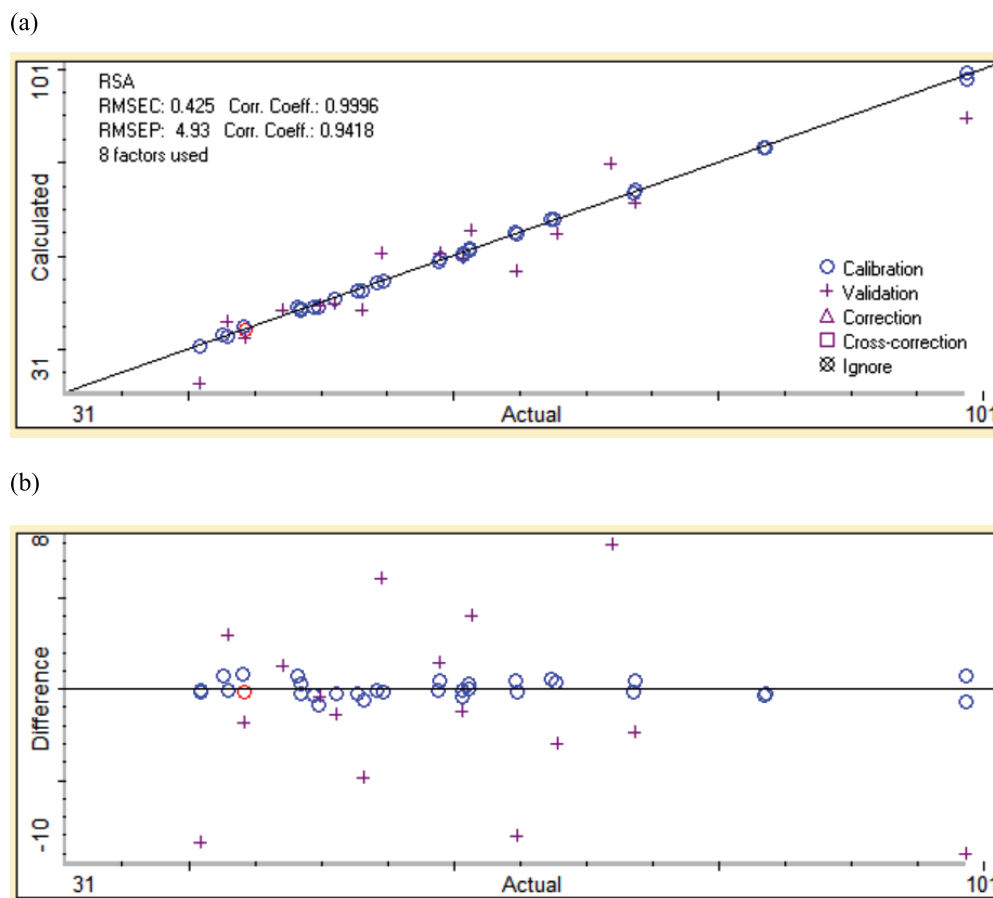
PSO was used to build a calibration model for the prediction of radical scavenging activities in PSO. In this study, to obtain the best prediction model, the wavenumber regions (3,900–650, 3,500–650, 1,700–650, 1,130–700, and combined wavenumbers of 3,050–2,800 and 1,790–650  $\text{cm}^{-1}$ ) and FTIR spectral treatment (normal, first derivative, and second derivative) were optimized. Spectra derivatization is able to produce better resolution (Windarsih *et al.*, 2020). Table 5 shows the optimization of the calibration model using the PLS calibration model. The selection of optimum conditions was based on the highest coefficient correlation and the lowest root mean square error of calibration (RMSEC) and the root mean square error of prediction (RMSEP) (Irnawati *et al.*, 2019). Finally, the first derivative FTIR spectra at wavenumber region at 3,500–650  $\text{cm}^{-1}$  were selected for the prediction of RSA in PSO with  $R^2$  value of 0.9996 and 0.9418 in calibration and validation models, while RMSEC and RMSEP were 0.425% and 4.93 %, respectively (Fig. 5a). Figure

**Table 5.** Multivariate calibration of PLSmodel for the prediction of radical scavenging activity in PSO.

Multivariate calibrations	Wavenumber ( $\text{cm}^{-1}$ )	Spectra	Calibration		Validation	
			$R^2$	RMSEC	$R^2$	RMSEP
PLS	3,900–650	normal	0.6316	11.700	0.6455	11.000
		1st der	0.9994	0.536	0.9403	4.900
		2nd der	0.9956	1.410	0.8985	6.260
	3,500–650	normal	0.6286	11.800	0.6429	11.100
		1st der	0.9996	0.425	0.9418	4.930
		2nd der	0.9945	1.590	0.9165	5.690
	1,700–650	normal	0.5060	13.000	0.5643	11.700
		1st der	0.9813	2.910	0.9173	5.730
		2nd der	0.5382	12.700	0.4284	13.000
	1,130–700	normal	0.3558	14.100	0.4240	12.900
		1st der	0.6700	11.200	0.6287	11.100
		2nd der	0.5179	12.900	0.4028	13.200
	3,050–2,800 and 1,790–650	normal	0.6316	11.700	0.6455	11.000
		1st der	0.9994	0.536	0.9403	4.900
		2nd der	0.9956	1.410	0.8985	6.260

5b shows the residual analysis between actual and predicted value. It is clear that all point differences are located above and below zero value; therefore, the systematic error during PLS modeling between actual values of antioxidant activities and FTIR predicted values could be negligible. The capability of FTIR spectroscopy combined with chemometrics to predict the antioxidant activities is not surprising because FTIR spectra are fingerprints in nature so that the analyst can select the peaks in FTIR spectra which correlated with the antioxidant activities to be predicted with antioxidant activities. Because of its benefits, in the future, it is hoped that FTIR spectra combined with chemometrics techniques of multivariate calibration can be used as an alternative method for determining the antioxidant activity of PSO *in vitro*.





**Figure 5.** PLS calibration model (a) and residual factor (b) for prediction of radical scavenging activity in PSO. The x-axis showed the actual radical scavenging activity in PSO; the y-axis showed the calculated radical scavenging activity of PSO.

## CONCLUSION

FTIR spectra combined with chemometrics techniques are effective means for the classification of PSO from different origins and for the prediction of antioxidant activities. PCA and CA were successfully applied for the classification of PSOs from different origins as indicated by clear separation among the evaluated samples. In addition, PLS regression was also accurate and precise for predicting the antioxidant activities as indicated by a high coefficient of determination ( $R^2$ ) and low errors. The developed method is considered a green analytical technique due to the use of less solvent and with minimum sample preparation; therefore, the method could be considered an alternative method for determining antioxidant activities.

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of

data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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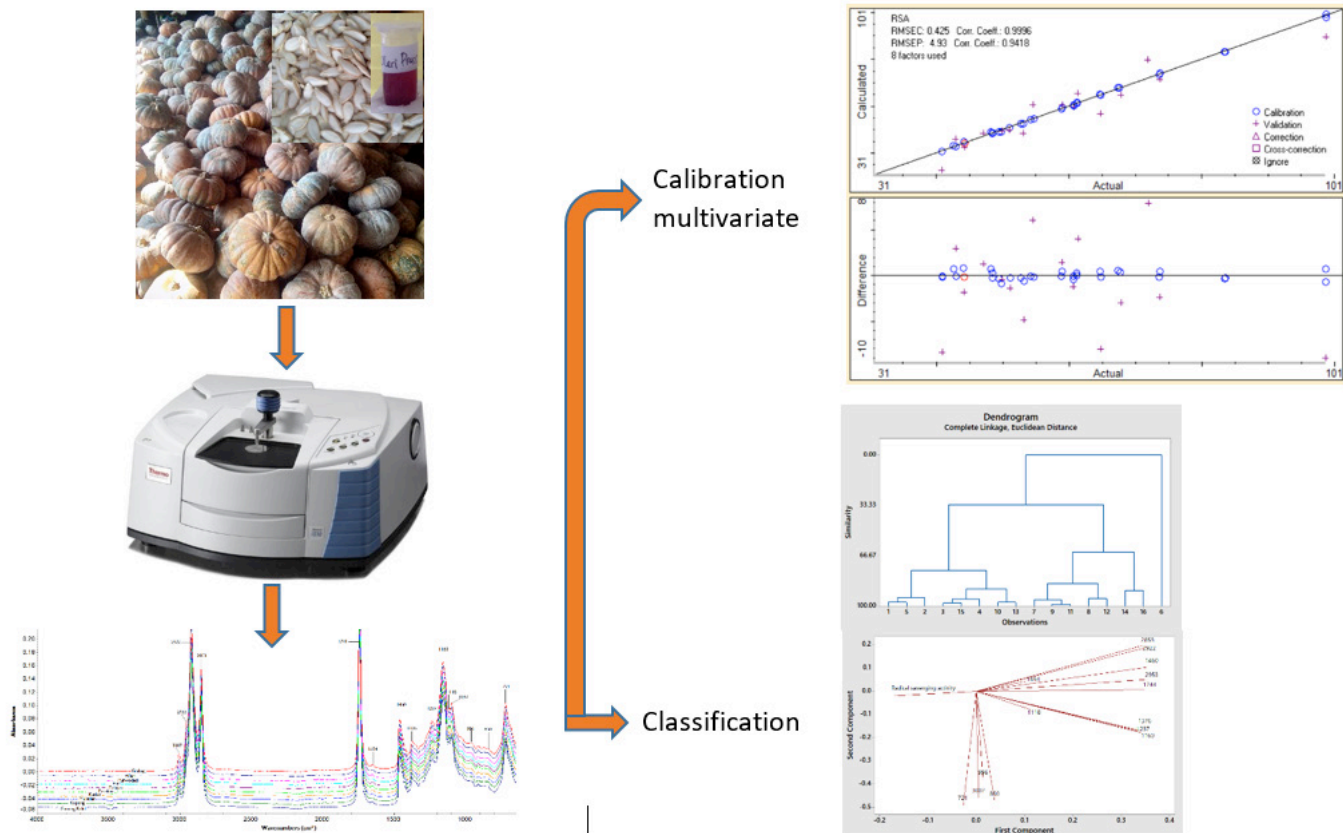
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## GRAPHICAL ABSTRACT



## SUMMARY

1. FTIR spectra combined with chemometrics techniques are effective means for the classification of PSO from different origins and for the prediction of antioxidant activities.
2. PCA and CA were successfully applied for the classification of PSOs from different origins.
3. PLSR was also accurate and precise for predicting the antioxidant activities.