Food Research 8 (1) : 166 - 173 (February 2024)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

METHOD DEVELOPMENT

Development of FTIR Spectroscopy and multivariate calibration for authentication beef meatballs from pork meat

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Article history:
Received: 10 February 2022
Received in revised form: 30 March 2022
Accepted: 3 October 2022
Available Online: 28 January 2024

Keywords:
Pork,
Adulteration,
Halal authentication,
PLSR,
Discriminant analysis

DOI:
https://doi.org/10.26656/fr.2017.8(1).092

Abstract

In several countries, especially Indonesia, meatballs are one of the favorite foods made from meat. The high price of beef is a reason for adulterated beef meat with pork to gain a significant profit. All food products must not contain pig derivatives such as pork in Muslim societies. This study aimed to develop Fourier Transform Infrared (FTIR) spectroscopy in combination with linear discriminant analysis (LDA) for classifying pork in beef meatballs and multivariate calibration for qualitative and quantitative analysis methods using partial least square regression (PLSR) and principal component regression (PCR). The lipid component from meatballs containing pork and beef in different concentrations was obtained by employing three lipid extraction methods, namely Bligh-Dyer, Folch, and Soxhlet methods, and subjected to attenuated total reflectance (ATR) spectral measured at wavenumbers of 4000-650 cm^{-1}. LDA at 3800-800 cm^{-1} was able to differentiate between beef meatballs and meatballs containing pork meat (PM). Additionally, PLSR using second derivative FTIR spectra at wavenumbers of 3100-900 cm^{-1} delivered great region for predicting pork levels in beef meatballs extracted using Folch methods with coefficient determination (R^2) values was > 0.99 and 0.02% for root mean square error of calibration (RSMEC) and root mean square error of prediction (RSMEP) values, respectively. PCR using absorbance values of 1st derivative FTIR spectra at wavenumbers 1400-800 cm^{-1} for Bligh-Dyer method and 3800-800 cm^{-1} for Soxhlet was offered the R^2 value was> 0.99 with low RSMEC and RSMEP values for prediction of pork fat in beef meatballs, respectively. It can be concluded that FTIR spectroscopy on the Savitzy-Golay derivatization method in combination with chemometrics was an accurate and quick approach for authenticating pork adulteration in beef meatballs.

1. Introduction

The research on halal products developed along with increasing awareness of Muslim people about what they are eating (Wahyudi, 2020). Meatballs are one of the favorite food products with the main ingredient of meat such as beef meat which is added seasonings to produce meatballs with royal taste. The high price of beef is the trigger for unethical producers to replace or adulterate beef with cheaper meat such as pork to get great profits (Yang *et al*., 2018). Authentication of beef meatballs from pork contaminants is very important to guarantee food products do not contain non-halal components and also to halal product certificated regulations. In some countries such as Indonesia, Malaysia, and the Middle East countries, halal-certified is a must, and it will increase the marketability of products (Peristiwo, 2019).

Fat components from beef and pork have the same content. The difference in both of them is just in the small composition. Many methods have been developed to detect, validate, qualitatively, and quantitatively analyze the presence of a non-halal component in food products, especially meatballs, through physicochemical and molecular biology approaches (Martuscelli *et al*., 2020). DNA-based methods using polymerase chain reaction are methods that are rapid in detecting DNA components in food products. Several studies such as simultaneous detection of porcine DNA in pharmaceutical gelatine capsules by duplex (Nikzad *et*

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

al., 2017), quantitative detection of pork meat by EvaGreen real-time PCR (Amaral *et al*., 2016), and DNA determination of bovine, porcine, and fish in gelatine mixture, food product and dietary supplement using TaqMan probe-based multiplex (Sultana *et al*., 2020), have been reported shown a capability for authentication beef from pork adulterations. Protein-based methods are also used for the identification of pork adulteration such as Fourier Transform Infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), electronic nose, and chromatography (Rohman *et al*., 2020).

Fourier Transform Infrared (FTIR) spectroscopy is a fast, reliable, and simple method that can be tested directly without complicated sample preparation. FTIR is widely used for authentication, and qualitative and quantitative analysis in several fields, including pharmaceutical, food, and biological fluid due to its ability to display absorption in the specific fingerprint area (Rohman, 2013; Rohman *et al*., 2020). Currently, FTIR spectroscopy is combined with chemometrics for food product authentication. Chemometrics is the mathematics and statistics applied to process chemical information infrared spectra. Principle Component Analysis (PCA), Discriminant Analysis, Partial Least Square Regression (PLSR), and Principal Component Regression (PCR) The types of chemometrics are the type of chemometrics methods used in analysis using FTIR spectroscopy (Pebriana *et al*., 2017; Ahda *et al*., 2020; Irnawati *et al*., 2021). In this study, three lipid extraction methods, namely Bligh-Dyer, Folch, and Soxhlet, were used. The different fat extraction methods produced different total fat even if applied to the same sample. This difference can be observed from FTIR spectra combined with multivariate calibration for authentication of beef meatballs from pork as adulteration.

2. Materials and methods

2.1 Materials

The beef was obtained from the supermarket around Padang, West Sumatera, and the pork was obtained from local people in Padang, West Sumatera. All samples were stored in a refrigerator (-4^{o}C) before manufacturing a meatball. All solvents used for extracting lipids and analysis were of pro-analytical grade.

2.2 Preparation of meatballs

Meatballs were prepared according to Rohman *et al*. (2011). A total of 90% of the delicate ground meat (beef and or pork) was emulsified with 10% starch, salt, onion, and pepper powder, and then shaped into a small ball. It is cooked in boiling water for 20 mins. Before being subjected to lipid extraction, meatballs were ground using a chopper and extracted with three lipid extraction methods.

2.3 Preparation of calibration and validation samples

Meatballs were also prepared for calibration and validation by mixing pork and beef meat in varying concentrations at 10%, 20%, 30%, 40%, 50%, and 75%. Meatballs containing 100% beef and 100% pork were also prepared to observe the differentiation of spectra. Lipid was obtained separately from three lipid extraction methods used for FTIR spectral measurement.

2.4 Lipid extraction by Bligh-Dyer method

Lipid form meatballs were extracted using Bligh-Dyer method (Pebriana *et al*., 2017). A 20.0 g of ground meatball sample was mixed with 60 mL chloroform: methanol (1:2 v/v) in Erlenmeyer, stirred at 60^{o}C for 30 mins. The mixture was filtered with filter paper. The filtrate was mixed with 25 mL of distilled water in a separating funnel and shaken vigorously. The lower phase is a chloroform phase. It was separated and dried using anhydrous Na_2SO_4. After being filtered using filter paper, the chloroform was evaporated using a vacuum rotary evaporator (Rotavapor R-210 Buchi) at 40^{o}C until the solvent was completely removed. The lipid extract was transferred into a vial and stored in a refrigerator (-4^{o}C) before being subjected to scanning FTIR spectra.

2.5 Lipid extraction by Folch method

Lipids from meatballs were extracted using Folch methods (Rahayu *et al*., 2018). A 20.0 g ground meatball sample was mixed with 400 mL of chloroform: methanol (2:1 v/v) in Erlenmeyer and stirred for 30 mins. The mixture was filtered with filter paper. Then, the filtrate was mixed with 25 mL of distilled water in a separating funnel and shaken vigorously. The lower phase is a chloroform phase. It was separated and dried using anhydrous Na_2SO_4. After being filtered using filter paper, the chloroform was evaporated using a vacuum rotary evaporator (Rotavapor R-210 Buchi) at 40^{o}C until the solvent was completely removed. The lipid extract was transferred into a vial and stored in a refrigerator (-4^{o}C) before being subjected to scanning FTIR spectra.

2.6 Lipid extraction by Soxhlet method

Lipids from meatballs were extracted using Soxhlet methods (Rahmania *et al*., 2015). A 50.0 g of ground meatball sample was wrapped using filter paper and placed into the Soxhlet apparatus. A 437.5 mL of hexane was used as an extracting solvent. The extraction was run for 8 hrs at 100^{o}C (50 cycles). The hexane was dried using anhydrous Na_2SO_4. After being filtered using filter

https://doi.org/10.26656/fr.2017.8(1).092

paper, the hexane evaporated using a vacuum rotary evaporator (Rotavapor R-210 Buchi) at 60°C, and the fat obtained from the extraction was subjected to FTIR spectral measurement.

2.7 FTIR spectral measurement

All lipids obtained from three lipid extraction methods were subjected to attenuated total reflectance (ATR) spectral measured at wavenumbers of 4000-650 cm^{-1} using The Nicolet iS10 FTIR Spectrophotometer. All spectra data was detected using DTGS (deuterated triglycine sulfate) detector using air as background and scanned at a resolution of 8 cm^{-1} with 32 scannings. After each scanning, a new reference air background spectrum was taken. FTIR spectra were connected to OMNIC Software. All spectra were scanned as absorbance values at each data point in triplicate (Candoğan *et al*., 2021).

2.8 Statistical analysis

FTIR spectra of all lipid extract samples were analyzed using TQ analyst software (version 6, Thermo electron Corporation, Madison, WI) for chemometrics analysis using LDA and multivariate calibration (PLSR and PCA). The statistical parameters were done using a value of root mean square error of calibration (RMSEC), root mean square error of prediction (RSMEP) and coefficient of determination (R^2) (Khotimah *et al*., 2021).

3. Results and discussion

In this study, FTIR spectroscopy was combined with chemometrics such as Discriminant Analysis, Partial Least Square Regression (PLSR) and Principal Component Analysis (PCA) developed for simple predicting procedures lard in a beef meatball. Lipid from meatballs was extracted using three lipid extraction methods, namely Bligh-Dyer, Folch, and Soxhlet methods. The amount of solvent used and the temperature used varied among the three lipid extraction methods. For the Bligh-Dyer, Folch, and Soxhlet methods, the solvent ratios were 1:3, 1:20, and 1:8,75, respectively. Soxhlet methods were the simplest and most accessible of the three lipid extraction methods compared to Bligh-Dyer and Folch methods. However, the Folch method yielded more lipid extract due to the high solvent-to-sample ratio (Ulmer *et al*., 2018).

Triacylglycerols (TAG) were reported it be the most common edible fat and oils in the lipid components isolated from beef and pork meatballs. Because fats and oils are essentially single component systems of triglycerides (TGs), they might be applied directly in the intended form to ATR crystal, FTIR spectroscopy can be a suitable approach for analyzing them (Rohman, 2019). The FTIR spectra characteristics of edible fats and lipids are fingerprint features. The FTIR spectra revealed tiny variations in peak intensities, which can be used to select the wavenumber areas (peaks) able to detect and quantify pork meat in beef meatballs. Each FTIR peak and shoulder of lipid components isolated from meatballs suggested functional groups relating to TAG for infrared absorption in the mid-infrared range (4000-650 cm^{-1}) and the fingerprint region at wavenumbers of <1500 cm^{-1} in each lipid measured (Irnawati *et al*., 2021).

The profiles of lipid FTIR spectra acquired using three distinct approaches are comparable. The lipid spectra of beef and pork fat extracted using the Bligh and Dyer, Folch, and Soxhlet techniques are shown in Figure 1 and Table 1. A peak at around 3006 cm^{-1} indicated C-H in Cis C=CH stretching vibration. C-HCH stretching vibration was observed at 2922/2853 cm^{-1}. The functional group Carbonyl C=O ester was found at 1744 cm^{-1}. The peak at 1462 cm^{-1} revealed the C-HCH scissoring bending, while symmetrical $–CH_3$ bending was detected at 1376 cm^{-1}. The peaks at 1234/1159/1106 cm^{-1} were ascribed to the functional group C-O ester. The peak at 964 cm^{-1} was associated with –CH trans out of the plane, and at 755 cm^{-1} might explain the –CH rocking vibration out of the plane (Candoğan *et al*., 2021).

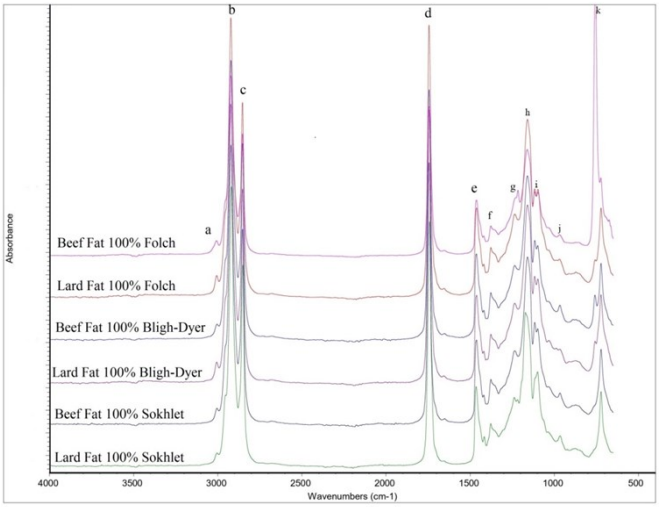

Figure 1. FTIR spectra of beef fat and lard fat were scanned using attenuated total reflectance (ATR) mode in the infrared region (4000-650 cm^{-1}).

The qualitative analysis for differentiating pork meat as an adulterant in beef meatballs using linear discriminant analysis (LDA). LDA is the most extensively used as a supervised pattern recognition method for differentiating two or more categories of objects (Rohman and Putri, 2019). The FTIR spectra of lipid components isolated from beef meatballs, pork meatballs, and beef meatballs containing PM using Bligh and Dyer, Folch, and Soxhlet techniques at the whole wavenumbers region (4000-650 cm^{-1}) were used as

https://doi.org/10.26656/fr.2017.8(1).092

Table 1. Functional group and modes of vibration of FTIR Spectra of lipid component extracted from pork meatballs and beef meatballs (Candoğan *et al*., 2021).

Assignment	Wavenumber (cm^{-1})	Functional Group	Intensity
A	3006	Cis C=CH stretching	Medium
B	2922	C-HCH stretching vibration	Very strong
C	2853	C-HCH stretching vibration	Very strong
D	1744	Carbonyl C=O ester	Very strong
E	1462	C-HCH scissoring bending	Medium
F	1376	$-CH_3$ Symmetrical bending	Medium
G	1234	C-O ester	Weak
H	1159	C-O ester	Medium
I	1106	C-O ester	Medium
J	964	-CH out plane trans	Weak
K	755	$-CH_2$ rocking vibration out plane	Very Strong

variables in LDA modelling. Based on Mahalanobis distance to form Cooman's plot, Figure 2 shows the discrimination spectra between beef meatballs, pork meatballs, and their mixture with an accuracy level of 100% and without any misclassification. Unsuccessful in LDA modeling caused by misplacing the variable of peaks in selected wavenumber region. The best discrimination of pork in beef meatballs can be obtained by improving the selection of peaks to be used in LDA modelling (Leng *et al*., 2020).

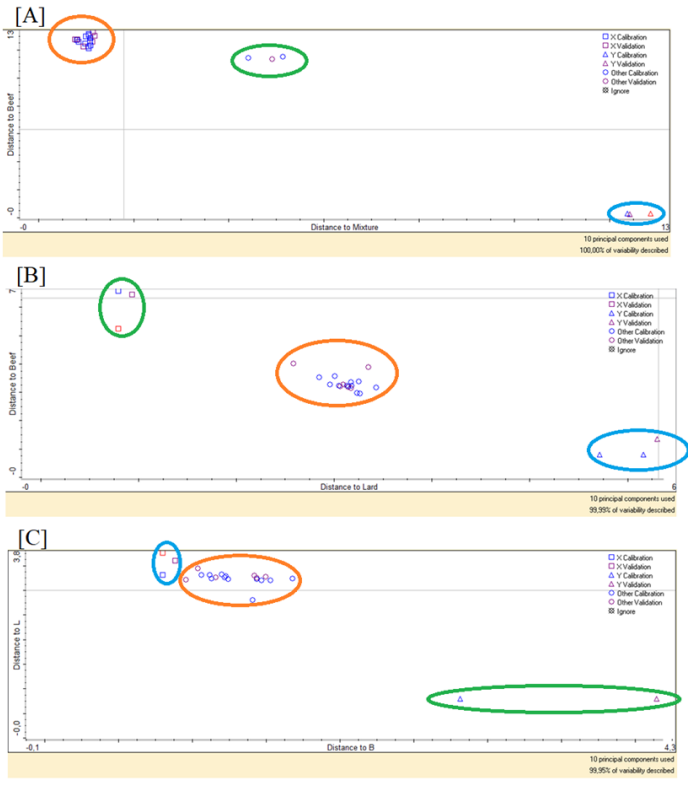

Figure 2. The Cooman plot for discriminant analysis of lipid components extracted from 100% beef meatballs (blue), 100% pork meatballs (green) and beef meatballs mixed with pork meat at different concentrations (orange) using Bligh-Dyer (A), Folch (B), dan Soxhlet (C) methods.

The quantitative analysis of pork meat as an adulterant in beef meatballs used the multivariate calibration of PLSR and PCR. FTIR spectra were pre-processed using the Savitzy-Golay derivatization method (first and second derivative). The best model for predicting lard as an adulterant was by comparing standard and derivative FTIR spectra at specific wavenumber regions and multivariate calibrations. Derivatization of FTIR spectra may increase the resolution of nearby peaks, resulting in improved calibration model performance; nevertheless, the higher order of spectra derivative may reduce model sensitivity (Fadzlillah *et al*., 2014). For the accuracy evaluation, the coefficient of determination (R^2) between actual values and FTIR predicted values, as well as root, mean square error of calibration (RMSEC), and root, mean square error of prediction (RMSEP) for precision evaluation, were utilized as statistical parameters. FTIR spectral condition was chosen because of its potential to develop the best predictability model of high R^2 and low RMSEC and RMSEP values (Rohman *et al*., 2019).

Tables 2, 3, and 4 show the statistical performance of multivariate calibrations using absorbance values at selected wavenumbers regions for the prediction of the levels of beef and pork at eight concentrations (0,10,20,30,40,50,75,100%) through analysis of lipid components extracted using Bligh-Dyer, Folch, and Soxhlet. Based on optimization, PLSR using the second derivative at certain wavenumbers region of 3100-900 cm^{-1} for the Folch method offered a great model for correlating actual values of beef and pork meat in a meatball. The R^2 values were > 0.99 with low errors in both RSMEC and RSMEP of 0.02%, respectively. Figure 3 describes the relationship between actual values of beef and pork meat and FTIR predicted values using the PLSR model at optimum conditions. Furthermore, multivariate calibration of PCR using the first derivative at certain wavenumbers region of 1400-800 cm^{-1} for Bligh-Dyer method and 3800-800 cm^{-1} for Soxhlet method was selected for simultaneous quantitative analysis of beef and pork meat in meatballs. The R^2 values of > 0.99 were obtained in pork and beef meat, ith

https://doi.org/10.26656/fr.2017.8(1).092

Table 2. The statistical performance of multivariate calibrations using absorbance values at selected wavenumbers region for prediction of the levels of beef and pork through analysis of lipid components extracted using Bligh-Dyer.

Wavenumber (cm^{-1})	Multivariate Calibration	Spectra	Calibration		Prediction	
			RMSEC	R^2	RMSEP	R^2
1500-1000	PLS	Normal	0.1170	0.9285	0.1210	0.9226
		1st Derivative	0.1050	0.9432	0.0998	0.9484
		2nd Derivative	0.2350	0.6641	0.2410	0.6462
	PCR	Normal	0.0689	0.9757	0.0689	0.9758
		1st Derivative	0.0746	0.9715	0.0670	0.9815
		2nd Derivative	0.0649	0.9785	0.0401	0.9923
1800-800	PLS	Normal	0.0950	0.9531	0.0970	0.9517
		1st Derivative	0.0871	0.9609	0.0818	0.9659
		2nd Derivative	0.1350	0.9035	0.1380	0.8996
	PCR	Normal	0.0683	0.9762	0.0653	0.9783
		1st Derivative	0.0829	0.9647	0.0737	0.9728
		2nd Derivative	0.0786	0.9683	0.0651	0.9789
1400-800	PLS	Normal	0.1870	0.8042	0.1930	0.7922
		1st Derivative	0.2690	0.5171	0.2710	0.5142
		2nd Derivative	0.1740	0.8326	0.1730	0.8362
	PCR	Normal	0.0491	0.9877	0.0489	0.9879
	PCR	**1st Derivative**	**0.0031**	**1.0000**	**0.0121**	**0.9995**
		2nd Derivative	0.0043	0.9999	0.0118	0.9994
3700-3100	PLS	Normal	0.3120	0.1199	0.3120	0.1339
		1st Derivative	0.0856	0.9623	0.1390	0.9587
		2nd Derivative	0.1010	0.9474	0.1570	0.9576
	PCR	Normal	0.0410	0.9915	0.1060	0.9459
		1st Derivative	0.0898	0.9584	0.1490	0.9540
		2nd Derivative	0.0857	0.9622	0.1720	0.9462
3100-2700	PLS	Normal	0.2960	0.3417	0.2940	0.3568
		1st Derivative	0.0322	0.9947	0.0540	0.9862
		2nd Derivative	0.2990	0.3802	0.2980	0.3246
	PCR	Normal	0.0496	0.9875	0.0538	0.9861
		1st Derivative	0.0445	0.9900	0.0548	0.9855
		2nd Derivative	0.0448	0.9898	0.0881	0.9613

The selected condition is in **bold**.

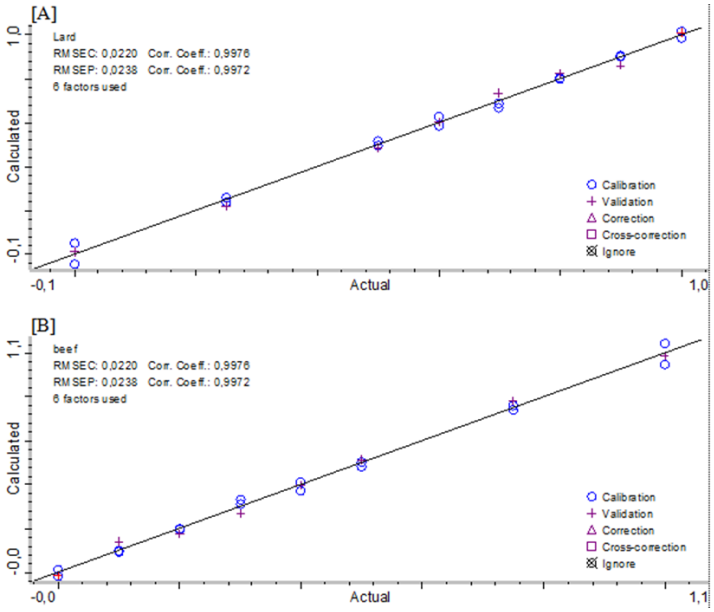

Figure 3. The correlation model between actual values of lipid components of lard [A] and beef meat [B] and FTIR predicted values using partial least square regression (PLSR) extracted using Folch using the variable of 2nd derivative FTIR spectral absorbances at wavenumbers of 3100-900 cm^{-1}.

https://doi.org/10.26656/fr.2017.8(1).092

Table 3. The statistical performance of multivariate calibrations using absorbance values at selected wavenumbers region for prediction of the levels of beef and pork through analysis of lipid components extracted using Folch.

Wavenumber (cm^{-1})	Multivariate Calibration	Spectra	Calibration		Prediction	
			RMSEC	R^2	RMSEP	R^2
1500-1000	PLS	Normal	0.2890	0.3921	0.2890	0.3992
		1st Derivative	0.2420	0.6381	0.2400	0.6539
		2nd Derivative	0.0814	0.9660	0.0950	0.9713
	PCR	Normal	0.0700	0.9749	0.175	0.9217
		1st Derivative	0.0465	0.9890	0.1070	0.9743
		2nd Derivative	0.0544	0.985	0.0978	0.9828
1800-800	PLS	Normal	0.274	0.4891	0.273	0.4973
		1st Derivative	0.2860	0.4178	0.2890	0.3993
		2nd Derivative	0.2680	0.5223	0.2740	0.4965
	PCR	Normal	0.0392	0.9922	0.0313	0.9964
		1st Derivative	0.1080	0.9396	0.0979	0.9528
		2nd Derivative	0.1010	0.9471	0.0848	0.9991
1400-800	PLS	Normal	0.2930	0.3626	0.2920	0.3759
		1st Derivative	0.1160	0.9301	0.1110	0.9372
		2nd Derivative	0.2380	0.6541	0.2350	0.6687
	PCR	Normal	0.0537	0.9853	0.0776	0.9784
		1st Derivative	0.0727	0.9729	0.0876	0.9680
		2nd Derivative	0.0560	0.9840	0.0755	0.9788
3100-900	**PLS**	Normal	0.266	0.5320	0.269	0.5202
		1st Derivative	0.287	0.4132	0.286	0.4316
		2nd Derivative	**0.0220**	**0.9976**	**0.0238**	**0.9972**
	PCR	Normal	0.0318	0.9949	0.0616	0.9969
		1st Derivative	0.0400	0.9919	0.0562	0.9952
		2nd Derivative	0.0295	0.9956	0.0312	0.9951
3100-2700	PLS	Normal	0.1860	0.8067	0.2010	0.8007
		1st Derivative	0.2000	0.7724	0.2150	0.7414
		2nd Derivative	0.1930	0.7894	0.2160	0.7425
	PCR	Normal	0.0825	0.965	0.109	0.9673
		1st Derivative	0.0731	0.9727	0.105	0.9648
		2nd Derivative	0.0610	0.981	0.1290	0.9675

The selected condition is in **bold**.

RMSEC values of 0.003%, 0.01%, and RSMEP values of 0.01%, and 0.1%, respectively. High R^2 and low RMSEC and RSMEP suggested that combining FTIR spectra and PLSR and PCR might be an efficient approach for predicting pork meat as an adulterant in beef meatballs with accurate and precise results (Kazemi *et al*., 2022).

4. Conclusion

Pork meat as an adulterant in beef meatballs could be determined by using FTIR spectroscopy on the Savitzy-Golay derivatization method combined with Linear Discriminant Analysis. PLSR and PCR as multivariate calibration for quantitative analysis of pork and beef in meatballs also provided an accurate and quick approach without the need for specialized equipment and sample preparation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This research was funded by the Faculty of Pharmacy, Andalas University through funding for basic research and lecturers development number 16/ UN16.10.D/PJ.01./2021. The authors also thank the Center of Excellence, Institute for Halal Industry and System, Gadjah Mada University for the Collaborative research program with contact number: 001/PUI.IHIS/ X/2021.

https://doi.org/10.26656/fr.2017.8(1).092

Table 4. The statistical performance of multivariate calibrations using absorbance values at selected wavenumbers region for prediction of the levels of beef and pork through analysis of lipid components extracted using Soxhlet.

Wavenumber (cm^{-1})	Multivariate Calibration	Spectra	Calibration		Prediction	
			RMSEC	R^2	RMSEP	R^2
1500-1000	PLS	Normal	0.1810	0.8173	0.2050	0.7724
		1st Derivative	0.1950	0.7849	0.2120	0.7514
		2nd Derivative	0.1940	0.7885	0.2160	0.7444
	PCR	Normal	0.0266	0.9964	0.075	0.9774
		1st Derivative	0.0287	0.9958	0.1070	0.9561
		2nd Derivative	0.0212	0.9977	0.1400	0.9063
1800-800	PLS	Normal	0.243	0.6352	0.242	0.6390
		1st Derivative	0.0443	0.9901	0.1020	0.9536
		2nd Derivative	0.2120	0.7384	0.2220	0.7175
	PCR	Normal	0.0258	0.9966	0.0585	0.9839
		1st Derivative	0.0230	0.9973	0.0923	0.9685
		2nd Derivative	0.0456	0.9894	0.1250	0.9429
1400-800	PLS	Normal	0.1820	0.8162	0.2060	0.7710
		1st Derivative	0.1960	0.7825	0.2150	0.7458
		2nd Derivative	0.1960	0.7835	0.2190	0.7367
	PCR	Normal	0.023	0.9973	0.0726	0.9804
		1st Derivative	0.0290	0.9957	0.1150	0.9507
		2nd Derivative	0.0462	0.9892	0.1440	0.9183
3800-800	PLS	Normal	0.1870	0.8041	0.2130	0.7507
		1st Derivative	0.2030	0.7648	0.2170	0.7302
		2nd Derivative	0.1950	0.7857	0.2160	0.7387
	PCR	Normal	0.0161	0.9987	0.0863	0.9662
		1st Derivative	**0.0138**	**0.999**	**0.107**	**0.94715**
		2nd Derivative	0.0138	0.999	0.1170	0.9349

The selected condition is in **bold**.

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