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Authentication analysis of goat milk from cow milk using Fourier Transform Infrared spectroscopy and chemometrics

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Abstract

The authentication of higher quality milk such as goat milk (GM) from the lower price of cow milk (CM) is a big issue in the milk industry, because unethical producers may get economic profits from the adulteration practice. This study intended to apply Fourier Transform Infrared (FTIR) spectroscopy and chemometrics for the authentication analysis of GM from CM. The characterization of CM and GM was performed by determining fatty acid composition using gas chromatography. The binary mixtures of GM and CM were prepared for making calibration and validation models and were subjected to FTIR spectral measurement using an attenuated total reflectance (ATR) accessory. The model was optimized by selecting multivariate calibrations of partial least square regression (PLSR) and principal component regression (PCR) along with spectral modes (normal and derivatives) and wavenumbers regions. The results showed that absorbance values at 2nd derivative spectra at wavenumber regions of 1500-800 cm^{-1} could provide the best accuracy and precision of the developed model. Principal component analysis (PCA) at selected wavenumbers and linear discriminant analysis (LDA) at 3800-800 cm^{-1} could discriminate authentic GM and GM adulterated with CM without any misclassification objects observed. It concluded that FTIR spectra combined with chemometrics techniques could be used as a reliable method for the authentication analysis of milk products.

1. Introduction

The world's goat milk production has increased recently, accumulating approximately 20% annually. It is in line with the increased demand for goat milk which is motivated by its unique characteristics compared to cow milk. Goat milk has some functional properties with beneficial health effects on physiological functions to be used as the nutrition of children and elderly people. GM had high digestibility and low allergenicity without negative effects on people suffering from cow milk allergy (Hodgkinson *et al*., 2012; Pinto *et al.,* 2017). From an economic point of view, goat milk (GM) is more expensive than cow milk (CM), consequently, unethical milk players can substitute GM with CM driven by economic profits. The most common adulteration practice typically met in the milk industry is the replacement of high-priced milk with lower ones with partial or total substitution without correct labelling. The adulteration practice of milk products can affect the market and consumer confidence leading to negative impacts on the dairy economy. The main milk adulterant of GM is cow milk because of its lower cost and greater abundance (Pereira *et al*., 2020). GM and CM have similar physicochemical characteristics, consequently, there is a difficulty in identifying GM and CM in the mixtures.

Several analytical methods have been reported for the identification of adulteration practices involving high price milk such as GM with lower milk, including DNA-based methods using real-time polymerase chain reaction with specific primers (Pinto *et al*., 2017) and real-time fluorescent multiplex loop-mediated isothermal amplification (LAMP) (Yu *et al*., 2021), protein-based methods employing the enzyme-linked immunosorbent assay (ELISA) (Song *et al*., 2011), liquid

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chromatography hyphenated with electrospray ionization mass spectrometry (HPLC/ESI-MS) (Chen *et al*., 2004), gas chromatography-mass spectrometry (GC-MS) in combination with chemometrics analysis (Scano *et al*., 2014), and rapid capillary electrophoresis (Trimboli *et al*., 2017). However, such techniques have some drawbacks, including high cost, skilful analysts, time-consuming, laborious, and requiring various steps of sample pretreatment. Therefore, some simple methods based on molecular spectroscopy have been proposed and developed for the authentication of goat milk.

Molecular spectroscopy based on the interaction between electromagnetic radiations in certain frequencies with the analyte(s) at the molecular level has emerged as a rapid and reliable technique for authentication analysis of milk products (Rohman and Windarsih, 2020). Vibrational spectroscopy in combination with multivariate data analysis or chemometrics, including the near-infrared (NIR) spectroscopy method (Mabood *et al*., 2017; Pereira *et al*., 2020), mid-infrared (MIR), and Raman spectroscopy (Yaman, 2020), has been successfully applied for authentication of GM. In this study, the application of Fourier infrared (FTIR) spectroscopy in combination with chemometrics for authentication of GM from CM was investigated.

2. Materials and methods

2.1 Materials

For constructing the multivariate modelling either in classification or quantification, the representative samples of cow milk and goat milk with varied sources of raw materials were available from different farmers located in West Nusa Tenggara and Yogyakarta, Indonesia. All samples were stored in a refrigerator at -4^{o}C before being used for analysis.

2.2 Fatty acid compositional analysis of goat milk and cow milk

The fatty acid composition of fats extracted from pure GM and CM was carried out using gas chromatography with a flame ionization detector (FID) following the procedure in Man *et al*. (2011) with modification. The 50 mg of fats extracted from milk were added with 1 mL hexane and 0.2 mL 1 M $NaOCH_3$ prepared by adding NaOH into CH_3OH until the solution was saturated. The mixture was then added with saturated NaCl and vigorously shaken for 60 s with a vortex mixer. After that, 1 µL of the clear supernatant was injected into gas chromatography (Agilent Technologies 7890 B, USA), using a DB-WAX column (0.25 mm internal diameter, 100 m length, and 0.2 µm film thickness). The oven was prepared at 100^{o}C (hold for 5 mins), then increased to 200^{o}C (4^{o}C /min), and finally held at 240^{o}C for 15 mins. The flame ionization detector (260^{o}C) used carrier gas He and make-up gas N_2 at 30-40 mL/min. The Injector was set up to 260^{o}C with a split ratio (1:10) and split flow (17.5 mL/min). Thirty-seven standard FAME (Sigma, St. Louis, MO) were used as authentic samples to calculate the percentage of fatty acids based on peak area. Quantification of FAME was performed using an internal normalization technique.

2.3 Preparation of calibration samples

For the quantification of cow milk (CM) as an adulterant in goat milk (GM), a series of 25 calibration samples consisting of GM and CM in binary mixtures were prepared. The concentration range of CM in GM was in the range of 0-100%. A set of independent samples to be predicted using the calibration models called validation samples consisting of CM and GM spanning the concentration ranges in calibration models were also prepared. Table 1 compiles the binary composition of CM and GM. All samples were subjected to FTIR spectral measurement.

Table 1. The binary mixture of samples containing goat's milk and cow's milk.

Sample	Cow's Milk	Goat's Milk
1	10	90
2	17	83
3	63	37
4	33	67
5	62	38
6	82	18
7	50	50
8	39	61
9	87	13
10	79	21
11	21	79
12	58	42
13	69	31
14	23	77
15	85	15
16	52	48
17	11	89
18	67	33
19	75	25
20	84	16
21	90	10
22	29	71
23	34	66
24	100	0
25	0	100

2.4 Scanning FTIR spectra

Spectrophotometer FTIR (FTIR Nicolet iS20) using detector DTGS (*deuterated triglycine sulfate*) connected to OMNIC software. The samples were directed and placed into multi-bounce attenuated total reflectance

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(ATR) crystal and scanned using the resolution of 8 cm^{-1} and the number of scanning 64. All spectra were measured in the mid-infrared region (4000–650 cm^{-1}) using air as background. All spectra were recorded in absorbance mode to facilitate quantitative analysis according to Lambert-Beer law. The data obtained were managed using the software of TQ Analyst, Minitab, and Orange Data Mining.

2.5 Chemometrics analysis

Chemometrics analyses were Linear Discriminant Analysis (LD), Principal Component Analysis (PCA), and multivariant calibrations (PCR and PLSR). LDA was used for discrimination between goat milk (GM) and GM adulterated with cow milk (CM). The Coomans plot based on Mahalanobis distance was constructed for the discrimination between authentic GM and GM adulterated with CM. Meanwhile, PCA was predicted and classified GM, CM and GM adulterated with CM which is an item belonging to their class based on Euclidean distance. The samples consisting of pure GM and GM blended with CM at different concentrations covering 1-100% called a training set were prepared. The independent samples called test samples were evaluated using training sets. In addition, multivariate calibrations were evaluated by the root mean square error of calibration (RMSEC), root mean square error of prediction (RMSEP), and coefficient of determination (R^2).

3. Results and discussion

In this study, the application of FTIR spectroscopy in combination with chemometrics was studied for authentication analysis of goat milk (GM) from an adulterant of cow milk (CM). The fatty acid compositional analysis was employed using GC-FID to characterize both kinds of milk. The fatty acids were non -volatile. Therefore, the fatty acid methyl esters (FAMEs) were derivatized before being injected into the GC instrument. The FAME profiles were fingerprints in nature, therefore, fatty acids are appropriate for milk characterization. Table 2 lists fatty acid profiles of GM and CM in which pentadecanoate acid (C15:0), oleic acid (C18:1), linolenic acids (C18:3), and methyl cis-11-eicosenoate (C20:1(n-9)) were present in a higher amount than other fatty acids. Figure 1 shows and classifies GM, CM, and other milk as horse's milk (HM) based on different fatty acids of composition milk using Orange Data Mining software. The result showed that HM, CM, and GM could be clearly distinguished using PCA, indicating the difference in fatty acids composition used as variables. This information is critical to building the prediction model for authentication of GM adulterated with CM in the range of 0-100% using FTIR spectroscopy in combination with chemometrics because there must be differences in the fatty acids composition of adulterated samples.

The authentication of GM with CM was carried out

Table 2. The composition of fatty acid from cow's milk, goat's milk and horse's milk.

Fatty Acids	Fatty acid composition (%)	
	GM	CM
Methyl Butyrate (C4:0)	1.332	0.000
Methyl Hexanoate (C8:0)	1.216	0.825
Methyl Undecanoate (C11:0)	0.243	0.159
Methyl Tridecanoate (C13:0)	0.000	0.000
Methyl tetradecanoate (C14:0)	0.214	0.000
Methyl Pentadecanoate (C15:0) *	13.441	10.883
Methyl Palmitate (C16:0)	1.297	0.996
Methyl Heptadecanoate (C17:0)	0.000	0.147
Methyl stearate (C18:0)	0.770	0.000
Methyl Heneicosanoate (C21:0)	3.001	0.000
Methyl Lignocerate (C24:0)	0.200	0.111
Methyl Palmitoleate (C16:1)	0.276	0.000
Methyl cis-oleic (C18:1) *	35.525	33.156
cis-10-Pentadecenoic AME (C15:1 n-5)	1.107	0.634
trans-9-Elaidic AME (C18:1 trans-9)	0.455	0.407
Linolelaidic AME (C18: 2 trans-9, trans-2)	0.415	0.324
Methyl Linolenate (C18:3) *	0,000	50.796
cis-11,14-Eicosadienoic (C20:1, cis11,14)	0.151	0.002
cis-11,14,17-Eicosatrienoic (C20:3n3)	0.002	0.652
gamma-Linolenic (C18:3-6)	0.000	0.000
cis-8,11,14-Eicosatrienoic AME (C20: 3n6)	0.000	0.606
cis-4,7,10,13,16,19-Docosahexaenoate (DHA)	0.135	0.123
Methyl cis-11-eicosenoate (C20:1(n-9)) *	39.262	0.000

*Higher amount of fatty acid profiles on GM and CM.

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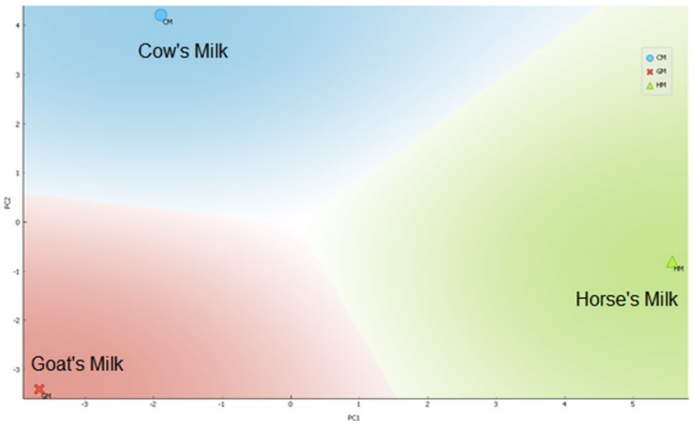

Figure 1. The PCA models of goat's milk/HM (×), cow's milk/CM (O) and other milk as horse's milk/HM (Δ) based on different fatty acids of composition milk using Orange Data Mining software.

Table 3. IR absorption bands in milk product

No.	Wavenumber (cm^{-1})	Functional Group	Assignment
A	3320	-OH	Water in milk
B	2922	$-CH_2$ stretch	Fatty Acid
	2852	-CO ester	
	1462	$-CH_2-CH_3$	
C	1635 and 1454 (1700-1500)	Amide I and II (CO and NH)	Protein
D	1134-1018	-CO	Lactose

Source: Rohman *et al*. (2020), Nicolaou *et al*. (2010)

using FTIR spectroscopy in conjunction with chemometrics due to its capability of FTIR spectra as fingerprinting properties, especially at wavenumbers of 1500-650 cm^{-1} in authentication analysis (Man *et al*., 2011). Figure 2 reveals attenuated total reflectance (ATR)-FTIR spectra of GM and CM at the mid-infrared region (4000-650 cm^{-1}) in which each peak in this region corresponds to functional groups which exhibited the characteristics of protein peaks. It is not surprising because the main component in milk is protein characterized by peptide bonds. The peak at wavenumbers (1/λ) of 3320 corresponded to the stretching vibration of bonded -OH coming from water contents in the studied milk. The wavenumbers of 1700-1500 cm^{-1} corresponded to amide groups of amides I and amides II which are specific in nucleic acids and proteins. The amide bands can be characterized by the presence of absorption peaks at 1635 cm^{-1} and 1455 cm^{-1} in Table 3 (Rohman *et al*., 2020).

Two multivariate calibrations of PLSR and PCR were compared using variables of absorbance values in different wavenumbers and different spectral modes (normal and derivatives) in terms of their capability to provide the best prediction model of CM as an adulterant in GM (Table 4). The derivatization process of FTIR spectra is intended to improve the resolution of adjacent peaks which may offer better performing models. However, the use of high-order FTIR spectral derivative may reduce the model sensitivity (Fadzlillah *et al*., 2014). In PLSR and PCR modelling, some parameters were assessed for the coefficient of determination (R^2) for evaluation of model accuracy as indicated by the closeness between actual values and FTIR predicted values, root mean square error of calibration (RMSEC) for the evaluation of precision in the developed analytical method in calibration models, while the root mean square error of prediction (RMSEP) was used for a precision indication in validation model. The selection of the calibration model relied on its capability to provide high R^2 with low values of RMSEC and RMSEP (Rohman *et al*., 2019). PLSR using a variable of absorbance values of 2nd derivative spectra at selected fingerprint regions at wavenumbers of 1500-800 cm^{-1} provided the best model for the relationship between actual values of cow milk (CM) as an adulterant in goat milk (GM) and FTIR predicted values, therefore PLSR was more preferred than PCR. The R^2 values obtained

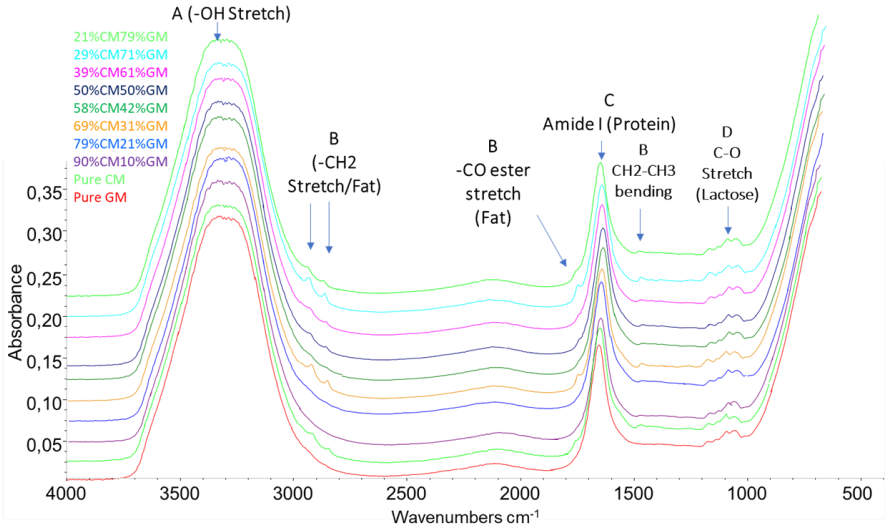

Figure 2. FTIR spectra of goat's milk and cow's milk scanned using attenuated total reflectance (ATR) mode in the infrared region (4000-650 cm^{-1}).

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Table 4. The optimization of wavenumbers region of multivariate calibration for authentication of goat's milk in binary mixture with cow's milk

Wavenumber (cm^2)	Multivariate Calibration	Spectra	Calibration		Prediction	
			RMSEC	R^2	RMSEP	R^2
3800-900	PLS	Normal	0.2520	0.4789	0.2510	0.4870
		1st Derivative	0.0133	0.9989	0.0711	0.9713
		2nd Derivative	0.0877	0.9520	0.1360	0.8906
	PCR	Normal	0.0312	0.9941	0.0392	0.9918
		1st Derivative	0.0721	0.9679	0.0903	0.9556
		2nd Derivative	0.1670	0.8119	0.1840	0.7720
3800-800	PLS	Normal	0.2520	0.4794	0.2500	0.4874
		1st Derivative	0.0237	0.9966	0.0722	0.9705
		2nd Derivative	0.0862	0.9538	0.1350	0.8916
	PCR	Normal	0.0326	0.9935	0.0403	0.9911
		1st Derivative	0.0693	0.9703	0.0892	0.9565
		2nd Derivative	0.1570	0.8357	0.1790	0.7883
1500-800	**PLS**	Normal	0.2600	0.4253	0.2590	0.4309
		1st Derivative	0.0751	0.9651	0.0697	0.9727
		2nd Derivative*	**0.0099**	**0.9994**	**0.0795**	**0.9612**
	PCR	Normal	0.0252	0.9961	0.0377	0.9919
		1st Derivative	0.0491	0.9853	0.0555	0.9836
		2nd Derivative	0.1040	0.9326	0.1320	0.8903
1500-900	PLS	Normal	0.2590	0.4302	0.2580	0.4365
		1st Derivative	0.0408	0.9898	0.0497	0.9860
		2nd Derivative	0.0303	0.9944	0.0706	0.9704
	PCR	Normal	0.0288	0.9950	0.0377	0.9915
		1st Derivative	0.0414	0.9895	0.0469	0.9880
		2nd Derivative	0.0947	0.9439	0.1110	0.9242
1500-1000	PLS	Normal	0.2570	0.4468	0.2550	0.4552
		1st Derivative	0.0427	0.9888	0.0513	0.9848
		2nd Derivative	0.0374	0.9915	0.0776	0.9652
	PCR	Normal	0.0247	0.9963	0.0374	0.9920
		1st Derivative	0.0424	0.9890	0.4980	0.9859
		2nd Derivative	0.0832	0.9570	0.1010	0.9367
1600-1000	PLS	Normal	0.0453	0.9874	0.0604	0.9778
		1st Derivative	0.0465	0.9868	0.0550	0.9822
		2nd Derivative	0.0560	0.9807	0.0864	0.9545
	PCR	Normal	0.0268	0.9956	0.0399	0.9906
		1st Derivative	0.0485	0.9856	0.0526	0.9838
		2nd Derivative	0.1040	0.9324	0.1250	0.9021

Bold indicates selected condition.

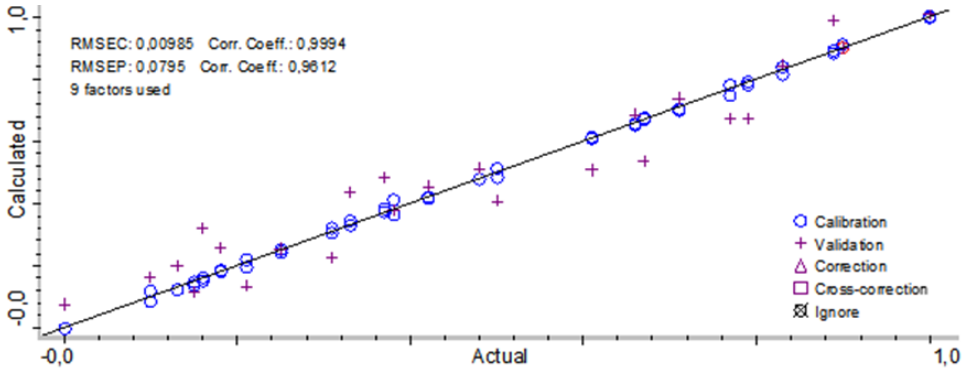

Figure 3. The PLS model for authentication goat's milk and adulterants (cow's milk) at wavenumbers region of 1500-800 cm^{-1}.

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using this optimum condition for calibration and validation models obtained were 0.9994 and 0.9612 respectively with RMSEC and RMSEP values of 0.0099 and 0.0795. High values of R^2 and low values of RMSEC and RMSEP indicated that the developed model was acceptable in terms of its accuracy and precision. Figure 3 reveals the linear relationship between actual values of CM (x-axis) and FTIR predicted values (y-axis) using the optimum condition either in calibration or validation models. From residual analysis (Figure 3), it is clear that the difference values between actual and predicted were around zero (0) points. Therefore, errors occurring during PLSR modelling are random errors, not systematic errors (Jamwal *et al*., 2020).

For discrimination between authentic GM and GM adulterated with CM, Linear discriminant analysis (LDA) was used applying absorbance values of FTIR normal spectra at wavenumbers of 3800-800 cm^{-1}. In this study, LDA is applied to predict the class membership of unknown samples (authentic GM and GM adulterated with CM) (Messai *et al*., 2016) based on Mahalanobis distance to form Cooman's plot. Both groups are clearly separated and discriminated with no classification objects observed (Figure 4). This indicated that LDA was successful in discriminating authentic GM from adulterated ones with CM. Misclassification may occur because of the close similarities in chemical composition among groups (Rohman and Che Man, 2009).

The FTIR spectra of GM and GM adulterated with CM were classified and authenticated using PCA models at 4000-650 cm^{-1} in Figures 5 and 6. The PCA technique identified the most important variables in the data and explored the clustering of samples based on spectral differences. The selected wavenumbers were highly correlated with other predictors, they were 650, 800, 850, 900, 950, 1001, 1039, 1042, 1050, 1075, 1100, 1150, 1200, 1251, 1301, 1351, 1454, 1462, 1635, 2015, 2105, 2116, 3267, 3303, and 3320 (Figure 7).

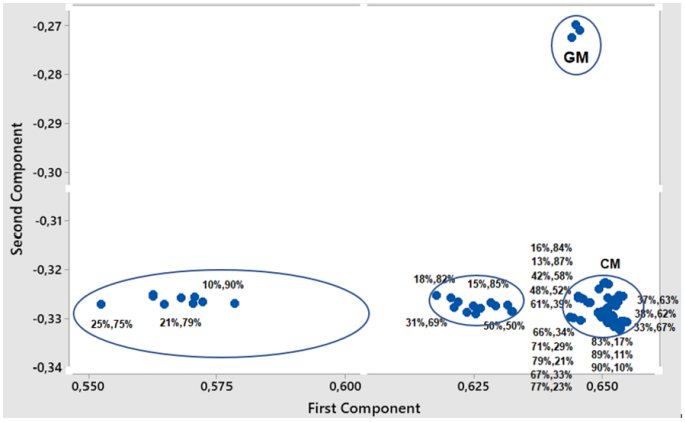

Figure 6. The score plot of PCA models to authentic GM and GM adulterated CM in binary mixture (GM%:CM%) at 3320-650 cm^{-1} using Minitab software.

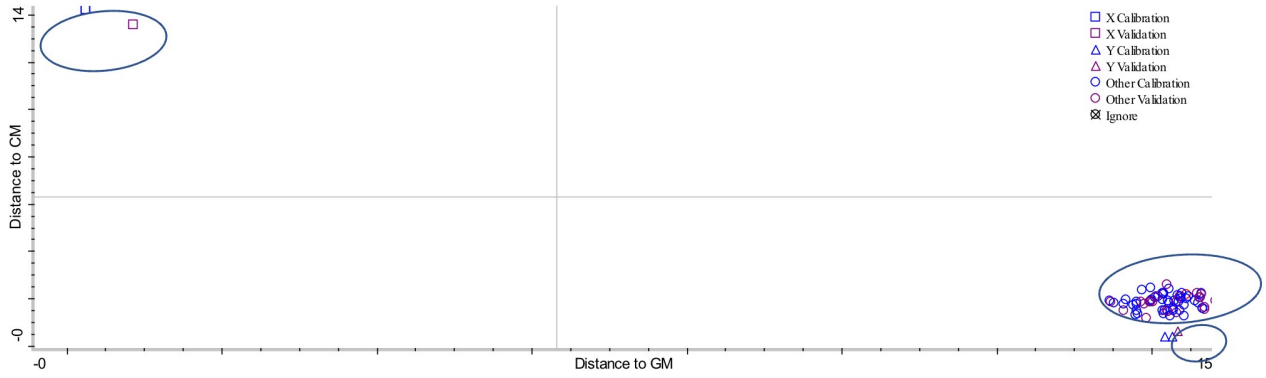

Figure 4. The Cooman's plot for discrimination between goat milk (□), cow milk (Δ) and goat milk adulterated with cow milk (O) at wavenumbers region of 3800-800 cm^{-1}.

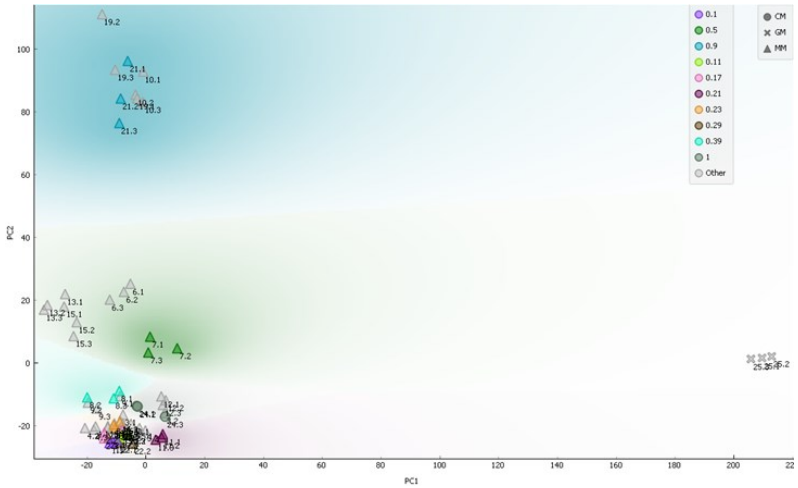

Figure 5. The PCA models of goat's milk (×), cow's milk (O) as adulterants and binary mixture (Δ) at wavenumbers region of 4000-650 cm^{-1} using Orange Data Mining software.

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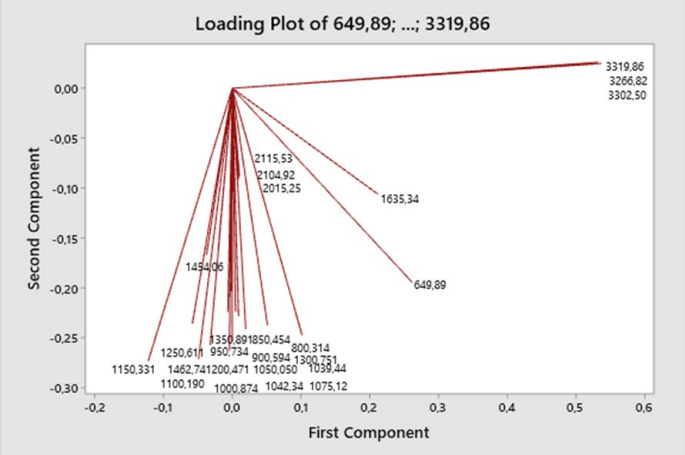

Figure 7. The loading plot of PCA models to authentic GM and GM adulterated CM at 3320-650 cm^{-1} using Minitab software.

4. Conclusion

The combination of FTIR spectroscopy and chemometrics offered a powerful and reliable technique for the authentication analysis of GM from CM. PLSR using 2nd derivative spectra at wavenumbers of 1500-800 cm^{-1} provided the best quantification model of CM in GM. In addition, linear discriminant analysis was fruitfully employed for the discrimination between authentic GM and GM adulterated with GM without any misclassification (accuracy level of 100%). The developed method is a fast and green analytical technique because the use of chemicals and solvents could be hindered.

Conflict of interest

The authors declare no conflict of interest.

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