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Combining Fourier Transform Infrared Spectroscopy with Chemometrics for Gelatin Content Analysis in Imported Soft Candy Products

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ABSTRACT

BPS-Statistics Indonesia recorded an increase in processed food and beverage imports, two of the most brought-in products from abroad. An example is soft candy that contains gelatin, which can be sourced from pork or beef. Fourier transform infrared spectroscopy is one of the techniques used to analyze food ingredients. This study was conducted to evaluate the ability of FTIR spectroscopy in combination with chemometrics to detect and distinguish between bovine and porcine gelatins in soft candy products. In this non-experimental research, protein precipitation was utilized to isolate gelatin from soft candies to be further tested for functional groups using FTIR. In the FTIR, partial least square (PLS) was employed to optimize the range of wavenumbers in which the model's degree of errors was calculated using calibration, cross-validation, and external validation. Using principal component analysis (PCA), the gelatin samples were classified according to their source (pork, beef, or others). FTIR detected five functional groups in the gelatin: O-H, aliphatic CH, C=O, N-H, and C-N. PLS found that gelatin could be optimally detected in the 1600–1621.92 cm^{-1} region. The calibration model had RMSEC = 0.188 and R^2 = 0.999, the cross-validation showed RMSECV = 2.891 and R^2 = 0.990, and the external validation had RMSEP = 1.652 and R^2 = 0.998. Clustering with PCA shows that samples Hrb and Y were in the same quadrant as bovine gelatin, while C was outside the quadrants of bovine and porcine gelatin. In conclusion, FTIR spectroscopy alone cannot distinguish between the sources of gelatin in soft candy; the analysis must be complemented by PLS and PCA.

Keywords: Chemometrics, FTIR, Gelatin, Soft candy

INTRODUCTION

Data from the BPS-Statistics Indonesia in 2018 show that food and beverages were the most imported processed products by households from 2015 to 2018, with import values increasing from USD2,260.0 million in 2015 to USD2,965.0 million in 2016, USD2,804.3 million in 2017, and then USD4,125.7 million in 2018. These products include soft candy, which, according to the Indonesian National Standard SNI 3457-2-2008, is a type of solid snack food made of sugar or a mixture of sugar and other sweeteners, with or without the addition of other food ingredients and permitted food additives (*bahan tambahan pangan*, BTP), and is relatively soft or becomes soft when chewed.

Because not all imported soft candies are halal-certified, the halal status of the components used in their making should be tested. The chewy texture of soft candy comes from gelatin, which can be made from beef or pork (Karim & Bhat, 2008). For Muslims, who comprise the largest share of the population in Indonesia, it is essential to know whether or not the consumed edibles are halal. As explained in verse 3 of Quran Surah Al-Maidah (5:3), "It is forbidden for you (to eat) carrion, blood, pork, (animal meat) that is slaughtered in the name of other than Allah, that is killed by strangling, beating, a fall, or by being gored to death, and that is (partly) eaten by wild animals, except for those that you could slaughter, and (unlawful for you) those that were sacrificed on altars."

Several analytical techniques have been developed to identify porcine and bovine gelatin, for instance, by analyzing animal fat profiles using Fourier transform infrared (FTIR) spectrophotometry (Hermanto *et al.*, 2008; Salamah *et al.*, 2022). Hermanto *et al.* (2008) used gas chromatography-mass spectrometry (GCMS) to identify the composition of fatty acids in animal fats. Meanwhile, Salamah *et al.* (2022) used the combination of FTIR and chemometrics, i.e., partial least square (PLS) and principal component analysis (PCA), to identify the functional groups of gelatin samples isolated from commercial soft candies with different techniques.

The current non-experimental study also used FTIR spectroscopy. For gelatin detection, FTIR can differentiate the spectra of gelatin samples more simply and precisely than protein analysis. However, bovine and porcine gelatins may share a similar infrared spectral profile, which makes FTIR unable to differentiate them alone. Therefore, it should be combined with chemometrics, comprising PLS (calibration and validation) and PCA to accurately distinguish between bovine and porcine gelatins to meet the halal requirement (Nemati *et al*., 2004; Rohman *et al*., 2020). This study is expected to provide a reference for the public in selecting halal soft candies imported from Asian countries and for the halal status analysis of food products.

MATERIALS AND METHODS

This non-experimental study combined FTIR spectroscopy with chemometrics to analyze porcine and bovine gelatins. The research was conducted at the Pharmacy Laboratory of the Ahmad Dahlan University and FMIPA Laboratory and Integrated Laboratory of the Indonesian Islamic University, Yogyakarta, Indonesia.

The materials used were standard bovine gelatin (Brataco), standard porcine gelatin (Sigma Aldrich), citric acid, tartrazine, acetone (Merck), aquadest, sugar, and samples of imported soft candies from three commercial brands: C, Y, and Hrb. The equipment included an FTIR/UATR spectrophotometer (Spectrum Two by Perkin Elmer and Nicolet Avatar 360 IR), magnetic stirrer, beakers, tubes, dropper pipettes, a water bath, filter papers, thermometers, an analytical balance, and a hot plate. Statistical data analyses with PCA and PLS were processed using Minitab 18 and OriginLab programs.

Principal Component Analysis (PCA)

Principal component analysis (PCA) was performed in Minitab 18 to create clusters of the spectrum data. First, the data obtained from the FTIR analysis (gelatin concentration and spectral properties) were inputted into the program. Then, the PCA analysis was run by clicking *Stat* > *Multivariate* > *Principal Components*. Afterward, the selected wavenumber range was entered into the variable field on the PCA window. C*orrelation* was selected as the *Type of Matrix*. After clicking *Graphs* > OK, the analysis results appeared on the screen (Rohman, 2013; Rohman & Salamah, 2018).

Partial Least Square (PLS)

Partial least square (PLS) was run using the following steps: Cross-validation with the leave-one-out feature. After selecting S*tat* > *Regression* > *Partial Least Square*, the column Responses on the PLS window was filled with Levels, and the column Model was filled with the wavenumbers from the fingerprint region (i.e., the wavenumber region containing the absorbance value for optimal gelatin detection). After selecting *Options* > *Cross-Validation* > *Leave One Out* > *OK*, the analysis results appeared on the screen (Rohman, 2013). The actual and predicted values obtained were inputted into Ms. Excel 2019 to create a regression model using Data Analysis. Then, the predicted value was inputted into the resulting regression equation $y = bx + a$, where y is the predicted value, x is the actual value, b is the slope gradient, and a is the intercept. The root mean square error (RMSE) was calculated using the equation below (Danzer *et al*., 2004):

$$RMSE = \sqrt{\frac{\sum_{t=1}^{n}(A_t - F_t)^2}{n}}$$

where A_t is actual values, F_t is predicted values, and n is the number of data.

RESULTS AND DISCUSSION

Reference Soft Candies

Reference soft candies were made for calibration and validation. Gelatin was dissolved in aquadest in a water bath, with the temperature maintained at no higher than 60°C. To prevent thickening, the completely dissolved gelatin should not be left too long; it should be mixed immediately with regular sugar (not sucrose or glucose). Then, it was mixed with the coloring agent tartrazine (orange color) and the widely used flavoring agent in candies, citric acid (orange flavor).

Citric acid also prevents sugar crystallization. This process produced eight reference soft candies with different gelatin types and concentrations: 100% bovine, 100% porcine, 95% porcine, 80% porcine, 65% porcine, 50% porcine, 35% porcine, and 20% porcine.

Gelatin Samples Isolated from Reference and Commercial Soft Candies

Protein compounds of gelatin were isolated from eight reference soft candies and three commercial soft candies using protein precipitation. This method was used because it is suitable for analyzing samples with high proteins (Fic *et al*., 2010; Salamah *et al*., 2023), such as gelatin, which contains around 84–86% protein (Hastuti & Sumpe, 2007). Acetone was used as the solvent in the precipitation because it precipitates more protein than other organic solvents like methanol and chloroform (Fic *et al*., 2010). Besides, gelatin is insoluble in both solvents. The precipitation produced a supernatant that contained polar compounds dissolved in acetone. This supernatant was then centrifuged to see if there was any residual precipitate. Gelatin was sedimented at the bottom of the tube, collected, washed, and then dissolved in distilled water. The resulting solution was later analyzed using FTIR.

Functional Groups of Gelatin from Reference Soft Candies

The FTIR spectroscopy of protein isolates from eight reference soft candies and three commercial soft candies was conducted in the wavenumber range of 400–4000 cm^{-1}. The analysis produced infrared (IR) spectral peaks with functional group information, as presented in Table I and Figure 2. Peaks 1 to 5 indicated five functional groups: O-H, C-H, C=O, N-H, and C-N. These correspond to Zain & Nugraha (2018), which found free O-H, C-H, N-H, C=O, N-H, and C-N in the spectral profile of protein isolates. The OH stretching vibrations were detected in the range of 3200–3600 cm^{-1} (peak 1), preceded by the C-H vibration with a weak absorption in the range of 2800–3000 cm^{-1} (peak 2). Both peaks usually appear in hydrocarbon compounds and biomolecules. These results are in line with Pavia *et al*. (2001), which explained that proteins can be spectrally detected from free OH bonds and C-H at 2900 cm^{-1}.

Information on secondary protein and the source of gelatin can be seen in the 1600–1200 cm^{-1} region. As shown in Figure 2, C=O stretching from 1600 to 1690 cm^{-1} indicated a carbonyl double bond. C=O absorption is generally detected in the 1630–1680 cm^{-1} region. The C=O group confirms the presence of an acidic group, i.e., an amino group, which is one of the building blocks of protein. N-H bending and C-N stretching absorptions were observed in the 1560–1335 cm^{-1} region. Peaks in the 1240 – 670 cm^{-1} region were thus detected as N-H and C-N. Pavia *et al*. (2001) explained that proteins also contain the functional groups C=O, N-H, and C-N.

The absorption intensity of porcine gelatin was generally lower than bovine gelatin because the former had a higher water content than the latter. This also indicated that FTIR can be used to detect gelatin. However, further analysis, i.e., chemometrics, is needed to distinguish between bovine and porcine gelatins.

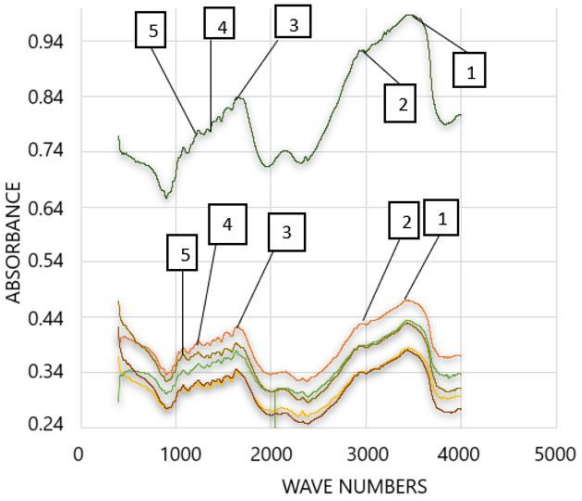

Figure 1. IR spectra of isolated gelatin samples from the reference soft candies made with different gelatin compositions: 95% porcine (red), 80% porcine (yellow), 65% porcine (green), 50% porcine (brown), 35% porcine (light brown), and 20% porcine (blue) (1–5 show spectral peaks in Table I).

Functional Groups of Gelatin from Imported Soft Candies

Protein isolate solutions from three commercial soft candies produced in Malaysia and Thailand (C, Y, and Hrb) were analyzed using FTIR-ATR in the wavenumber range of 400–4000 cm^{-1}. This FTIR-ATR tool can directly analyze the test solution by placing a small sample drop onto the ATR crystal. The sample must not contain excess water because this will affect the instrument's reading.

Table I. Spectral peak positions of gelatins isolated from the reference and commercial soft candies

Peak	Porcine 100 %	Porcine 95%	Porcine 80%	Porcine 65%	Porcine 50%	Porcine 35%	Porcine 20%	Vibration	Bonding
1	3428.86	3427.17	3443.40	3427.47	3427.64	3433.13	3434.23	Stretching	O-H
2	2910.77	2910.09	2980.20	2900.10	2980.26	2920.40	2910.60	Stretching	C-H
3	1634.40	1643.53	1642.68	1632.97	1633.61	1634.68	1637.70	Stretching	C=O
4	1448.10	1445.53	1448.58	1446.19	1444.57	1443.76	1442.56	Bending	N-H
5	1235.22	1235.65	1233.74	1236.66	1234.11	1233.03	1259.00	Stretching	C-N

Peak	Sample Y	Sample C	Sample Hrb	Vibration	Bonding
1	3374.29	3339.72	3351.22	Stretching	O-H
2	2910.20	2970.00	2940.90	Stretching	C-H
3	1644.88	1641.43	1642.99	Stretching	C=O
4	1422.53	1422.71	1423.14	Bending	N-H
5	1234.76	1237.44	1237.45	Stretching	C-N

The reference bovine and porcine gelatins had the same spectral pattern but different absorbance (Figure 1). According to Rahmawati *et al*. (2015), there is no significant difference in the spectral profile of bovine and porcine gelatins because they have similar amino acid content, although in different concentrations. Therefore, solely using FTIR cannot distinguish between the two gelatins. Moreover, variations in absorbance values may stem from differences in the physical-chemical properties of soft candies sold in the market and those made in the laboratory. The reference soft candies were made from sugar, gelatin, acids, and coloring and flavoring agents. However, the commercial ones usually contain other components that cause incomplete protein isolation. The functional groups detected from the commercial samples were those of gelatin's protein isolates (OH, CH, C=O, CN, and NH), which correspond to Zain & Nugraha (2018) (Table I and Figure 1).

Partial Least Square (PLS) Analysis

PLS was employed to first optimize wavenumbers for calibration and validation models before applying PCA to classify the reference and commercial soft candies according to the source of the gelatin used. Minitab cannot process the entire spectrum; therefore, only the fingerprint regions were selected for the chemometric model. This model was further tested for accuracy based on the coefficient of determination (R^2), root mean square errors of calibration/validation (RMSEC/RMSECV), and the ratio of performance to deviation (Kusumiyati *et al*., 2021). The wavenumbers selected for the calibration and validation models were decided from the region with the highest R^2 and the lowest RMSEC. In addition, the ideal regression coefficients for the model would be a = 0 and b = 1 (Danzer *et al*., 2004) (Table II).

It can be concluded that the wavenumber range of 1600–1621.92 cm^{-1} was the most optimal because it had the highest R^2 (0.999) and the lowest RMSEC (0.188) (Table II). This region represents the spectral profile of the C=O group of amide (Pavia *et al*., 2001). Similarly, Rahmawati *et al*. (2015) confirmed that the 1200–1660 cm^{-1} region shows the presence of protein functional groups and peptide bonds. Therefore, the wavenumbers to be used in the PCA were in the range of 1600–1621.92 cm^{-1}.

The calibration model between the actual absorbance of the reference gelatin obtained from FTIR (*x*-axis) and calculated/predicted values (*y*-axis) for the selected wavenumber region produced the regression equation y = 0.999x + 0.003 (Figure 2). The slope (b) = 0.999 was close to 1, and the intercept (a) = 0.003 was close to 0, indicating an ideal line intersection (Danzer *et al*., 2004). The R^2 for the optimal wavenumber range was 0.999. R^2 is a statistical measure between two variables (Mahyudin *et al*., 2014) (Table II). R^2 close to 1 demonstrates a very strong correlation, meaning the predicted values closely correspond to the actual values. Root mean square error of calibration (RMSEC) shows the degree or error of the calibration results. Generally, there is no ideal standard for RMSEC. However, as a rule of thumb, the smaller the RMSEC or the closer it is to 0, the more accurate the prediction results (Mahyudin *et al*., 2014). RMSEC was the lowest (0.188) for wavenumbers in the 1600–1621.92 cm^{-1} region, confirming that the predicted data would be the most accurate in this optimal range.

Cross-validation or internal validation determines the accuracy of data classification and finds the optimal pattern in the distribution of reference data and test data (Okfalisa *et al*., 2017). Root mean square error of cross-validation (RMSECV) is the most commonly used parameter in evaluating a model. When the measurement level was set at 8, there were two with very high cross-validated residual values, resulting in high RMSECV. Therefore, after omitting them, only six were selected for the regression model. This process produced the regression equation $y = 0.990x - 2.135$, with RMSECV = 2.891. $R^2 = 0.990$ means a very strong correlation between the two variables, although not perfect. However, with RMSECV scoring far above 0, the model is not very accurate.

Table II. Optimized wavenumbers and the calibration model using the PLS multivariate analysis

Wave-numbers (cm^{-1})	R^2	Regression equation	RSMEC (%)
403.5–460	0.997	y=0.996x+0.220	1.755
522.64–582.43	0.999	y=0.999x+0.040	0.751
900–1040	0.894	y=0.893x+7.238	9.396
1517.78–1544.78	0.970	y=0.970x+2.042	5.335
1600– 1621.92	0.999	y=0.999x+0.003	0.188

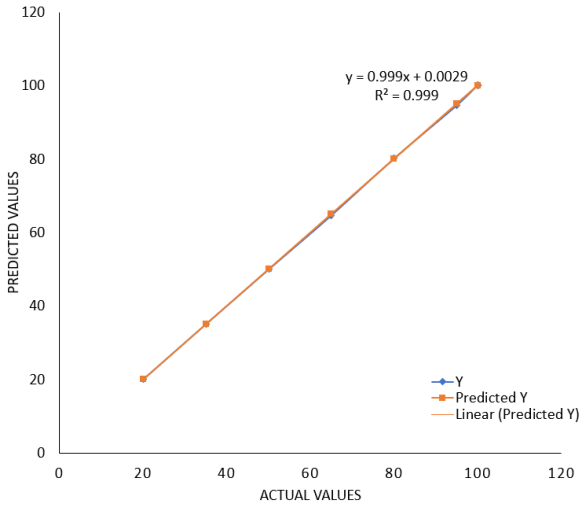

Figure 2. Calibration curve between the actual and predicted values at wavenumbers of 1600 to 1621.92 cm^{-1}.

In addition to cross-validation, gelatins from reference and commercial soft candies were also tested with PLS external validation. When the measurement level was set at 10, there were three with high residuals. Therefore, after omitting them, only seven were used to calculate the root mean square error of prediction (RSMEP) and R^2. Results show RMSEP = 1.652 and the regression equation y = 0.998x – 4.098.

Table III. Summary of the validation and calibration models using PLS and leave-one-out.

Step	Validation Parameter	Parameter Value
Calibration	RMSEC	0.188
	R^2	0.999
	Intercept (*a*)	0.003
Cross Validation	RMSECV	2.891
	R^2	0.990
	Intercept (*a*)	-2.315
External Validation	RMSEP	1.652
	R^2	0.998
	Intercept (*a*)	-4.098

Summarizes of the calibration, cross-validation (leave-one-out), and external validation results (Table III). From the high R^2 (> 0.990) and low RSMEC, RMSECV, and RMSEP, it can be inferred that the model can be used to determine the source of gelatin of commercial soft candies.

Sample Clustering with Principal Component Analysis (PCA)

The principal component analysis (PCA) classified commercial soft candy samples according to the source of the gelatin used by plotting them into the quadrants of porcine gelatin, bovine gelatin, or others. In addition, PCA was also used to sort and reduce data dimensionality from many to fewer variables. The variables referred to here were wavenumbers and absorbances.

PCA is generally run in three stages. First, data standardization is conducted if the data have different units of measurement (e.g., units of length: cm, km, dm) to reduce errors in the matrix. The second step is calculating the covariance or correlation matrix, which is the input data used to obtain eigenvalues and eigenvectors. Eigenvalues state how much variance a principal component (PC) can explain. The third and final stage is dimensional reduction. Only PCs with eigenvalues higher than 1 are selected.

In this study, PCA produced two principal components, each with an eigenvalue > 1 (PC1 = 7.087, PC2 = 4.085). The cumulative sum of PCA up to PC2 was 98.4%. As seen in Figure 3, the resulting score plot shows that porcine gelatin was in quadrant III. This is similar to Zilhadia *et al*. (2018), where PCA also placed porcine gelatin from

experimental vitamin C candies in quadrant III. Meanwhile, the bovine gelatin was in quadrant II. None of the three commercial soft candy samples were in the same quadrant as porcine gelatin. Samples Y and Hrb were in the same quadrant as bovine gelatin, while sample C belonged to neither the bovine nor the porcine gelatin group.

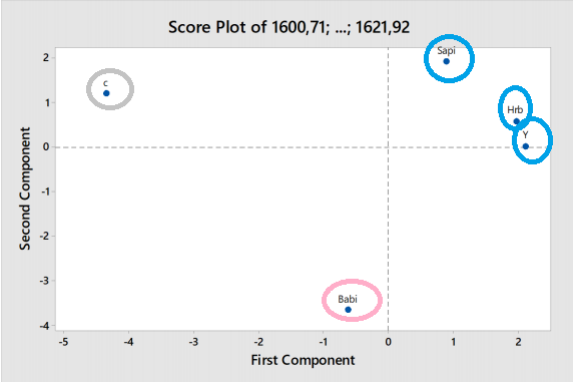

Figure 3. Score plot of gelatins from reference and commercial soft candies (Sample C: a sour soft candy from Malaysia; Y: Yupi Mixed Pastilles from Malaysia; Hrb: Haribo Starmix from Thailand; Sapi: 100% bovine gelatin from the reference soft candy; Babi: 100% bovine gelatin from the reference soft candy.

CONCLUSION

Fourier transform infrared (FTIR) spectroscopy produces the same spectral pattern for both bovine and porcine gelatins. It also shows that bovine gelatin generally has a higher absorbance than porcine gelatin. This analysis, however, only proves that the reference soft candies indeed contain gelatin, but it cannot determine its source. Therefore, this study proposes combining FTIR with chemometrics: partial least square (PLS) and principal component analysis (PCA). Based on PLS, wavenumbers in the range of 1600–1622 cm^{-1} have been detected as the optimal region for gelatin detection, as evidenced by high R^2 and low RMSEC. The resulting regression equation is y = 0.999x + 0.003, with RMSEC = 0.188, RMSECV = 2.891, and RMSEP = 1.652. Further, using PCA, the source of gelatin used in selected commercial soft candies (C, Y, Hrb) has been determined based on their positions in the quadrants. Samples Hrb and Y are in the same quadrant as the reference bovine gelatin, meaning the gelatin they use is made of beef. In contrast, sample C is made with gelatin of an unknown source (neither bovine nor porcine).

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CONFLICT OF INTEREST

The authors declared no conflict of interest in the manuscript.

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