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Gelatin Analysis in Local Soft Candy Products using Fourier Transform Infrared (ATR-FTIR) Combined with Chemometrics

(Analisis Gelatin pada *Soft Candy* Produk Dalam Negeri Menggunakan *Fourier Transform Infrared* (FTIR) dengan Kombinasi Kemometrika)

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Abstract: Gelatin is widely used in the food industry, including soft candy. It is generally sourced from cows and pigs. However, adding porcine gelatin to food will be problematic for the Muslim community due to its prohibition in their beliefs. Therefore, the soft candy's components should be analysed to determine whether the functional groups are of bovine or porcine gelatin. From three local soft candy products in Indonesia, gelatin was isolated with acetone by vortexing and centrifugation. The supernatant was analysed with FTIR to determine its spectral profile. Furthermore, the wavenumbers and absorbances of gelatin's functional groups were analysed in Minitab 18 with multivariate PLS and PCA. FTIR showed that gelatin contained proteins with O-H, aliphatic C-H, C=O, N-H, and C-N groups. PLS and PCA were conducted in the wavenumber range of 1576–1481 cm^{-1}. The calibration yielded R^2 = 0.9997 and RMSEC = 0.9376%. The internal validation showed R^2 = 0.9998 and RMSECV = 1.29%, while the external validation produced R^2 = 0.9996 and RMSEP = 1.04%. Clustering with PCA revealed that the gelatin sample from one soft candy was in the same quadrant as the reference bovine gelatin, while the other two were in different quadrants from both references.

Keywords: Bovine gelatin, chemometrics, FTIR, porcine gelatin, soft candy.

Abstrak: Gelatin banyak digunakan dalam industri pangan, salah satunya pada produk permen lunak (*soft candy*). Gelatin umumnya berasal dari sapi dan babi. Namun, penggunaan gelatin babi akan bermasalah bagi umat Islam karena adanya larangan dalam keyakinan mereka. Oleh karena itu, perlu dilakukan analisis komponen *soft candy* untuk mengetahui perbedaan gugus fungsi antara gelatin sapi dan babi. Gelatin diisolasi dengan aseton menggunakan metode *vortexing* dan sentrifugasi. Supernatan yang dihasilkan kemudian dianalisis dengan spektrofotometer FTIR untuk mengetahui profil spektrumnya. Selanjutnya, dilakukan analisis multivariat *partial least square* (PLS) dan *principal component analysis* (PCA) menggunakan software Minitab 18 terhadap bilangan gelombang dan absorbansi gugus fungsi gelatin. Hasil analisis FTIR menunjukkan bahwa senyawa penyusun gelatin merupakan protein yang terdiri dari gugus O-H, C-H alifatik, C=O, N-H, dan C-N. Analisis PLS dan PCA dilakukan pada bilangan gelombang 1576-1481 cm^{-1}. Hasil kalibrasi menunjukkan nilai R^2 = 0,9997 dan RMSEC = 0,9376%. Hasil validasi internal menunjukkan nilai R^2 = 0,9998 dan RMSECV = 1,29% dan validasi eksternal menghasilkan R^2 = 0,9996 dan RMSEP = 1,04%. Analisis PCA menunjukkan bahwa satu produk *soft candy* berada dalam satu kuadran dengan *soft candy* yang digunakan sebagai referensi gelatin sapi, sedangkan dua sampel lainnya berada di luar kuadran referensi gelatin sapi dan babi.

Kata kunci: FTIR, gelatin babi, gelatin sapi, kemometrika, soft candy.

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INTRODUCTION

GELATIN was widely used in the production of food, such as candy, cake, ice cream, cheese, jelly, and chocolate products. It has unique and beneficial functions, including as a gelling, thickening, and stabilising agent[1]. Gelatin was mainly composed of proteins, varying from 85 to 92%, with some mineral salts and water remaining after the drying that can be found in small amounts[2]. The proteins are derived from collagen and connective tissues in animal bones and skin by hydrolysis[3]. The most common sources were cows and pigs because they were easy to obtain and had good-quality proteins.

The food industry draws on gelatin's binding and gelling properties to produce the chewy texture in confectionery, including soft candy. Soft candy was a processed food that had a soft texture when first chewed and could adhere to the teeth. This candy was made of sugar, water, and gelling components like gelatin. Porcine gelatin was often used to replace bovine gelatin because of its similarity in function but more affordable production costs[4,5]. However, this will be problematic for the Muslim community because their beliefs prohibit the consumption of pork and its derivatives. Thus, it was necessary to analyse the source of gelatin used in this widely liked confectionery product, which can be achieved using Fourier-transform infrared (FTIR) spectroscopy.

Infrared spectroscopy displays information on functional groups through wavenumbers and absorbance. FTIR analyses the fingerprint or characteristic area of a test compound. Vertical and horizontal shifts in absorbance often overlap and cannot be quickly defined. Therefore, a multivariate analysis using chemometrics was needed to see the compound grouping more clearly. For specific grouping, multivariate calibration can be performed using partial least squares (PLS) and principal component analysis (PCA)[6]. This study aimed to distinguish between the functional groups of porcine and bovine gelatins using the combination of FTIR and chemometrics and use the results to determine the type of gelatin isolated from local soft candy products in Indonesia.

MATERIALS AND METHODS

MATERIALS. Bovine gelatin and porcine gelatin were purchased from Sigma-Aldrich (St. Louis, United States). Samples of commercial soft candies from three local brands (MintZ, Relaxa Twish, and Relaxa PLAY) from local supermarket. Sugar, tartrazine dye, citric acid, aquadest, and acetone were purchased from Merck KGaA (Darmstadt, Germany).

Equipments. Analytical balance GR-200 (AND Company Ltd., Tokyo, Japan), vortex (KA Eurostar, Staufen im Breisgau, Germany), centrifuge (IKA Eurostar, Staufen im Breisgau, Germany), FTIR spectrophotometer (Perkin Elmer Spectrum One, Beaconsfield, United Kingdom), and a computer equipped with Minitab 18 software (Informer Technologies, Lps Angeles, California, America).

METHODS. Preparation of Reference Soft Candies. Bovine or porcine gelatin powder was weighed according to the formulation in Table 2 and then moistened with distilled water while stirred in a water bath heated to 50–60°C. In a separate container, 35 g of sugar was moistened with distilled water and added slowly to the moistened gelatin, followed by 1 mL of citric acid. The mixture was stirred, added with one drop of tartrazine dye, and stirred again until homogeneous, as marked by even colour and no lumps. Afterward, it was poured into a mould and left until it stabilised into soft candy[7]. This process produced reference soft candies, from which reference bovine and porcine gelatins were isolated.

Isolation of Gelatin from Soft Candies: References and Commercial Samples. Soft candy weighing 5 g was put into 5 mL of distilled water heated at 60°C then stirred until dissolved. Afterward, 3 mL of this solution was removed to a separate container and added with 12 mL of acetone at -20°C. The mixture was vortexed for 5 minutes and then stored in a freezer for 24 hours at -20°C. The formed precipitate was collected and placed in a separate container, while the supernatant was centrifuged at 6000 rpm for 25 minutes to collect any remaining precipitate. Afterward, all these precipitates were combined and washed three times with 3 mL of acetone at -20°C. Then, the washed precipitate were dissolved by adding two drops of distilled water at 60°C[8].

FTIR Spectroscopy of Gelatin: References and Commercial Samples. At this stage, the research had produced a reference bovine gelatin, a reference porcine gelatin, and three gelatin samples isolated from commercial soft candies; all were prepared in solutions. Each gelatin solution was scanned with an FTIR spectrophotometer in the wavenumber range of 4000 to 400 cm^{-1}. This process produced a spectrum for each reference and sample, from which the spectral profile of gelatin comprising absorbance, wavenumber, and functional group was determined[9,10].

Data Analysis. Absorbance values and wavenumbers were analysed with partial least squares (PLS) and principal component analysis (PCA) in the Minitab 18 programme. PLS and PCA are used if the spectral profile contains intercorrelated variables. PLS analysis included wavenumber optimisation, calibration,

and validation (internal and external), which all used the same method to determine actual and predicted values in Minitab 18. The only difference lies in the calculation of RMSE. PLS and PCA steps were as follows: Several ranges of wavenumbers containing the functional groups were selected for optimisation. Next, the wavenumber and absorbance data were inputted into Minitab 18. After selecting Start menu > Regression > Partial Least Squares in the programme, the PLS window would appear on the screen. On the PLS window, the Responses column was filled with the dependent variable (y), the Model column was filled with predictors (x), and the Categorical predictors column was filled with the number of principal components (PCs) to be calculated or left empty. After clicking Options > Leave-One-Out > OK, the results would appear as worksheet columns showing predicted and actual values[11]. Then, the r-value, regression equation, and RMSE were calculated in Microsoft Excel. After selecting the Data menu > Data Analysis > Regression, x and y were inputted into the Input X and Y Range columns. Then, after ticking the Standardised Residuals box and clicking the OK button, the r value and intercept would appear. The RMSE was calculated as the square root function of actual values (yi), predicted values (), and the number of data points (n), as presented below[12]:

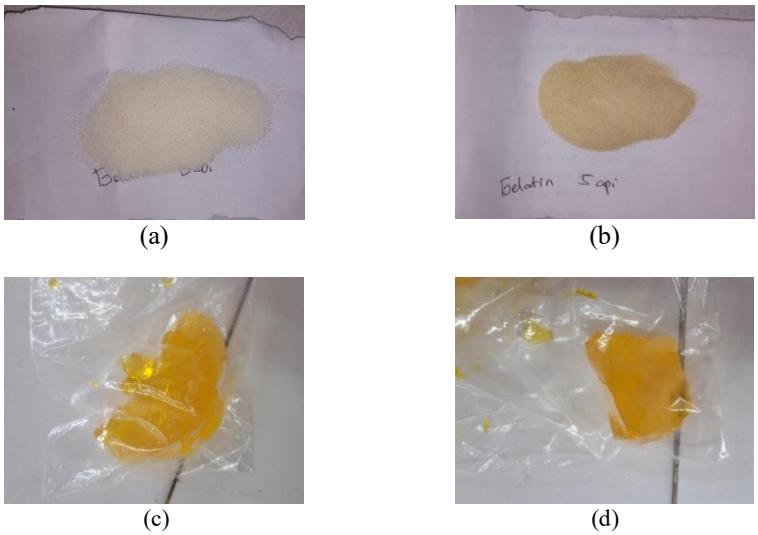
$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)2}{\text{n}}}$$

Furthermore, the previously optimised wavenumbers and absorbance values were inputted into Minitab 18 for PCA. The steps were as follows: After clicking Start menu > Multivariate > Principal Components, the PCA window would appear. The Variables column was filled with the ranges of wavenumbers obtained from FTIR spectroscopy, and covariance was selected as the type of matrix used. After clicking graphs and completing a final check, these results would appear: score plot, eigenvalue, proportion, and cumulative values[12, 13].

RESULTS AND DISCUSSION

FTIR Spectroscopy Results: Gelatin's Spectral Profile Analysis. The reference soft candy was created using a similar method to the one available on the market, except that the main ingredients only consisted of gelatin, sugar, citric acid, and tartrazine colouring[14]. The resulting soft candies made with porcine and bovine gelatin were yellow and had a soft and chewy texture. There were no significant physical differences, even though the gelatin powder had different colours: the porcine gelatin was bone white, while the bovine gelatin was pale yellow, as seen in Figure 1.

Gelatin analysis using FTIR spectroscopy aimed to determine the chemical bond profiles at the peaks of the spectrum and to identify differences between the spectra of the references and samples used. FTIR was a fast, reliable, and relatively simple method. After isolating the main component (i.e., compound) to be observed, it can be input into an FTIR spectrophotometer. In this study, gelatin was isolated with acetone at -20°C; this temperature was selected because it could denature proteins[15]. FTIR then scanned the inputted sample in the mid-IR region (from 4000 to 400 cm^{-1}) to produce spectral profiles from which the functional groups of gelatin were determined.



(a) (b) (c) (d)

Figure 1. Visual appearances of (a) porcine gelatin powder, (b) bovine gelatin powder, and reference soft candies made with (c) porcine gelatin and (d) bovine gelatin.

Figure 2 shows the spectral readings of porcine and bovine gelatins. While they had visually identical patterns (i.e., five peaks at the same wavenumbers), further quantitative analysis found that their absorbance values differed. In FTIR, gelatin was read as a protein compound. Based on Table 1, bands a for both reference gelatins showed peaks at 3281 and 3279 cm^{-1}, indicating the absorption bands of the O-H group[16]. Further, band b of each reference peaked at 2939 and 2935 cm^{-1}, showing a C-H aliphatic with stretching vibration[17]. This corresponds to the asymmetric C-H stretching vibration on the spectrum of type II gelatin analysed in a previous study by Jeevithan, *et al*[18]. Band C of each reference peaked at 1632 and 1629 cm^{-1}, often referred to as the Amide I region, with the stretching vibration of the C=O group on the peptide bond. The following peaks were at 1334 and 1333 cm^{-1} or within the Amide II region, with a bending N-H vibration[18]. Amide I and II were the regions used to analyse secondary proteins and gelatin sources[19]. The last peaks on bands E appeared at 1233 and 1236 cm^{-1}, identified as the C-N group[20].

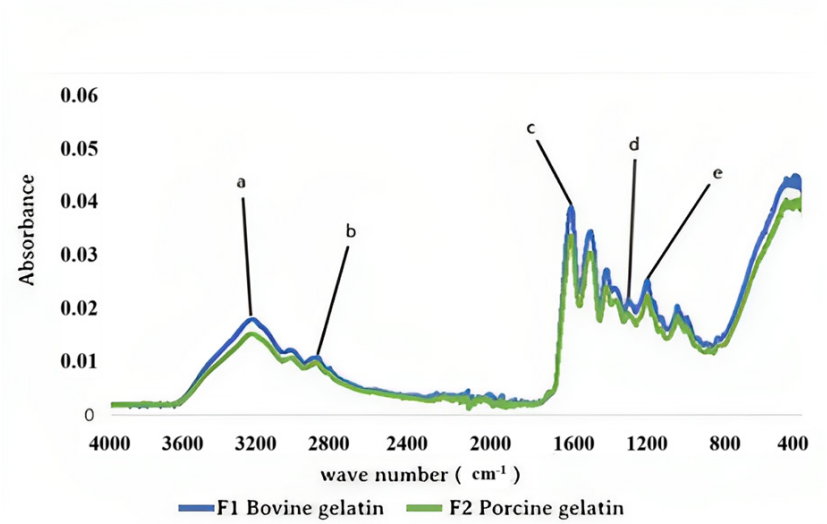


Figure 2. FTIR spectra of the reference bovine and porcine gelatins at 100% concentration.

Table 1. Functional groups of the reference bovine and porcine gelatins at 100% concentration.

Band	Wavenumber (cm^{-1})		Functional group	Vibration model	Intensity
	Bovine gelatin	Porcine gelatin			
a	3279	3281	O-H	Stretching	Medium
b	2939	2935	C-H aliphatic	Stretching	Medium
c	1632	1629	C=O	Stretching	Strong
d	1334	1333	N-H	Bending	Strong
e	1236	1233	C-N	Stretching	Strong

Gelatins were obtained from three local soft candy products: MintZ, Relaxa Twish (rm), and Relaxa PLAY (rs). Solutions of the isolated gelatin were analysed using an FTIR spectrophotometer in the wavenumber range of 4000 to 400 cm^{-1}. As seen in Figure 3, the resulting spectra of the three gelatin samples had similar peak positions to those of the reference bovine and porcine gelatins but with different absorbances. Table 2 summarises the wavenumbers, functional groups, and vibrations of the spectral peaks. FTIR readings showed five functional groups: O-H, C-H aliphatic, C=O, N-H, and C-N. The O-H group appeared in the wavenumber range of 3300 to 3200 cm^{-1}. The aliphatic C-H group generally shows at 2900 cm^{-1}; thus, the three peaks at 2939, 2938, and 2936 cm^{-1} were also considered indicative of this group. Slightly higher wavenumbers than 2900 cm^{-1} were likely due to increased cross-linking between protein-protein molecules through hydrogen bonding of low molecular weight peptides[19]. The protein's carbonyl group (C=O) was detected at different peaks close to 1600 cm^{-1}. Shifts in the peak of the C=O group to lower wavenumbers can be attributed to heating in the isolation process. The last bonds showed the bending N-H and the stretching C-N vibrations from 1300 to 1200 cm^{-1}. This region contains vibrations that correspond to groups found in proteins, fatty acids, polysaccharides, and phosphate derivatives.

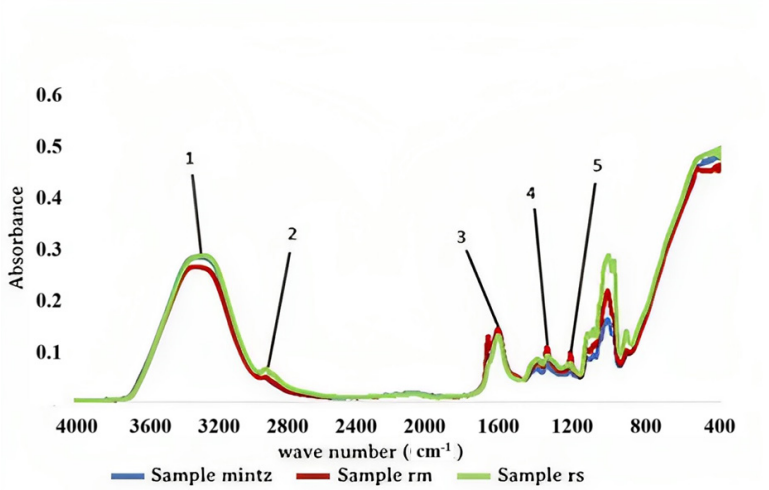


Figure 3. FTIR spectra of gelatin samples isolated from local soft candy products.

Table 2. Functional groups of gelatin in soft candies from three local brands: MintZ, Relaxa Twish (rm), and Relaxa PLAY (rs).

Band	Wavenumber (cm^{-1})			Functional group	Vibration model
	Mint Z	rm	rs		
1	3306	3341	3272	O-H	Stretching
2	2939	2938	2936	C-H aliphatic	Stretching
3	1694	1696	1640	C=O	Stretching
4	1369	1369	1368	N-H	Bending
5	1238	1237	1238	C-N	Stretching

PLS Calibration and Validation Models. Without distinctive features that could determine whether the gelatin samples were bovine or porcine, the spectral profiles were further analysed using PLS for calibration and validation and PCA for quadrant grouping[21]. PLS and PCA were multivariate analyses used to classify similar properties of a material or substance[14].

PLS was one of the quantitative multivariate analysis methods. In concept, it selects highly correlated variables that can effectively project the response variable[22]. Accordingly, it can predict unknown concentrations by reconstructing spectra from the loading spectra[14]. It uses parameters to correlate predicted values (*x*-axis) with actual values (*y*-axis). PLS modelling uses a linear regression equation $y = bx + a$, where y was the predicted value obtained from FTIR spectroscopy, b was the gradient slope, x was the actual analyte content, and a was the intercept, i.e., the intersection between the y-axis and the slope of the line[22].

Before performing PLS, the wavenumbers to be modelled for calibration and validation should be optimised. The selected wavenumbers were in the regions that contain functional groups, called fingerprint regions. The wavenumbers chosen for the optimal calibration model had the highest coefficient of determination (R^2), or the closest to 1, and the lowest root mean standard error of calibration (RMSEC)[21]. Suppose R^2 was close to 1. In that case, there was a strong correlation between the independent and dependent variables. At the same time, the smaller the RMSEC, the less error the calibration model has[9].

As seen in Table 3, the optimised wavenumbers for calibration and validation were in the range of 1576 to 1481 cm^{-1}. The selected wavenumbers can differ from one study to another, even with the same sample (gelatin). For instance, Cebi *et al.* classified gelatin from gummies using ATR-FTIR combined with PLS, HCA, and PCA[17]. They used wave numbers in the 1734-1528 cm^{-1} region for PLS calibration and validation. The calibration model produced the equation $y = 0.9997 + 0.0515$ with $R^2 = 0.9997$ and RMSEC 0.9376%. This suggests that the independent variable (*x*-axis) can explain 99.97% of the dependent variable (*y*-axis). The correlation curve between the actual (*x*-axis) and predicted values (*y*-axis) of the calibration model in the wavenumber range of 1576–1481 cm^{-1} was shown in Figure 4.

The predictive model was validated using the leave-one-out feature in Minitab 18, which removes one piece of data and uses the remaining data to create a new model. This validation was also known as internal validation. A predictive model was considered valid if the resulting root mean square error of cross-validation (RMSECV) was low, with R^2 close to 1[12]. Figure 5 shows the correlation curve between the actual (*x*-axis) and predicted values (*y*-axis) using the new model. The curve equation was y = 0.9998x - 0.31104, with R^2 = 0.9998 and RMSECV = 1.29%. The smaller the RMSECV, the better or more accurate the predicting ability of the built model.

In addition, the external validation of the regression model produced a correlation curve with the equation y = 0.9996x - 0.68705, R^2 = 0.9996, and root mean square error of prediction (RMSEP) = 1.12%, as seen in Figure 6. These figures indicated a strong correlation between the predicted and actual values. In other words, with these R^2 and RMSEP values, the model could accurately project the data[12].

Table 3. Wavenumber optimization for multivariate calibration with partial least squares (PLS).

Wavenumber $(cm)^{-1}$	Coefficient of Determination (R^2)	RMSEC	Equation
1691–1601	0.9666	5.9328	y = 0.9666x + 2.2850
1576–1481	0.9997	0.9376	y = 0.9997x + 0.0515
1302–1228	0.9991	0.9506	y = 0.9991x + 0.0586
1576–1228	0.9962	1.9831	y = 0.9962x + 0.2554
1200–1001	0.9936	2.5805	y = 0.9936x + 0.4323
1011–901	0.9727	5.3634	y = 0.9727x + 1.8675

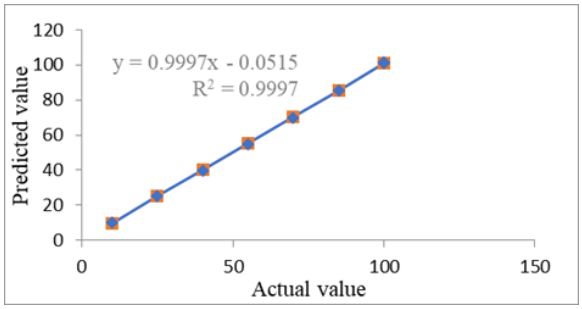


Figure 4. Correlation curve between actual (*x*-axis) and predicted values (*y*-axis) derived from the calibration model at wavenumbers 1576–1481 cm^{-1}.

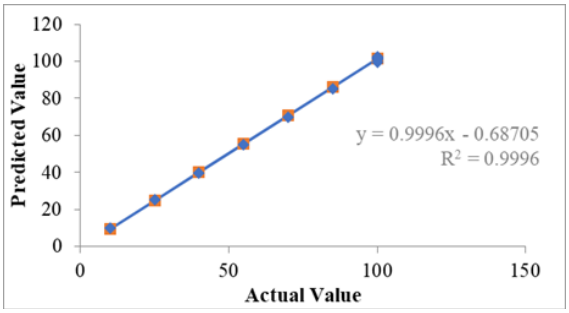


Figure 6. Correlation curve between actual (*x*-axis) and predicted values (*y*-axis) derived from the external validation model at wavenumbers 1576-1481 cm^{-1}.

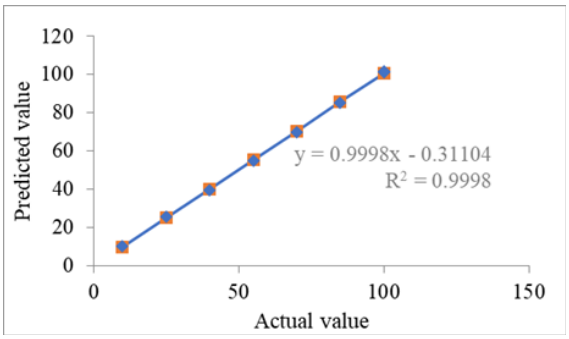


Figure 5. Correlation curve between actual (*x*-axis) and predicted values (*y*-axis) derived from the internal validation model at wavenumbers 1576-1481 cm^{-1}.

Data Processing Using PCA. PCA groups data based on the correlation between variables. PCA works in this way: if objects with principal components (PCs) have similar physicochemical properties, clustering will occur [21].

The absorbance data of the reference bovine and porcine gelatins and three gelatin samples were input into PCA. This process produced a score plot and PC values. PCs were also called *eigenvectors*; the technique to find them was called *eigenanalysis*. The result of *eigenanalysis* was *eigenvalues*, which indicate the amount of variance in the data explained by PC[6]. The eigenvalue will decrease as the number of the main component (PC) increases. Very low eigenvalues mean the matrix has very little or even no variance. Table 4 shows the number of PCs and their respective eigenvalues, proportions, and cumulative proportions. The *proportion* was the amount of variance explained by each PC as a proportion of the total, and the *cumulative proportion* refers to the accumulated proportion[14]. As seen in the table, one principal component, i.e., PC1, could obtain 99.1% of the variation in the data. This was confirmed by the eigenvalue of PC1, which was positive and close to zero.

Table 4. Principal component analysis (PCA) of the reference bovine gelatin, reference porcine gelatin, and gelatin samples.

	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
Eigenvalue	0.014765	0.000104	0.000027	0.000009	0.000	0.000	0.000
Proportion	0.991	0.007	0.002	0.001	0.000	0.000	0.000
Cumulative	0.991	0.998	0.999	1.000	1.000	1.000	1.000

Figure 7 shows the score plot produced in the PCA, which grouped the references and samples of porcine and bovine gelatins into one of the four quadrants. The reference bovine gelatin was in quadrant II, while the reference porcine gelatin was in quadrant III. One of the samples, Mintz, was in quadrant II, suggesting that this soft candy contains gelatin with the same physicochemical properties as the reference bovine gelatin[6]. In other words, this product uses bovine gelatin as the gelling agent. On the contrary, the other two samples labelled with rm and rs were quadrant IV and I, respectively. Both quadrants contained no reference gelatins. Therefore, it can be said that both rm and rs have no common physicochemical properties with either the reference porcine or the reference bovine gelatin[15].

The reference soft candy was prepared with a simple technique and fewer ingredients than the ones made in the factory. As a result, the gelatin in each sample might have different compositions. In addition, there were many brands of gelatin on the market, meaning the gelatin used in the reference soft candy might not be the same as the one added to the commercial soft candy products. This potentially affects the collagen properties of the gelatin and, consequently, the score plot resulting in the PCA[23]. According to Zilhadia et al., the conformation of gelatin can also change when it goes through heating and stirring or is combined with additional substances like polysaccharides (sugars). Changes in gelatin structure will produce different PCA results.

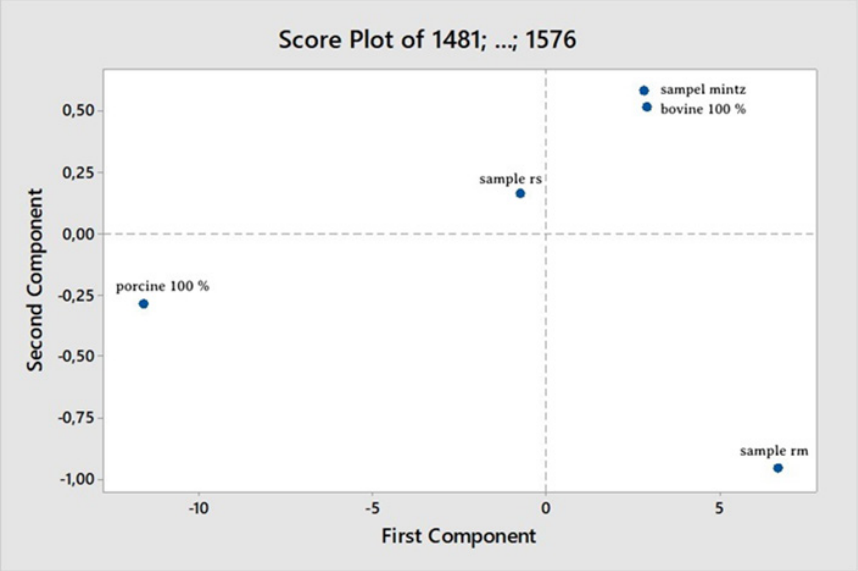


Figure 7. Score plot of the reference bovine gelatin (100 %), reference porcine gelatin (100 %), and gelatin samples from three local soft candy products (Mintz, rm, and rs).

CONCLUSION

Fourier-transform infrared (FTIR) spectroscopy can be used to distinguish the functional groups of bovine and porcine gelatins by identifying their respective chemical compounds, which are components of proteins: O-H, C-H aliphatic, C=O, N-H, and C-N groups. Partial least squares (PLS) calibration at the optimised wavenumbers, 1576 to 1481 cm^{-1}, produced a coefficient of determination (R^2) = 0.9997 and RMSEC = 0.9376%. The internal validation results showed R^2 = 0.9998 and RMSECV = 1.29%, and the external validation yielded R^2 = 0.9996 and RMSEP = 1.12%. Clustering using a chemometric method, i.e., principal component analysis (PCA), has detected that one soft candy product uses bovine gelatin and that the other two products use neither bovine nor porcine gelatin.

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