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Rapid Detection of Pork Gelatin in Hard Shell Capsules on Supplement Products Using FTIR and PCA

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A B S T R A C T

This study aimed to develop a fast and low-cost detection method to determine the difference between hard shell capsules on supplement products containing pork or non-pork gelatin using Fourier Transform Infrared (FTIR) spectroscopy and Partial Component Analysis (PCA). Twelve hardshell capsules were used, among which Eight samples were pure made from pork and some adulterated with cow. The samples were detected before using *real-time* PCR to determine the identity of the samples. All the gelatin samples were measured with the FTIR spectrophotometer in the reflection mode. Spectra were collectedin the wavenumber range from 4000-650 cm^{-1}. The results show that the PCA model with the data was pre-processed before PCA was performed. The absorbance data from FTIR were pre-processed using the *Savitsky-Golay* smoothing technique and continued with normalization by peak normalisation before being subjected to PCA. This newly developed method is fast, involves simple sample preparation and islow-cost.

1. INTRODUCTION

1.1. Research Background

A highly processed protein called gelatin is frequently used as a thickening and gelling agent (E441). Collagen comes from items like bones, hides, and skins from animal slaughterhouses and is hydrolyzed to create gelatin [1]. Controlled basic or acidic hydrolysis of connective tissue raw material, high-temperature water extraction, sterilization, and drying are all steps in gelatin manufacturing. These procedures are not standardized and impact the final gelatin product's characteristics, making them challenging to distinguish. Numerous studies on analytical techniques that can identify the difference between porcine and bovine gelatins have been published. For instance, real-time PCR [2,3,4,5,6,7,8].

1.2. Literature Review

The real-time PCR technique is an advancement over traditional PCR techniques. Despite the simplicity of the traditional PCR procedure, quantitative information cannot be obtained from the PCR results [9]. Salamah, in 2021, studied the use of real-time PCR combined with the primer of cyt-b to distinguish the gummy bear made from porcine and bovine. The developed technique is precise and trustworthy for acknowledging gelatin sources in food and pharmaceutical procedures [9].

Bovine and porcine gelatins can also be distinguished by amino acid analysis and enzyme-linked immunosorbent assay (ELISA) [2,4,6,7]. However, both techniques require repeated results and experience because the sample preparation is so difficult and sensitive. Instead of focusing on gelatin identification, most reported approaches have emphasized meat species identification. However, as damaged proteins and nucleic acids result from prolonged heat processing, it can be challenging to recover high-quality DNA, a necessary need. The unfortunate thing about this method is that it comes from the cost of detection—the sample preparation is also one of many disadvantages things in PCR-based analyses [2,3,4,5,6,7,8].

Knowing the origins of gelatin is crucial because Muslim nations expressly restrict the use of pig products and because there are worries about possible disease transfer to humans [10,11]. Methods that rely on physicochemical properties, such as infrared spectroscopy, have been proven suitable for differentiating a pork mixture.

For instance, it has been demonstrated that Fourier Transform Infrared Spectroscopy (FTIR) is a very effective technique for determining various adulteration issues in food products, such as lard content in various food processing products [12, 13, 14].

Suad *et al.* (2023) concluded that NIR spectroscopy in reflectance mode and PLS-DA may be used for a quick, inexpensive and non-destructive approach to detecting ice cream samples containing non-Halal (pork) gelatin. Based on a compromise between the lowest value of RMSECV = 0.147 for the calibration set and the lowest value of RMSEP = 0.098 for six factors with 100% classification among the samples of both samples containing Halal and Non-Halal gelatins for both the calibration and prediction set of samples, the results showed that the PLS-DA models with unit vector normalisation are the suitable method [13].

1.3. Research Objective

From the previous study, we used reflectance FTIR spectroscopy in combination with principal component analysis (PCA) as a quick, inexpensive, and non-destructive detection approach to distinguish between hard shell capsules containing halal (pork) and non-halal (pork) gelatin.

2. MATERIALS AND METHODS

2.1. Preparation of substrates

Gelatin capsules from supplement products with *halal* logos from different countries were purchased and used in this study. The samples were tested by real-time PCR to determine the identity of the sample, and the suspected result was continued to the FTIR method. Twelve samples were chosen from twenty samples (Table 1).

Table 1. Result of Real-Time PCR for Gelatin Capsules

Sample	Concentration (ng/µl)	Remarks
	Mean ± SD	
S1	4.85 ± 0.21	Mixture (M1)
S2	3.23 ± 0.34	Bovine (B2)
S3	2.95 ± 0.05	-
S4	3.90 ± 0.02	Mixture (M4)
S5	5.08 ± 0.06	Porcine (P5)
S6	6.14 ± 0.82	Porcine (P6)
S7	4.61 ± 1.07	-
S8	4.41 ± 0.91	-
S9	2.50 ± 0.17	Mixture (M9)
S10	3.23 ± 0.01	-
S11	5.01 ± 0.14	Mixture (M11)
S12	4.72 ± 0.16	Mixture (M12)
S13	4.43 ± 0.01	Bovine (B13)
S14	4.67 ± 0.01	Bovine (B14)
S15	3.05 ± 0.41	-
S16	2.16 ± 0.18	-
S17	1.94 ± 0.07	Bovine (B17)
S18	3.78 ± 0.54	Mixture (M18)
S19	4.82 ± 0.33	-
S20	4.30 ± 1.02	-

The capsules were cleaned using distilled water, dried, weighed to 0.65 g, and put in a centrifuge tube for FTIR analysis.

2.2. FTIR analysis

Gelatin can be analyzed using FTIR in one of two ways: (1) by comparing the spectrum of a gelatin standard (Figure 1) with a synthetic gelatin capsule spectrum pattern. (2) Calculate the absorbance values deduced from the spectrum's created peaks. The interaction of energy and matter is the basis for FTIR spectroscopy. The frequency range used for the analysis was 4000-650 cm^{-1}. The total absorbance value in the FTIR spectrum would be used for the cluster analysis utilising PCA.

Using Unscrambler X 10.3, PCA methods were used to evaluate the data. The primary goal of the PCA technique is to differentiate the amino acid content of the gelatin capsule in the supplement product. Before PCA was used, the data were pre-processed. Prior to being subjected to PCA, the FTIR absorbance data were pre-processed using the *Savitsky-Golay* smoothing approach and peak normalisation.

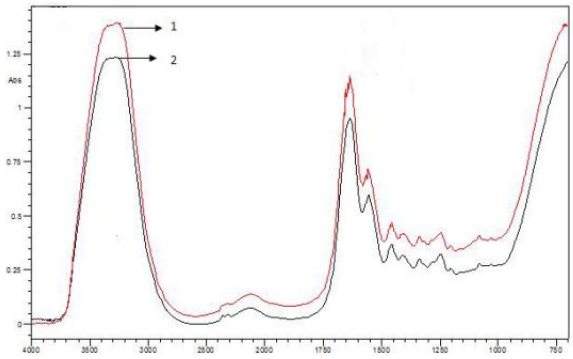

Fig. 1. FTIR Spectra for (1) Bovine Gelatin and (2) Porcine Gelatin [15]

3. RESULT AND DISCUSSION

The importance of IR spectroscopy for qualitative analysis comes from much information obtained and the possibility of assigning certain absorption bands related to the functional groups. In gelatin, most of the peaks and shoulders of the spectrum are attributable to specific functional groups [16]. Figure 2 shows the FTIR spectra of gelatin capsules from the previous study. The spectra look very similar and show a typical absorption band of gelatin structure (Figure 1). Four regions involved are 3600-2300 cm^{-1} (Amide A), 1656-1644 cm^{-1} (Amide I), 1560-1335 cm^{-1} (Amide II) and 1240-670 cm^{-1} (Amide III). A typical gelatin capsule spectrum showed low intensities of Amides A, I, II and III bands, with the Amide III band almost non-existent in a few samples (Figure 2). This is consistent with changes expected because of the denaturation of collagen to gelatin. A very low intensity showed for the Amide III region is associated with loss of triple helix state during high-temperature gelatin extraction [14].

The Amide A (3600-2300 cm^{-1}) region is donated by hydrogen-bonded amide groups' N–H bond-stretching mode. The absorption is polarised parallel to the N–H bond, parallel to the helix axis in a-helical structures and perpendicular to the polypeptide chain in b-sheets.

The band might be shifted to a lower frequency when the hydrogen bonding strength increases [17]. The carbonyl C=O

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double bond-stretching mode, with contributions from in-phase bending of the N–H bond and stretching of the C–N bond, occurs in the frequency range 1660-1620 cm^{-1} region, often referred to as the Amide I band. The frequency range 1660–1650 cm^{-1} was known as a-helical and 1640-1620 cm^{-1} as b-sheets structures.

The frequency range of 1550-1520 cm^{-1} is due to Amide II with a-helical structure between 1550-1540 cm^{-1} and b-sheets at 1525-1520 cm^{-1}. The Amide II vibration is caused by the deformation of the N–H bonds. Fischer *et al.* (2005) and Lagant *et al.* (1983) attributed 1500-1200 cm^{-1} to CH_2 deformation. It is known that this region contains vibrations corresponding to groups present in fatty acids, proteins, polysaccharides and phosphate derivatives [18,19].

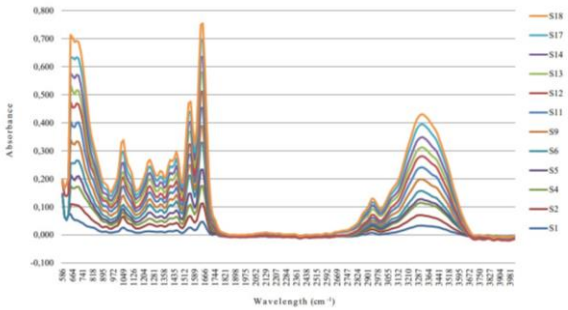

Fig. 2. FTIR Spectra of Gelatin Capsules. The figure showed the enlarged FTIR spectra at fingerprint regions. The different peaks in terms of peak intensity were used for selecting the spectral regions for the quantification and classification of gelatin capsules in supplement products.

Spectral region (frequency) selection is the major problem in FTIR analysis because the chosen frequency regions must be chosen so that the ones that describe the most characteristic analytes are to be determined and provide non-interfered data for the analytes. The FTIR spectra showed that the maximum absorption in gelatin capsules is at the Amide A and Amide I regions. This can be easily used to differentiate these compounds and identify the chemical forms of the supplement's gelatin ingredients, porcine and bovine. The chemometrics of Principal Component Analysis (PCA) classified gelatins with porcine and bovine. The wave number regions for PCA were also optimised based on their capability to separate between pig and adulteration present in gelatin capsules. The optimal wave numbers used for quantitative analysis (Amide A, I and II), was chosen for PCA (Table 2).

Table 2. The Absorbance Value of FTIR Spectroscopy in Selected Regions

Sample		Wavelength (cm^{-1})							
		694	1250	1342	1404	1450	1558	1636	3271
S1	Absorbance	0.053	0.013	0.011	0.014	0.016	0.026	0.046	0.032
S2		0.054	0.026	0.022	0.025	0.027	0.041	0.064	0.037
S4		0.066	0.020	0.017	0.021	0.022	0.041	0.064	0.040
S5		0.035	0.022	0.016	0.019	0.023	0.040	0.059	0.013
S6		0.059	0.028	0.022	0.028	0.032	0.063	0.097	0.028
S9		0.068	0.019	0.020	0.022	0.023	0.037	0.059	0.040
S11		0.066	0.025	0.020	0.024	0.026	0.040	0.064	0.041
S12		0.069	0.022	0.017	0.022	0.024	0.035	0.058	0.038
S13		0.047	0.026	0.022	0.024	0.028	0.045	0.068	0.029
S14		0.055	0.022	0.019	0.022	0.025	0.035	0.055	0.035
S17		0.063	0.023	0.020	0.023	0.026	0.037	0.059	0.043
S18		0.058	0.023	0.019	0.022	0.025	0.035	0.056	0.034

Direct observations of spectra highlights are frequently used. However, statistical comparison of spectra can also be useful. Numerous multivariate techniques are available to allow statistical and mathematical comparison of the spectra of the same substance under various circumstances. Overlapping band components and challenges with band assignments from different complex amino acids, such as proline, hydroxyproline, and glycine, are two of the limitations of FTIR in drawing quantitative findings. Principal component analysis (PCA) may be used to solve this issue and analyse the spectra. A multivariate PCA approach reduces the number of dimensions by grouping numerous related variables and spectral data points [20]. PCA is a factor-based, chemometric method. In this case, the factors would be the relevant wavenumbers (Table 2).

To investigate the structural characteristics of polymeric substances like gelatin capsules, infrared spectroscopy is used. However, in many instances, other interfering bands make it challenging to determine the wavelength and absorbance of specific bands. Differentiating IR spectra can be used to solve such issues. Smoothing and normalising data are the most often used preprocessing techniques for multivariate analysis or calibration. There are several methods for mathematically separating a spectrum. The most popular and common smoothing calculation is via the *Savitzky-Golay* method. The method proposed and described by Savitzky and Golay (1964) calculates up to the ninth derivative and adjusts a convoluting function to give a desired derivative order and degree of smoothing [21]. These different derivatives of all the spectra lay a foundation or hold some significance, to be used as a reference and guide for data input into statistical programs such as PCA. The normalisation of the data to the area under the entire spectrum was applied due to the nonexistence of a most intense yet static spectral band during treatment and varying concentrations of gelatin capsules. Normalisation is a common pre-processing step and is required for adequate PCA results. This treatment is employed because it deletes differences between spectra due to different amounts of sample and path length variation [22,23]. Normalising spectra does not substantially affect the relative intensities within a spectrum.

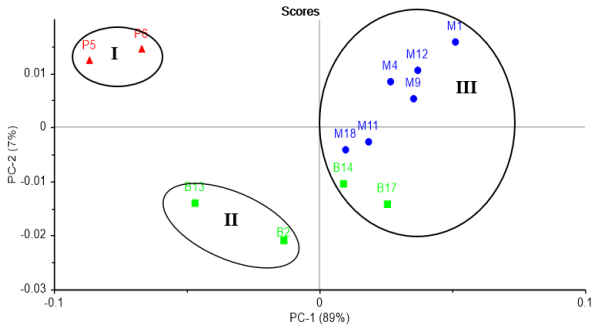

Fig. 3. PCA Scores Plot of FTIR Spectra of Gelatin Capsule

Supplements. PC1 vs. PC2. PCA was pre-treatment with *Savitsky-Golay* and area normalization, and the S code would be changed to the specific classification where number P is code for porcine, B is code for bovine; and M is code for the mixture.

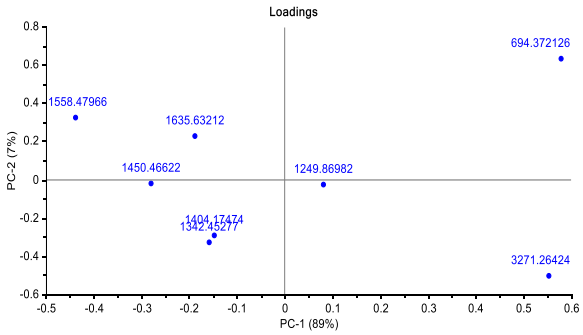

Fig. 4. PCA loading plot (PC1 vs. PC2)

Figure 3 depicts the normalization and flattening of the gelatin capsules' FT-IR spectra. To show the variables on an x, y coordinate system, PCA manipulates the data of variables (peak height of amino acids). PCA achieved this by determining principal components (PC1), a linear combination of the original variables. The first principal component, or PC1, accounted the majority of the variation in the data, while the second principal component, or PC2, explained the remainder. PC1 and PC2 were orthogonal. Figure 3 exhibited the PCA score plot of porcine and bovine gelatins from commercial capsule shells. Bovine and porcine gelatins were separated. PC1 described 89% variation of data, while PC2 and PC3 account for 7% and 3% variations, respectively. Therefore, more than 90% of variation can be described only by three PCs. Figure 4 illustrates the loading plot for determining variables (wavenumber) contributing to the differentiation and separation of the samples. The projection of variables in the same plane as the PCA loading plot specified the score plot. The loading plot's absolute value in wavenumber explains the significance of each amino acid's contribution. As a result, an amino acid's contribution to the PCA model increases with wavenumber distance from the origin of the variable point [24,25]. Figure 4 showed that the factors with the greatest influence on PC1 were 694 and 3271 cm-1, whereas PC2 was more influenced by 1558 and 1635 cm^{-1}. Based on Figure 4, it was seen that the PCA plot showed that profiles were significantly different in the region 694, 3271, 1558 and 1635 cm^{-1}. 3271 cm^{-1} in PC1 contribution indicates the presence of an aliphatic N-H stretching region in a peptide bond. The second difference lies in the frequency of 1558 cm^{-1} and 1635 cm^{-1}, which indicate a C-N-H bending and C=O stretching of the peptide bonds [25].

4. CONCLUSION

Some capsule shells available in numerous pharmacies were investigated by determining the level of amino acid contents and subsequently subjected to PCA. The results showed that PCA could not distinguish the adulteration in capsule shells, as indicated by the irregular profile of the PCA score plot of capsule shell samples, where the sample was very close to bovine gelatin. It could be explained that the capsule shells assayed in the adulteration sample may have the same concentration of bovine gelatin. Hence, the adulteration has the same spot as bovine gelatin. As mentioned previously, the composition of the gelatin structure had similar vibrational frequencies, indicating similar content. A decrease and increase in intensity would indicate less amino acid material.

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