Chemical and Chemometric Methods for Halal Authentication of Gelatin: An Overview

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Abstract: The issue of food authenticity has become a concern among religious adherents, particularly Muslims, due to the possible presence of nonhalal ingredients in foods as well as other commercial products. One of the nonhalal ingredients that commonly found in food and pharmaceutical products is gelatin which extracted from porcine source. Bovine and fish gelatin are also becoming the main commercial sources of gelatin. However, unclear information and labeling regarding the actual sources of gelatin in food and pharmaceutical products have become the main concern in halal authenticity issue since porcine consumption is prohibited for Muslims. Hence, numerous analytical methods involving chemical and chemometric analysis have been developed to identify the sources of gelatin. Chemical analysis techniques such as biochemical, chromatography, electrophoretic, and spectroscopic are usually combined with chemometric and mathematical methods such as principal component analysis, cluster, discriminant, and Fourier transform analysis for the gelatin classification. A sample result from Fourier transform infrared spectroscopy analysis, which combines Fourier transform and spectroscopic technique, is included in this paper. This paper presents an overview of chemical and chemometric methods involved in identification of different types of gelatin, which is important for halal authentication purposes.

Keywords: chemical methods, chemometric, food analysis, food products, food science, gelatin, halal authentication, mathematical

Introduction

Food and pharmaceutical products are essential needs for human beings. In line with industrialization and globalization, these products have to meet the demand of society along with technological developments in biochemistry. The advancement of technology in the industries has led to fraud in the product and it may contain unknown ingredients including nonhalal substances. Some of the products in the market may have been labeled with incorrect information regarding the source of the ingredients (Montowska & Pospiech, 2010). This has become an issue for some religious adherents such as the Muslims, since the status of the raw materials used for the ingredients and the production process may not fulfill the religious requirements. The consumption of halal foods and products is compulsory to all Muslims (Al-Qaradhawi, 1994).

One of the nonhalal substances commonly found in food and pharmaceutical products is gelatin (Mursyidi, 2013). Gelatin has traditionally been produced and used as ingredients in food products such as desserts, jelly, and some dairy products (Nollet & Toldra, 2011). It is also commonly used in pharmaceutical industries for capsule manufacture (Duconseille, Astruc, Quintana, Meersman, & Sante-Lhoutellier, 2015; Jeevithan, Qingbo, Bao, & Wu, 2013; Zhang et al., 2013). Gelatin is a protein derived from collagen of animal through partial hydrolysis (Johnston-Banks, 1990). It has numerous applications in food and pharmaceutical products because of its gelling and thickening properties. The physical and structural properties of gelatin such as its gel strength and viscosity depend on their molecular weight distribution and amino acid composition (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). Gelatin has a unique sequence of amino acids. The amino acid composition and its sequence differ from one source to another. Gelatin is normally extracted from mammals, mainly porcine and bovine (Karim & Bhat, 2008). In recent years, fish gelatin has gained importance as an alternative to the mammalian gelatins and has become one of the main commercial sources of gelatin in halal market (Al-Mazeedi, Regenstein, & Riaz, 2013; Lin, Regenstein, Lv, Lu, & Jiang, 2017).

Non-Islamic countries are the major suppliers and manufacturers of gelatin, with Europe and America being the most prominent manufacturers along with Russia, India, China, and Southeast Asia (Cochrane, 2016). In Malaysia, the suppliers of Halal food products have to face major challenge in offering Halal products due to the possible presence of nonhalal gelatin in the products (Said, Hassan, Musa, & Rahman, 2014). The presence of undeclared prohibited ingredients in food products has raised concerns among Muslims about the halal status of their food (Lubis, Mohd-Naim, Alizul, & Ahmed, 2016). According to Al-Qaradhawi (1994), the *Quran* has stated the raw materials which Allah prohibited and one of them includes pork or flesh of pigs. Hence, it is essential for Muslims to authenticate the sources of the raw materials used, particularly porcine source since it is prohibited for Muslims consumption. Authentication is the process by which a product is verified as fulfilling with its label description (Dennis, 1998). Authentication tests usually involve analytical techniques, which are applicable in dealing with particular samples, depending on the state of the sample itself (Hargin, 1996). In Malaysia, for example, since the majority of the population is Muslim, the Malaysian government is concerned with the religious principles and obligations (Al-Nahdi, Ismail, Haron, & Islam, 2008). Hence, the Dept. of Islamic Development Malaysia (JAKIM) has launched a Halal Assurance System as a guidance that is manifested in the form of Malaysia halal certification for each food product (JAKIM,

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2015). Several laboratories in Malaysia have been selected as Halal Panel Laboratories, including an exclusive halal laboratory for halal authentication test known as Malaysia Halal Analysis Centre (MyHAC) (Ahmad, Abidin, Othman, & Rahman, 2018). These laboratories are responsible for verification process in applying for halal certification. However, suitable techniques and more sensitive and robust detection methods are needed to support the halal authentication process (Van der Spiegel et al., 2012).

Since gelatin is derived from various sources including porcine, bovine, and fish, it is paramount to develop a fast and robust method to distinguish these types of gelatin for halal authentication. One way to identify the different gelatins is through the study of their intramolecular structures using chemical techniques (Hermanto, Sumarlin, & Fatimah, 2013). A number of review articles on chemical techniques for differentiation of gelatins have been published (Ali et al., 2016; Nhari, Ismail, & Che Man, 2012; Rohman & Che Man, 2012). To our knowledge, a review study on chemometrics involving mathematical and statistical methods is not yet available. Hence, this paper presents an overview of chemical and chemometric methods on identification of gelatin for halal authentication purposes.

Chemical Methods

Analytical methods are becoming increasingly important for halal authenticity in food and pharmaceutical products. Numerous analytical methods have been developed to deal with the emerging issues as they are the only means of scientific validation for the halal authentication so far. Few studies have been published to differentiate the sources of gelatin, mainly bovine and porcine due to the concerns on halal status of the products and health issue involving bovine. Some of the main chemical methods used for the authentication analysis are biochemical, chromatography, mass spectrometry, electrophoresis, and spectroscopic techniques.

Biochemical technique

Hidaka and Liu (2003) used a chemical analysis called pH drop method to study the effects of different gelatins on calcium phosphate precipitation. The study was conducted to differentiate bovine bone gelatin from porcine skin gelatin and commercial gelatin products, Akyo and cooking jelly. The study is concerned with the involvement of bovine bone gelatin which may lead to bovine spongiform encephalopathy (BSE) disease. The samples were analyzed based on induction time and concentration effect in transformation of hydroxyapatite (HAP), and reaction rates of amorphous calcium phosphate (ACP), both after calcium phosphate precipitation. The method was able to distinguish the gelatins by measuring the effects on induction time and analyzing the peak concentration for stimulation of HAP transformation. The induction time for bovine bone gelatin was significantly longer compared to the porcine skin gelatin, while different peak concentrations were recorded for bovine and porcine gelatins at 0.5 and 4.0 mg/mL, respectively. The significant differences were determined by statistical method called principal component analysis (PCA). Since this study is one of the earliest attempts on differentiating the bovine and porcine gelatins, it can be referred to as an alternative to halal authentication study in identifying the presence of porcine. However, the analysis on ACP formation was unable to create clear results. Besides, this method is limited for analysis of gelatin from bovine bone and porcine skin and incapable of differentiating various sources of gelatin in food products. The pH drop method based on the ACP formation and HAP transformation may not be able to effectively differentiate and identify the sources of gelatins in food products due to their reactivity toward the possible interferences in the food products.

Venien and Levieux (2005) introduced the enzyme-linked immunosorbent assay (ELISA) method to identify bovine and porcine gelatins. The method was developed in response to the BSE disease issue and prohibition by some religions on the use of porcine gelatin for human consumption. It is a biochemical technique, which involves the quantification of biologically molecular interaction and normally used in immunology to determine the presence of antibody and antigen. In the study, bovine-specific antibodies obtained from immunized rabbits against species-specific sequences of the bovine collagen alpha 1(I) chain were employed for identification of bovine and porcine gelatins. The study selected putative species-specific sequence from the N-terminal sequence as peptide 1 and peptide 2 were selected from the central region of the bovine alpha 1(I) chain. Two approaches of ELISA method, namely indirect ELISA and competitive indirect ELISA, were then developed for the differentiation analysis. An indirect ELISA involves the use of primary antibody which incubated with specific antigen and an enzyme-linked secondary antibody which causes chromogenic or fluorogenic substrate to produce a signal. The results in indirect ELISA indicated that antipeptide 2 antibodies show strong reactivity compared to antipeptide 1 when tested against gelatin and collagen. The antipeptide 2 antibodies were shown to be highly reactive with bovine gelatin compared to porcine. This approach could be used to identify the origin of gelatins in raw materials. However, the method is not capable of identifying the origin of gelatins in mixtures due to the possibilities of changes in the structure of the mixed molecules during coating. Hence, competitive indirect ELISA was used to analyze the same mixtures of gelatins in which high sensitivity of bovine gelatin was detected in the mixture. This ELISA approach could be used as an alternative to differentiate bovine and porcine gelatins. However, the process in preparing for the sample analysis is time-consuming and requires tedious incubation procedures. Hence, it is not efficient for a rapid identification test. Moreover, other factors involving possibilities of contaminations and sensitivity of the antibodies could affect the efficiency of the analysis.

Another analytical method in biochemistry that has been widely used by numerous researchers in identification of gelatin for halal authentication purposes is polymerase chain reaction (PCR). The PCR technique is used in detecting the presence of deoxyribonucleic acid (DNA) and quantification of trace DNA (Nikzad, Shahhosseini, Tabarzad, Nafissi-Varcheh, & Torshabi, 2017). It is widely used in the identification of porcine and pork DNA as well as other animals in gelatin, food, and pharmaceutical products. Sahilah et al. (2012) and Sahilah, Liyana, Aravindran, Aminah, and Mohd Khan (2016) applied PCR analysis with utilization of southern-hybridization on chip to detect porcine DNA in capsules. This technique was able to detect a very low amount of porcine DNA in gelatin-based capsules. It is also potentially used in detecting porcine DNA in meatballs and surimi products (Aravindran, Sahilah, & Aminah, 2014). Real-time PCR is also one of the most common PCR techniques used to detect the presence of porcine DNA in food and pharmaceutical products (Al-Kahtani, Ismail, & Ahmed, 2017; Demirhan, Ulca, & Senyuva, 2012; Jannat et al., 2018; Sudjadi, Wardani, Sepminarti, & Rohman, 2016). The genetic differences among different species of animals were determined within the targeted sources. The detection of porcine DNA was indicated by the cycle threshold (ct) value and amplification curve of the samples DNA. Based on the detection results, the real-time PCR technique was able to identify the source of

gelatin. However, some amplification signals could not be detected due to the possible DNA denaturation and other occurrences. Other approaches of PCR technique that have been used in detecting porcine DNA in gelatin and pork identification in foods and pharmaceutical products are PCR-restriction fragment length polymorphism, species-specific PCR, and multiplex PCR (Ali et al., 2015; Amqizal, Al-Kahtani, Ismail, Hayat, & Jaswir, 2017; Murugaiah et al., 2009; Nikzad et al., 2017; Shabani et al., 2015). These researchers used different approaches in applying the PCR technique for identification of porcine gelatin and pork DNA in food and pharmaceutical products. However, several factors involving the high cost of real-time equipment and reagents for real-time PCR analysis and stability of the DNA may affect the efficiency of this technique. This technique requires sufficient amount of high-quality extracted DNA as an essential prerequisite for PCR analysis. The extraction of DNA is time-consuming and could take up to 6 hr depending on the sample composition (Lubis et al., 2016). In addition, the DNA may be denatured during manufacturing process and consequently causing difficulty in obtaining number of suitable DNA fragments for molecular and identification analysis (Teletchea, Maudet, & Hänni, 2005).

Chromatographic technique

Nemati, Oveisi, Abdollahi, and Sabzevari (2004), Zhang et al. (2009) and Widyaninggar, Triyana, and Rohman (2012) identified bovine and porcine gelatins using high-performance liquid chromatography (HPLC) method, while Azilawati, Hashim, Jamilah, and Amin (2015) applied the HPLC method to identify bovine, porcine, and fish gelatins. The HPLC method was used to profile the amino acid content in the gelatin in the form of chromatogram and the peak heights are observed to discriminate the sources of gelatin. The level of amino acids in all samples was quantified with HPLC with precolumn derivatization.

Nemati et al. (2004) and Azilawati et al. (2015) applied reversed-phase HPLC (RP-HPLC) for the amino acid analysis on the gelatin samples. The procedure of RP-HPLC method by Nemati et al. (2004) involves separation and determination of amino acids through precolumn derivatization using selected derivatization agent followed by reversed-phase HPLC. Nemati et al. (2004) used Orthophtaldialdehyde (OPA) and 4-chloro-7-nitro benzofurazane (NBD-Cl) as derivatizing reagents for the procedure and analyzed the samples of bovine and porcine gelatin. The chromatogram profiles of both types of gelatin were obtained and 20 peaks were observed for each type of gelatin. The chromatogram peaks of both gelatins were almost similar and only one slight difference at the 11th peak. Hence, this method requires further analysis involving chemometric or mathematical method for the differentiation of the gelatins. Similarly, Azilawati et al. (2015) applied the RP-HPLC method for amino acid analysis of gelatins, but with the use of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) as the derivatizing reagent instead of OPA and NBD-Cl. The method was used to differentiate bovine, porcine, and fish gelatins and the results from the chromatogram spectral showed only small differences which is less than 1% differences among the amino acids of the 3 gelatins. Widyaninggar et al. (2012) applied HPLC method with OPA and 3-mercaptoethanol (MCE) as derivatizing agent to differentiate bovine and porcine gelatin in capsule shells. The level of amino acids for both gelatins was computed using HPLC with precolumn derivatization. As a result, the chromatograms showed that the bovine and porcine gelatin can be differentiated based on the peak level of certain amino acids, namely aspartic acid, hystidine, phenylalanine, isoleusine, lysine, glutamic acid, asparagines, glycine, threonine, and tyrosine. However, the slight differences on the level of these amino acids may not provide enough accuracy and require further validation. The results from these HPLC studies showed that it is insufficient for the discrimination due to the same chemical properties among the gelatins. Thus, further analysis using statistical and chemometric methods is needed to differentiate them.

Zhang et al. (2009) applied HPLC method with tandem mass spectrometry (HPLC-MS/MS) to identify specific marker peptide for tryptic digests of bovine and porcine gelatin. The method was able to detect the specific marker peptide for digested bovine and porcine gelatins based on the sequence in MS/MS spectrum. Hence, this method may be used to detect marker peptides in different sources of gelatins for authentication purposes. However, the procedure involving the digestion process and chromatogram profiling is very time-consuming and not suitable for rapid authentication analysis. Cheng et al. (2012) classified 5 different sources of gelatin using another chromatography method called the ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC/Q-TOF-MS). The method was used to analyze tryptic peptides of donkey-hide, bovine-hide, and pig-hide gelatins, and tortoise shell glue and deerhorn glue. The time of flight mass spectrometry (TOF) method is an effective method for protein analysis and was applied in this study for further identification of gelatins. This method using the step-gradients, with the enhanced selectivity of TOF-MS detection and better chromatographic resolution, had shortened the amount of time for the profiling compared to the earlier study by Zhang et al. (2009). However, the profiling time is still considered long and the difficulty in differentiating the gelatins through the chromatogram profile still exists due to the large similarity of gelatins' composition which caused the marker peptides to be concealed within a larger number of tryptic peptides. Yilmaz et al. (2013) developed ultra-performance liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry (nanoUPLC-ESI-q-TOF-MS^E) method to differentiate bovine and porcine gelatin. Tryptic gelatin peptides from extracted gelatins were analyzed using the nanoUPLC-ESI-q-TOF-MS^E. However, most of the tryptic peptides were hardly differentiated, especially in gelatin mixtures. Hence, the method is insufficient in analyzing gelatin in foods or mixtures. In addition, the profiling time of peptides through chromatographic separation is lengthy and requires skill to perform the analysis (Azilawati et al., 2015). Sha et al. (2018) combined the HPLC method with linear-ion trap (LTQ)/Orbitrap high-resolution mass spectrometry to differentiate bovine, porcine, and donkey-hide gelatins. Sha et al. (2018) utilized LTQ/Orbitrap mass spectrometry in order to effectively discriminate the gelatins. The mass spectrometry tool provides a high resolution and better mass accuracy. However, the process of identifying the gelatin sequences is difficult due to the large similarity of the mass peptides. Moreover, the identification of peptides and fragments of the gelatin involves tedious process, which includes the selection and fragmentation of ions. Hence, it is very time-consuming and not effective for a rapid analysis. Besides, chemometric or mathematical tools are required to perform a better discrimination test for these chromatographic results for further verification of the sources and halal status of the products.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Nur Azira, Amin, and Che Man (2012) and Nur Azira, Che Man, Raja Mohd Hafidz, Aina, and Amin (2014) differentiated

bovine and porcine gelatin in processed foods and adulterated samples using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method. The SDS-PAGE is an electrophoretic method used for separation of molecules based on their molecular weight. The method is usually conducted according to the SDS-PAGE system first described by Laemmli (1970). It is commonly used to analyze proteins in complex extracts. Nur Azira et al. (2012) and Nur Azira et al. (2014) applied the method and examined the differences of electrophoretic polypeptides between porcine and bovine gelatin at molecular weight region ranged from 53 to 220 kDa (Nur Azira et al., 2012) and 50 to 220 kDa (Nur Azira et al., 2014). As a result, the porcine gelatin showed wider molecular weight distribution compared to bovine gelatin (Nur Azira et al., 2012) and polypeptides of porcine gelatin were more heterogeneous than those of bovine gelatin (Nur Azira et al., 2014). The distinct patterns between both gelatins estimated the origin of the gelatin and indicated the adulteration of the gelatin. Hermanto et al. (2013) also applied the SDS-PAGE method to differentiate bovine and porcine gelatins before and after pepsin hydrolysis. Gelatin, which composed of peptides with wide molecular weight distribution, was degraded into lower molecular weight after pepsin hydrolysis. The SDS-PAGE was used to differentiate the hydrolyzed gelatins by analyzing the molecular weight pattern. The result showed that the polypeptide bands for the porcine and bovine gelatin were similar at range 100 to 200 kDa. For molecular weight of 50 kDa and below, there were three different appearances observed in which two bands appear at 36.8 and 28.6 kDa in porcine, while none in bovine gelatin, and at below 28.6 kDa, different band appearances observed in both gelatins. These studies were able to differentiate the gelatin samples by using the SDS-PAGE method. However, there are few factors that may affect the efficiency of the results during the electrophoretic analysis such as difficulties in separating small peptides and molecules which will cause inaccurate measurement, and inability to estimate precise molecular weight of proteins with small molecular masses.

Fourier transform infrared spectroscopy

Another analytical method that has been employed by several researchers in identification of the sources of gelatin and other nonhalal substances is Fourier transform infrared (FTIR) spectroscopy. The FTIR spectroscopy is proven to be a useful technique in dealing with numerous adulteration problems in food products including lard or pig fat content in chocolate (Che Man, Syahariza, Mirghani, Jinap, & Bakar, 2005; Suparman, Sri Rahayu, Sundhaniv, & Dwi Saputri, 2015), lard in mixture of lamb, cow, and chicken fats (Rohman & Che Man, 2010), lard content in meatball (Kurniawati, Rohman, & Triyana, 2014; Rohman, Sismindari, Erwanto, & Che Man, 2011), and lard content in "rambak" crackers (Erwanto, Muttaqien, Sismindari, & Rohman, 2016). Lard is also one of the nonhalal ingredients that is commonly used in food products. These researchers used FTIR spectroscopy to analyze the lard and pig fat contents in various food products and to validate the halal status of the products. The halal authentication of these products is crucial especially for Islamic countries due to the possible presence of unknown and nonhalal source of ingredients. Che Man et al. (2005), Kurniawati et al. (2014), Rohman and Che Man (2010), Rohman et al. (2011), Suparman et al. (2015), and Erwanto et al. (2016) were able to detect minor differences on the FTIR spectra of products that contain lard and other pig derivatives. The differences were observed mainly at the fingerprint region based on the peak heights and intensities. The ability of FTIR analysis as fingerprint tool and its rapid performance are important strengths of this technique and it may be used for routine analysis for halal authentication. It is a powerful analytical method which has the ability to discriminate spectra between different samples through peak observations. The FTIR spectroscopy measures the absorption and transmission of infrared light of molecular vibrations over a range of wavelengths. It can determine some substances that are present in a food sample (Smith, 2011). In particular, it has been used to determine chemical and biochemical properties involving collagen and protein (Bryan et al., 2007; Muyonga, Cole, & Duodu, 2004; Paschalis et al., 2001; Paschalis et al., 2015).

Hashim et al. (2010) studied the utilization of the spectroscopic technique to identify the sources of gelatin. The FTIR analysis showed that bovine and porcine gelatin spectra were very similar to each other. Major peaks were observed at four regions namely Amide A, I, II, and III. These regions represent the fingerprint regions of gelatin which indicate the presence of certain functional groups. The FTIR spectra obtained from the analysis showed wide and overlapping absorption bands that cover multitude of different functional groups. Further analysis using chemometric and mathematical methods is needed to extract the information within the overlapping bands for the differentiation of gelatins. Hermanto et al. (2013) applied the FTIR technique to identify bovine and porcine gelatins before and after pepsin hydrolysis. The FTIR spectra of the hydrolyzed gelatins showed slight differences at region 2800 to 3000 cm^{-1}, 1543 cm^{-1}, and 1450 to 1300 cm^{-1}. The differences in these regions indicate the presence of different amino acid composition between the bovine and porcine gelatin especially for glycine, proline, and arginine. Cebi, Durak, Toker, Sagdic, and Arici (2016) used FTIR to identify the sources of gelatin involving bovine, porcine, and fish. The FTIR spectra of the three sources of gelatin showed similar pattern with major peaks on Amide region I, II, and III. Amide I absorption indicates the presence of carbonyl C=O stretching mode with a minor contribution from the C-N stretch, while Amide II absorptions were caused by N-H bend. These results were similar with those reported by Hashim et al. (2010).

The FTIR method is simple and accurate for discriminating spectra between samples compared to other analytical methods (Hashim et al., 2010). The result in the form of spectrum represents the molecular fingerprint of samples. The FTIR spectrum covers the details on functional groups and chemical compositions of samples. Each sample produces different spectrum and it is practical for the gelatin sources' identification and halal authentication. However, due to immense similarity of chemical properties and structures of the proteins between the gelatins, it is difficult to discriminate the sources accurately (Nemati et al., 2004). Thus, chemometric or mathematical methods are important to establish the differences between the gelatins in order to authenticate the sources and halal status of the products.

Chemometric Methods

A new subfield within chemistry called chemometrics which involves the application of mathematical and statistical methods has been evolved and used in providing maximum chemical information in data analysis (Varmuza & Filzmoser, 2016). Chemometric methods in data analysis are pervasive and important toward decision-making and problem-solving process. Chemical analysis deals with complex mixtures, compounds, and their properties, which are often very complicated to be analyzed. Development of computerized laboratory automation has led to the advancement of chemical data analysis with the aid of chemometric methods as tools for analyzing and structuring the data. Chemometric

Table 1–Authentication of gelatin using chemical and chemometric methods.

Chemical method	Chemometric method	Advantages	Limitations	References
pH drop method	Statistical Method: ANOVA and Scheffe's test	Chemical analysis: Able to differentiate bovine and porcine gelatins based on induction time and HAP transformation after calcium phosphate at concentration of 0.5 and 2.0 mg/mL for the induction time and at 0.5 and 4.0 mg/mL for maximum stimulation of HAP. Statistical analysis: Able to verify the results through significant differences using ANOVA and Scheffe's test by comparing the mean differences.	Chemical analysis: Limited for analysis of gelatin from bovine bone and porcine skin, and incapable of identifying the source of gelatins in food products due to reactivity toward possible interferences. Statistical analysis: Less accurate due to the assumption made by ANOVA on normal distribution of data and less statistical power in providing reliable results.	Hidaka and Liu (2003)
High-performance liquid chromatography (HPLC)	Principal component analysis (PCA)—MATLAB software (Nemati et al., 2004), Minitab software (Widyaninggar et al., 2012), Unscrambler X software version 9.7 (Azilawati et al., 2015)	Chemical analysis: Able to identify raw bovine and porcine gelatins (Nemati et al., 2004), in capsule shells (Widyaninggar et al., 2012), and bovine, porcine, and fish gelatins (Azilawati et al., 2015) based on chromatogram peaks. Statistical analysis: Able to classify the gelatins based on PCA score and loading plot which show the gelatins grouping and differences.	Chemical analysis: Insufficient for discrimination due to the same chemical properties and inconsistent level of amino acids. Statistical analysis: Less accurate due to the data manipulation and dimensionality decrease involving the number of variables in process.	Azilawati et al. (2015), Nemati et al. (2004), Widyaninggar et al. (2012)
Fourier transform infrared spectroscopy (FTIR)	Fast Fourier transform (FFT)—FTIRPrincipal component analysis (PCA)—TQAnalyst software (Hashim et al., 2010)OPUS software (Cebi et al., 2016)	Chemical analysis: A rapid analysis that is able to identify the spectra of bovine and porcine (Hashim et al., 2010) as well as fish gelatins (Cebi et al., 2016) and their functional groups. Statistical analysis: Able to analyze the selected spectra and categorize the gelatin through Cooman's plot (Hashim et al., 2010) and PCA plot (Cebi et al., 2016).	Chemical analysis: Large similarities of gelatin spectra and only slight differences observed at Amide I, II, and III regions due to the wide and overlapping bands. Statistical analysis: Less accurate due to the data manipulation and dimensional reduction involving the spectral data.	Cebi et al. (2016), Hashim et al. (2010)
Ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC/Q-TOF-MS)	Principal component analysis (PCA)—MassLynx software	Chemical analysis: Rapid analysis on bovine and porcine tryptic peptides compare to HPLC method. Statistical analysis: Able to discriminate 5 different sources of gelatin and identify significant markers.	Chemical analysis: Profiling time is long and the process for identification of marker peptides is tedious and requires previous data. Statistical analysis: Less accurate due to the data manipulation and dimensional reduction involving the spectral data.	Cheng et al. (2012)
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)	Principal component analysis (PCA)—Unscrambler 9.7 software	Chemical analysis: Able to differentiate bovine and porcine gelatin based on polypeptide patterns and molecular weight distribution. Statistical analysis: Able to classify the gelatins by sample grouping and signified their differences based on molecular weight regions.	Chemical analysis: Incapable of classifying the source of gelatin in complex mixture in processed foods and samples. Statistical analysis: Less accurate due to the data manipulation and dimensional reduction.	Nur Azira et al. (2012, 2014)
Real-time polymerase chain reaction (PCR) and mass spectrometry	Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA)—MATLAB 8.1.0 R2013a Software	Chemical analysis: Able to identify the source of gelatin based on the amplification curves of the extracted DNA. Statistical analysis: The PCA and PLS-DA model able to reveal several missed detection of porcine for the PCR analysis and correct identification for the mass spectrometry analysis.	Chemical analysis: Amplification signal could be missing due to the possible DNA denaturation and other occurrences. Statistical analysis: High possibility of the data being overfitted and less accurate due to the data manipulation and dimensional reduction.	Jannat et al. (2018)

methods involving the application of mathematical and multivariate statistical analysis are powerful tools, which are capable of solving problems involving classifications of different samples and determining the properties of a chemical compound (Christy et al., 2013). Chemometric and mathematical methods such as analysis of variance (ANOVA) and multivariate analysis, which includes PCA and discriminant analysis, are commonly used as tools to analyze chemical data (Kowalski, 2013). Data analysis through these approaches has proven to be efficient in supporting the chemical analysis in numerous studies on authentication of gelatin. The summary of the related studies involving chemometric methods in authentication of gelatin is presented in Table 1.

Statistical ANOVA test

Hidaka and Liu (2003) applied statistical methods called ANOVA and Scheffe's test to analyze the result obtained from the pH drop for bovine and porcine gelatins. In the study, the effects of bovine and porcine gelatins on induction time and HAP transformation after calcium phosphate precipitation were compared. The differences between bovine and porcine gelatins are considered significant when $P < 0.05$. The induction time for bovine is significantly different from porcine gelatins at concentration of 0.5 and 2.0 mg/mL. The effect of concentrations of bovine and porcine gelatins on the HAP transformation showed a significant difference at concentration of 0.5 mg/mL, while maximum stimulation of HAP was recorded at 0.5 mg/mL for bovine and 4.0 mg/mL for porcine. These statistical tests were able to determine the significant differences between the samples with the aid of statistical software. The Scheffe's test was conducted as a post hoc test in the ANOVA. Post hoc test is an additional hypothesis test that is performed after ANOVA to provide specific information on which mean differences are significant (Gravetter & Wallnau, 2016). The combination of ANOVA with Scheffe's test is simple and flexible for the analysis of mean differences compared to other post hoc tests (Kirk, 2012). However, ANOVA has the limitation that it assumes all data to be normally distributed (Norman & Streiner, 2008) and the Scheffe's test has less statistical power in determining significant and reliable results (Kirk, 2012). Thus, the results may not be accurate if it involves data which are not normally distributed and the methods are insufficient in providing reliable results for the significant differences.

Principal component analysis

The PCA is a nonparametric technique which is used to extract relevant information in data analysis (Pawar & Kamat, 2014). It is a chemometric tool, which is widely used in multivariate data analysis to extract information from data with multiple variables. The PCA method reduces the dimensionality of the data and extracts the most relevant information from the dataset. The mathematical computation of the PCA involves construction of principal components (PCs) from linear combination of the variables and determines the significant components through calculation of variance (Nollet & De Gelder, 2013). Results in the form of score and loading plots are visualized to identify the presence of clusters and outliers in the significant PCs and to compare and differentiate the samples.

Cheng et al. (2012), Nemati et al. (2004), Widyaninggar et al. (2012), and Azilawati et al. (2015) applied PCA to analyze the results obtained from chemical analysis by chromatographic methods. Nemati et al. (2004) applied PCA to analyze the HPLC chromatogram peaks and classify the gelatin according to their amino acid compositions. The result obtained from HPLC in the form of chromatograms showed slight difference at a peak and may not be sufficient for the discrimination of the sources of gelatin due to the large similarity of protein structures. In the study, PCA was executed using MATLAB for the data manipulation and peak determinations in order to extract the PCs for classification of bovine and porcine gelatins. The sources of gelatin are determined through PCA plot on a two-dimensional graph. Nine bovine and three porcine samples were analyzed first followed by another five bovine and two porcine samples which were used as prediction set. The results for the 19 samples were displayed on the two-dimensional graph according to their score values. The graph showed that the samples of bovine and porcine gelatins were distinguished by a line. As a result, Nemati et al. (2004) managed to classify the gelatins by using the PCA method. Widyaninggar et al. (2012) combined HPLC and PCA to classify the bovine and porcine gelatins in capsule shells. Based on chromatograms of amino acids from HPLC analysis, the study utilized PCA to manipulate the data, to compute the PCs to illustrate the data variations, and to visualize the results in the form of score and loading plot. The PCA score plot results showed that the bovine and porcine gelatins were well separated, while PCA loading plot showed the most significant amino acid composition which contributes to the differences of the gelatins. It is observed that leusine and serine+systein were the amino acids which contributed most toward principal component 1 (PC1), while tyrosine and lysine were responsible for principal component 2 (PC2). Azilawati et al. (2015) also combined HPLC method with PCA due to the large similarity of amino acid compositions between bovine, porcine, and fish gelatins. The dataset of gelatin samples was manipulated until a fit PC model is achieved. The PCA is performed by converting the dataset to a few PCs, PC1, PC2, and PC3 with significant variances among variables. The results were presented as score and loading plots. As a result, the PC1 and PC2 were able to differentiate the gelatins according to their origin based on the scores plot and the highest variance was described by PC1 at 72%, while the loadings plot established the correlation and significant amino acids which contributed to the differences in the score plot. These studies were able to differentiate the gelatins based on their origins.

Similarly, Cheng et al. (2012) analyzed tryptic peptides of 5 different sources of gelatin using UPLC/Q-TOF-MS method with combination of PCA. The five different sources of gelatin involved were donkey-hide, bovine-hide, pig-hide gelatins, tortoise shell, and deer-horn glues. However, due to the homology of collagen structures and properties, it was difficult to visualize the gelatins' differences through the UPLC/Q-TOF-MS chromatogram profiles. Therefore, PCA was used in their study to classify the five gelatins. The UPLC/MS dataset was converted into a 2D matrix using MassLynx software. The preliminary process of PCA includes examining the gelatins dataset to check for outliers and classification trends. The final results were presented through PCA score and loading plot. The PCA score plot showed that the five different types of gelatins were clustered among their respective origins, while PCA loading plot displayed the significant variables that caused the clusters. The results based on the PCA loading plot enhanced the possibility to identify the markers that play important roles in classification of gelatins. However, the amount of time taken to complete both chemical and statistical analysis is long and tedious since the identification of marker peptides requires previous data.

The PCA is also applied in combination with other chemical methods such as SDS-PAGE (Nur Azira et al., 2012, 2014) and PCR method (Jannat et al., 2018). Nur Azira et al. (2012, 2014)

applied PCA in combination with the SDS-PAGE method used in differentiating bovine and porcine gelatins. Based on the result from the score and loading plots, the sample groupings were consistent and significant molecular weight regions which contributed to it were 130 and 150 kDa on PC1, and 180 and 160 kDa on PC2 (Nur Azira et al., 2012) and 160, 96, and 87 kDa on PC1, and 145, 106, 83, and 53 kDa on PC2 (Nur Azira et al., 2014). The value of the loading plot based on the molecular weight regions signifies the influence of each variable on the gelatin classification. The application of PCA method showed that the presence of porcine and bovine gelatins can be detected and the differences can be identified. Jannat et al. (2018) applied PCA in analyzing the mass spectral data obtained from the combination of real-time PCR and mass spectrometry-based methods for identification of gelatins. The utilization of liquid chromatography–mass spectrometry (LC-MS) technique in the study provides sufficient data for classifying the gelatin samples. The PCA was combined with partial least squares discriminant analysis (PLS-DA) to determine the pattern of the gelatins. The PCA was employed to explore the distribution pattern and categorize the gelatins into different classes. As a result, 3 classes of gelatins comprised of fish, bovine, and porcine were visualized on the PCA score plot. The classes of gelatin were used for setting the PLS-DA parameters. The PLS-DA was applied to create a model for identifying the sources of gelatin. The final validated model was tested on real-time PCR and LC-MS analysis. The test revealed several missed detections of porcine for the PCR analysis and correct identification for the LC-MS analysis. Based on the results, the proposed methods are useful for identification of fish, bovine, and porcine gelatins. However, the PLS-DA model validation is often overlooked and there is high possibility of the data being overfitted (Gromski et al., 2015). Moreover, the PCA method applied in these studies is not suitable in analyzing a large dataset and the accuracy of the results may be affected since the dimension of the dataset is reduced during data manipulation.

Fourier transform infrared spectroscopy with principal component analysis

A mathematical technique called Fourier transform is applied in chemical analysis involving signal processing whereby beams of infrared light are emitted and enter the interferometer for modulation of the infrared frequency (Smith, 2011). The infrared beam then passes through the sample where the intensity of transmitted or reflected light is detected in the form of interferogram signal. The interferogram signal is then digitized and demodulated by Fourier transform to obtain a spectrum of intensity over wavelength or frequency (Smith, 2011). The spectroscopic technique is coupled with Fourier transform and is applied in numerous studies on characterization of lard and gelatin due to its ability in generating spectrum for chemical compositions in a sample. Kurniawati et al. (2014), Suparman et al. (2015), and Erwanto et al. (2016) applied FTIR with PCA method to analyze lard content in several food products for halal verification studies. The lard samples obtained from rendering process of pig tissue were analyzed using FTIR and compared with the spectra of commercial meatball (Kurniawati et al., 2014), chocolate (Suparman et al., 2015), and crackers containing cow skin (Erwanto et al., 2016). The FTIR results were further analyzed using PCA method and clear separation between the lard and food samples was observed on the PCA score plot. Besides food products, lard is also used in cosmetics (Rohman et al., 2014) and printing inks (Ramli et al., 2015). Rohman et al. (2014) applied FTIR in combination with PCA method to analyze the presence of lard in cream cosmetic product. Lipid components, which include lard, extra virgin olive oil (EVOO), and other nonpolar components, were extracted from the cream samples and analyzed using FTIR device. As a result, the FTIR spectra of lard and EVOO showed huge similarity and only few differences were observed at 3007, 1160, 1117, and 1098 cm^{-1}. Further analysis using PCA was performed to classify the lard and EVOO content in cream products and clear separation and classification were observed on the PCA score plot. Ramli et al. (2015) applied FTIR with PCA method to analyze the presence of lard in the printing ink used for food packaging films. Lard and printed packaging samples were analyzed using FTIR and further classified based on the PCA score plot. The PCA score plot showed that the lard and commercial ink samples were clustered separately and clear distinction was observed. Therefore, these studies on lard have proven that FTIR method with the aid of chemometric is capable of validating the nonhalal content in products.

Hashim et al. (2010) and Cebi et al. (2016) applied FTIR spectroscopy with combination of chemometric technique using statistical methods for spectrum analysis in authentication of sources of gelatin. Samples of FTIR spectra of bovine (B.G), porcine (P.G), and fish (F.G) gelatin at fingerprint region 1800 to 1200 cm^{-1}, with various concentrations between 4% and 20% (w/v), are included in this paper as shown in Figure 1. The spectra of the samples show slightly different patterns with respect to sample concentrations. Therefore, due to the large similarity in FTIR spectra of bovine, porcine, and fish gelatin, additional chemometric or mathematical tools are needed to identify them. Hashim et al. (2010) applied chemometric technique using PCA and discriminant analysis to analyze spectra of bovine and porcine and to characterize the gelatin compounds. The FTIR spectra of bovine and porcine gelatins showed similar patterns except at wavelength regions 3290 to 3280 cm^{-1} and 1660 to 1200 cm^{-1}. These 2 regions were used as calibration models in PCA in which PCs are calculated to describe the spectral information and spectral variance. Discriminant analysis was used to identify the gelatin samples based on the intensity of spectra using TQ Analyst software. The distances of porcine and bovine gelatins were visualized using Cooman's plot and clear distinction was observed. Cebi et al. (2016) applied PCA and cluster analysis to analyze FTIR spectra of bovine, porcine, and fish gelatins. Hierarchical clustering analysis was used to classify the gelatin samples based on their spectral diversity. The samples were analyzed using clustering method involving computation of Euclidean distances. Ward's algorithm was used after derivation and vector normalization of the data within the region of 1722 to 1487 cm^{-1}. The result in the form of dendrogram showed an apparent clustering scheme in which the sources of samples were discriminated. The result shows that fish gelatin is easier to be distinguished than bovine and porcine gelatins. Additionally, PCA analysis was used to determine the PCs in order to further classify the gelatins according to their origins. The analysis based on PCA plot showed that the sources of gelatins were clustered among their origins. In previous study, Hashim et al. (2010) managed to determine bovine and porcine gelatins; however, they did not include the study on fish gelatin. They used FTIR spectroscopy in combination with PCA in their study. These studies have proven that FTIR method with the aid of PCA, a chemometric tool, is capable of differentiating the sources of gelatins and may be used as routine analysis for halal authentication purposes. However, the application of PCA in these studies (Azilawati et al., 2015; Cebi et al., 2016; Cheng et al., 2012; Hashim et al., 2010; Jannat et al., 2018; Nemati et al.,

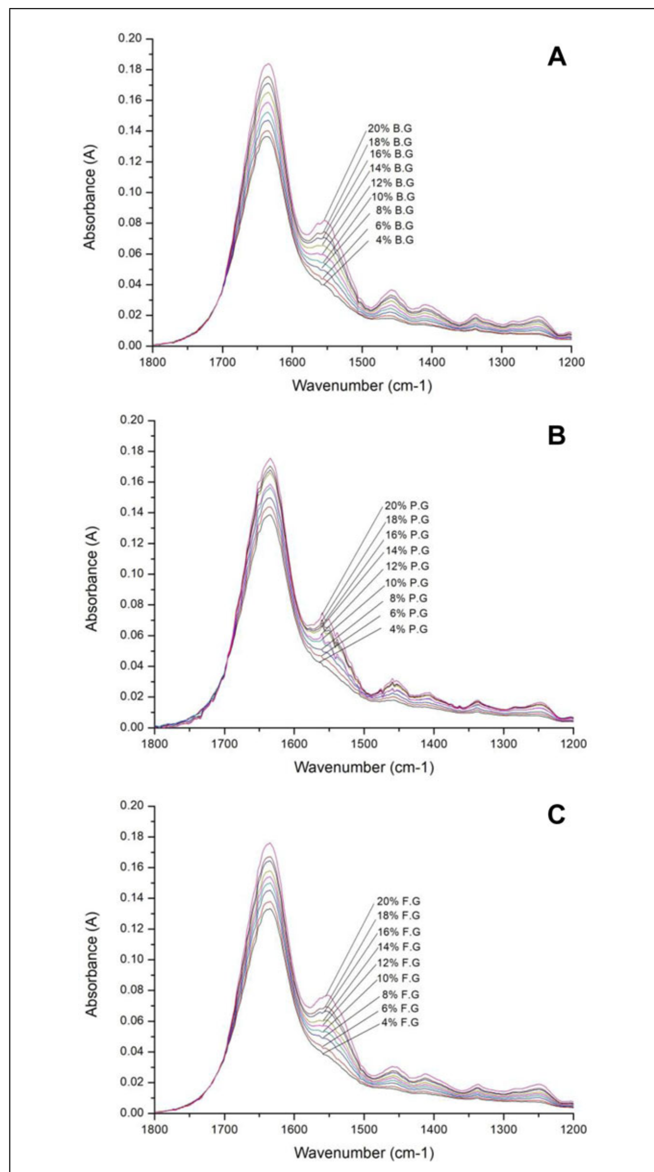

Figure 1–Concentration-dependent FTIR spectra of (A) bovine gelatin (B.G); (B) porcine gelatin (P.G), and (C) fish gelatin (F.G).

2004; Nur Azira et al., 2012; Nur Azira et al., 2014; Ramli et al. 2015; Rohman et al. 2014; Widyaninggar et al., 2012) may affect the accuracy of the results since the dimension of their dataset is reduced. The PCA method is used to emphasize variation by developing correlation structure between variables and transforming the correlated variables to uncorrelated PCs or eigenvectors. During the implementation of the PCA, the dataset is reduced by disregarding the components or eigenvectors of lesser significance. As a result, this method was able to classify the gelatins by examining the PCA score plot. However, imprecision during the data compression and dimensional reduction may affect the accuracy of the results.

Conclusion

Halal authentication of food and pharmaceutical products is important due to the concerns about the presence of prohibited and nonhalal substances. Numerous methods have been performed to differentiate bovine and nonhalal porcine gelatins and very few studies have considered fish gelatin. The most reliable chemical method observed is FTIR spectroscopy due to its ability in generating distinct spectra of samples. However, further analysis on bovine, porcine, and fish gelatin samples is needed due to the large similarity of FTIR spectra of the gelatins. The PCA is one of the chemometric methods that is useful in analyzing the chemical data of the gelatins. However, the data manipulation and dimensional reduction procedures in PCA may cause imprecision in the overall results and important information of the spectral data may be disregarded during the process. Hence, a robust chemometric tool with the application of mathematical method that deals with uncertainties and eigenvalues of overall spectral data is required to identify the significant difference and dominant functional group of the gelatins for halal authentication purposes.

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Author Contributions

Nurfarhana Hassan prepared and wrote the manuscript under the supervision of other authors. Tahir Ahmad and Norhidayu Muhamad Zain provided some inputs and reviewed the draft for publication.

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