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Analytical Methods for Gelatin Differentiation

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Abstract: Gelatin differentiation in edible products has been a debate over the years with respect to health and religious concerns. Researchers had performed various of studies in tackling this issue thus various of analytical methods for gelatin differentiation were developed. Such analytical methods mentioned involving several instruments and techniques including chromatography, molecular, electrophoresis, immunochemical and spectroscopy had been developed for finding the most accurate method in gelatin differentiation. However, with every method developed there will always be advantages and limitations. The present paper provides review on the recent method for gelatin differentiation as well as the advantages and disadvantages of each available method.

Keywords: Gelatin; Food adulteration; Food Analysis; Analytical methods

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Introduction

Gelatin is a heterogenous mixture of polypeptides obtained from the by-product of animal connective tissue collagens which undergo a process of partial hydrolysis (Zhang, 2009). It consists of 84 to 90 percent protein made from animal raw materials that contain collagen, 2 percent mineral salts and the rest is made from water (GME, 2018). The chemical structure of a gelatin is identical to the structure of collagen which is a triple helix structure, made up of repetition of three identical or non-identical polypeptide chains of -Gly-Xaa-Yaa-, in which the amino acid residues X- and Y-position are normally proline or hydroxyproline (Balian & Bowes, 1977; Engel & Bächinger, 2005). Gelatin consist of two types; Type A and Type B. Type A is derived using acid pre-treatment while Type B is derived from alkaline pre-treatment (Baziwane & He, 2003). The process of producing gelatin is a complex, multi-stage process with the used of high-tech equipment involving pre-treatment, extraction, purification, thickening and drying (GME, 2018).

Gelatin has been widely used around the world not only in pharmaceutical products such as making tablets, hard and soft capsule, plasma expanders and blood substitutes, but they can also be found in foodstuffs such as jellies, desserts, foam formation, ice-cream stabilizer and in meat products as an emulsion stabilizer (Hidaka & Liu, 2003; Karim & Bhat, 2009; Raja Mohd Hafidz, Amin, & Che Man, 2012). Gelatin is also added in food formulated for diabetic patients to reduce carbohydrate content (Karim & Bhat, 2009). In yogurt for example, it helps to decrease syneresis and to increase consistency (Cebi *et al.*, 2016).

Controversies rise in terms of health and religious issue due to the fact where gelatin is made up from bovine, pig skins, hooves and demineralized bones. It receives objection from Hindus which prohibits the usage of cow while vegetarian objects it due to animal-based products (Raja Mohd Hafidz *et al.*, 2012). Outbreak of bovine spongiform encephalopathy (BSE) also known as the mad cow disease had caught people's attention in objecting the use of bovine gelatin (Hidaka & Liu, 2003). Most of the gelatins made up from pork skins as well as hides and bones from slaughtered cattle. The different slaughtered laws causing Muslims and Jewish objections when it comes to using the product (Boran, Lawless, & Regenstein, 2010). Due to episodes of objections from society on mammal-derived gelatin, extensive research has been done and develop to find an alternative way to substitute it (Karim & Bhat, 2009). Recently, fish skin gelatin research has been a field of interest to replace gelatins made from bovine and porcine. Hence, the non-*halal* gelatin and health issues can be combat with the recent research. Boran *et al.* (2010) also stated that with the recent research, it may be able to reduce

fish waste leading to a better economy among fishers. Based on the statistics given by GME (2018), approximately 80 percent of gelatin comes from pork skin, 15 percent is produced from split obtained from a part of the cattle hide that contains collagen which lies between the epidermis and subcutaneous layer and the rest 5 percent of gelatin comes from porcine, fish and bovine bones (GME, 2018).

Food authentication is necessary to identify sources in foods such as gelatin. Several analytical methods have been developed and used to differentiate the origin of gelatin consisting of chromatographic, molecular, electrophoresis, immunochemical and also spectroscopic methods. Along with the use of analytical methods, chemometric methods had been developed to obtain better and precise results (Eryılmaz *et al*., 2017). Therefore, this paper reviewed and summarized about the analytical methods available for gelatin differentiation including its' advantages and disadvantages.

Analytical Methods

Chromatographic Method

There are several chromatographic techniques that have been established in order to distinguish the source of gelatin such as High-Performance Liquid Chromatography (HPLC) combined with linear-ion trap (LTQ)/Orbitrap (Sha et al., 2018), Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) with OPA/2-MCE and Principle Component Analysis (PCA) (Raraswati, Triyana, & Rohman, 2013), Ultra Performance Liquid Chromatography-Tandem Mass Spectrometer (UPLC-MS/MS) coupled with multiple reaction monitoring (MRM) (Guo et al., 2018), Liquid Chromatography-Mass Spectrometry (LC-MS) with Orbitrap (Kleinnijenhuis, van Holthoon, & Herregods, 2018) and Double Charged Selected Ion coupled with mass spectrometry (MS/MS) fragments monitoring (DCSI-MS/MS) (Cheng et al., 2014).

LC-MS is proven to be reliable in detecting the species origin of the gelatin due to its ability to apply multiple target peptides of the same species at the same time hence increasing its selectivity. However, the drawback of LC-MS method is that it is limited to only certain types of gelatin without taking account of the difference in sources of gelatin as well as the production process (Grundy et al., 2016).

Post-translational hydroxylation of proline in gelatin structure results in abundance of Hydroxyproline (Hyp) which leads to difficulty in identification of gelatin (Zhang, 2009; Song, & Mechref, 2013). However, with the help of mass spectrometer with high resolution and mass accuracy, identification of gelatin would be easier. Sha et al. (2018) combine the use of HPLC and LTQ/Orbitrap high-resolution mass spectrometry with high resolution and mass accuracy of more than 60,000 and 15 ppm, respectively. They successfully differentiate three types of mammalian gelatin including bovine-hide gelatin, donkey-hide gelatin and porcine-hide gelatin by the presence of marker peptides. At different mixing ratios, mass spectrometer is capable for detecting marker peptides without interference however the concentration of gelatin somehow contributes to the number of marker peptides observed. On the other hand, HPLC can also be very sensitive to the presence of sugar and oil hence a proper sample preparation is necessary to prevent any false positive results.

Composition of amino acid for bovine and porcine is said to be of different structure therefore the origin determination of gelatin can be determined by looking at their amino acid (Zhang, 2009). Raraswati et al. (2013) focuses on differentiating and classifying gelatin sources found in soft candy based on their amino acid profiles. They combined the use of RP-HPLC with fluorescence detection in separating amino acid and chemometrics PCA for discriminating and differentiating amino acid profiles (J. N. Miller, & J. C. Miller, 2005). The amino acid profiles for 7 samples of commercial soft candy and 3 laboratory-prepared soft candies were separated using RP-HPLC including a process of hydroxylation with ortho-phtalaldehyde and 2-mercaptoethanol (OPA-MCE). Results of the chromatogram shows peak height that can be used to differentiate between bovine and porcine gelatin however chemometrics analysis PCA is done for a better and reliable results. PCA is done by extracting significant variables of peak height for each amino acid. PCA managed to differentiate and classify the sources of gelatin in laboratory-prepared products however PCA were not able to differentiate between porcine or bovine sources in commercial food products. This is believed to be caused by modification of structure of gelatin in preparation technique of commercially processed food products or due to gelatin processed from different parts of animals.

Guo et al. (2018) conducted a study on using marker peptides for detection of porcine gelatin by UPLC-MS/MS in MRM mode. In their work, gelatin is detected by mass spectrometry and selected marker peptides for each gelatin is picked. Selected marker peptides are then monitored by LC-MS/MS in MRM mode mode at 3.2 kV multiplier voltage, following MRM transitions of [M + 2H]2+ precursor ions / product ions for identifying porcine gelatin and adulterations. The ability of MRM method in detecting porcine gelatin is tested by mixing 1%, 5% and 40% of horse-hide gelatin with porcine gelatin respectively. MRM chromatograms showed sensitive and reliable results of porcine gelatin in the mixture of porcine and horse-hide gelatin with a limit detection of 1%. UPLC-MS/MS coupled with MRM is proven to be a reliable method for detecting porcine gelatin at a very low concentration level. The use of marker peptide in classifying sources of gelatin by utilising mass spectrometry technique is a useful method in protein analysis (Nimptsch et al., 2011).

The objective for a study conducted by Cheng et al. (2014) is to distinguish different gelatin and identifying individual gelatins in mixture by observing the species-specific peptides of the gelatin. Analysis was done by using DCSI-MS/MS. Gelatins were digested by trypsin, undergo liquid chromatography separation, and peptide identification using DCSI-MS/MS. As claimed by Solazzo et al. (2008) also Huang and Henion (1990), tryptic peptides analysis is a powerful method for protein identification. A doubly charged ion is selected as the precursor by the MS detector and performing continuous MS/MS on one or more selected precursor during a time interval or along the whole run. Five precursor ions at m/z 765.8, m/z 641.8, m/z 924.5, m/z 758.8, and m/z 732.8 were analysed and recorded and the chromatogram traces for the fragment ions can be obtained. Different fragment ions are determined by the sequence of marker peptides and the average MS/MS spectra gives the perfect agreement with the peptide pattern. In this study, 20 different gelatins was tested including donkey-hide gelatins, bovine-hide gelatins, porcine-hide gelatins, deer-horn gelatins and tortoise shell glue. In sample preparation, the gelatin is digested by trypsin, undergo liquid chromatography separation and peptide identification by using DCSI-MS/MS. Twenty commercial samples obtained from the market was successfully identified by matching its peptide with the one corresponding to the reference

samples. Hence, DCSI-MS/MS scanning mode technique can be a great potential for peptide identification due to its rapid, simple and exclusive method.

Recently, Kleinnijenhuis et al. (2016) introduced the combination of UPLC-MS/MS with Orbitrap used in detecting traces of porcine gelatin in bovine gelatin and vice versa while taking several factors into considerations such as; the manufacturing process of gelatin and its raw material. This method was developed according to the Triskelion general workflow based on the previous study conducted by Kleinnijenhuis et al. (2018). Orbitrap has an advantage due to its high resolution thus providing the sequence of quantitative target peptides. Gelatin samples were prepared and undergo the process of solubilization, reduction and tryptic digestion. The method is very sensitive with a lower limit of quantification of 0.05% and provides quantitative analysis however it is a complex method which requires consideration of large variability of commercial gelatin involving molecular weight, production process and bloom value. Table 1 shows differentiation of gelatin using chromatographic methods.

Table 1. Differentiation of gelatin using chromatographic methods

Methods	Advantages	Limitations	LOD/LOQ*	References
HPLC combined with linear-ion trap (LTQ)/Orbitrap	Aim: Differentiate three mammalian gelatins. Detection: Success identification of mixture of bovine-hide gelatin, porcine-hide gelatin and donkey-hide gelatin. Advantages: Ability to differentiate mixture of gelatin by their marker peptides without strong peak interference.	Cannot detect low concentration of target gelatin in mixtures. Can be sensitive to some food ingredients e.g. presence of sugar and oil.	10% of gelatin in bovine-hide gelatin, porcine-hide gelatin and donkey-hide gelatin.	Sha *et al*., 2018
RP-HPLC with OPA-MCE and PCA	Aim: Differentiate and classify gelatin sources in soft candy based on amino acid profiles. Detection: Differentiate between porcine and bovine gelatins in laboratory-prepared food products. Advantages: Reliable method for gelatin detection.	PCA failed to distinguish between porcine and bovine gelatins in commercial food products.	Not stated.	Raraswati *et al*., 2013
UPLC-MS/MS coupled with MRM	Aim: Use of marker peptides for detection of porcine gelatin. Detection: Detect porcine gelatin by using UPLC-MS/MS coupled with MRM. Advantages: Highly sensitive for mixture of gelatin.	Further quantitative analysis can be done with HPLC and mass spectrometry.	Horse-hide gelatin mixed with 1% porcine gelatin	Guo *et al*., 2018
LC and DCSI-MS/MS	Aim: Distinguishing different gelatin and identifying individual gelatins in mixture by observing the species-specific peptides of the gelatin. Detection: Distinguished one gelatin from another by observing species-specific peptides. Advantages: Works efficiently in identification of commercial samples by comparing the specific peptides with reference.	Qualitative analysis from previous characterized peptide.	Not stated.	Cheng *et al*., 2014
UPLC-MS/MS with Orbitrap	Aim: Detect porcine gelatin in bovine gelatin and vice versa. Detection: Successful in detection of porcine and bovine gelatin. Advantages: Multiple target peptides can be applied at the same time to differentiate between samples.	Complex method which requires consideration of large variability for commercial gelatin.	0.05% of bovine in porcine gelatin and porcine in bovine gelatin	Kleinnijenhuis *et al*., 2018)

*LOD: Limit of Detection, LOQ: Limit of Quantitation

Molecular method

Nucleic-acid methods used for determining the species composition of food products and polymerase chain reaction (PCR) has been proven to be a great method in detecting gelatin samples by pattern recognition (Jannat et al., 2018). PCR-based assays are proven to be sensitive, highly specific and a useful technique for animal species detection compared to protein-based assay. The benefits of using this technique are inexpensive and rapid analysis (Aida et al., 2007). However, determining the species composition of some food products by DNA-based methods is not easy due to denaturation and removal of DNA fragments in highly processed food and the mixture of products during the production process. Therefore, the following parameters need to be considered when conducting a study on DNA-based methods including; PCR amplification success, DNA content and accuracy in identification of species (Muñoz-Colmenero et al., 2016).

PCR assay is an alternative method for detection of porcine gelatine due to high stability of DNA structured compare to protein which can be easily degraded when extreme pH and temperature is applied (Aida et al., 2007; Tasara, Schumacher, & Stephan, 2005). Shabani et al. (2015) employed a study by observing DNA using through conventional PCR assay. They have succeeded in identifying the animal origin of gelatin powders and gelatin used in food products and capsule shells by PCR assay. DNA is isolated from the gelatin powders, gelatine-containing products and capsule shells to obtain DNA fragments. However, the problem with highly processed food is that the DNA fragments can be very short and there will be issues when doing PCR amplification (Mafra, Ferreira, & Oliveira, 2008). It is compulsory to obtain a sufficient amount of DNA fragments when conducting PCR amplification. PCR amplification is the act of doing DNA replication in in vitro using primers to target DNA and DNA will be synthesised into millions of copies (Nakyinsige, Man, & Sazili, 2012). Hence, in this study, isolation of DNA is carried out using a commercial kit which is adjusted for maximal recovery of short DNA fragments. The results from their experiment shows eight samples that were labelled as bovine gelatin has been successfully detected containing only bovine gelatin and the absence of porcine gelatin. The minimum level of detection for bovine and porcine proven in this study is found out to be at 0.1% w/w when mixture of gelatin is tested.

Jannat et al. (2018) in their study have differentiated porcine, bovine and fish source of gelatin in commercial pure gelatin as well as drug and food products using PCR technique. They employed the use of species-specific primer and chemometrics analysis to obtain pattern which is helpful for discrimination of gelatin origins. Note that developing and selecting the right primers are compulsory to ensure successful discrimination of species in a sample (M. A. Sentandreu, & E. Sentandreu, 2011). PCA and PLS-DA methods are used in analysis of LC/MS data sets in achieving an obvious gelatin origin pattern. LC/MS method used in this study is proven to give advantage when DNA is denatured or destroyed or removed during the food manufacturing process. However, the method in this study has some limitations especially when it comes to denatured DNA or when DNA loss occurs in the products tested. Although this method is quite reliable and simple for gelatin determination however it is not applicable for processed food due to the degradation of DNA (Fajardo et al., 2010).

It has been a great challenge to researchers and food industries in finding the most accurate and reliable method in detecting porcine DNA. Moreover, mislabelling of products or mixing of cheaper products to a more expensive one is excessively found in the food industry (Amaral et al., 2017). Abdullah Amqizal et al. (2017) performed a study for Halal authentication of gelatin-containing food products and commercial pure gelatin by using conventional PCR method. Seven primers were tested for specificity and sensitivity using conventional PCR for choosing the most suitable primer. Porcine species-specific primer No. 2 was chosen as it can detect up to 12 out of 36 processed food samples. Although a cross-reactivity is shown towards bovine and have low specificity with porcine, it can minimize sensitivity to degraded DNA due to its short amplicon. Further confirmation test is carried on the 12 positive samples and 5 out of 12 positive samples were confirmed by using cloning, sequencing and blasting method. Negative results might occur due to the absence of targeted gene in the food samples.

DNA-based method which targets the amplified DNA sequences is a good technique for detection of DNA in food matrices as it is less affected by heat or manufacturing processed and has greater DNA stability (Poms, Klein, & Anklam, 2004; Lee et al., 2016). DNA can be easily obtained as it is presence in most of the tissues and only a small amount is needed to be extracted. Lee et al. (2016) reported a technique of employing DNA-based methods by using species-specific PCR assay in determining the species origin of capsule gelatin. They managed to obtain the information regarding the source of gelatin used in capsule production and confirmed that 27 out of 28 samples were labelled correctly according to the manufacturer label. One sample is labelled as tilapia gelatin however based on their PCR method it was found out to be made of bovine gelatin. For further confirmation, they conducted cloning and sequencing to the PCR product, and it was found that 98% sequence identity is matches the genes of bovine species. It was confirmed that the manufacturing company conducted fraudulent by substituting bovine gelatin to tilapia gelatin. Whole Genome Amplification kit (WGA) is used to amplify DNA extracted from the gelatin capsules to increase the quantity of DNA. They also successfully developed a technique by using species-specific primers and DNA amplifications of short fragments. No cross reaction was observed among bovine, porcine, fish and plant gelatin with limit of detection as low as 0.01 ng/μL DNA.

The issue regarding gelatin does not limit to just religion where certain religions forbid the consumption of several animals but also due to allergic reaction towards the presence of some gelatin ingredients (Tanabe et al., 2007; Raja Mohd Hafidz, et al., 2012). Hence, to solve this issue of gelatin, Mutalib et al. (2015) conducted a study to find a solution for a better method in detecting porcine DNA by comparing the sensitivity of PCR-southern hybridization technique and conventional PCR technique in gelatin capsules. Results from PCR-southern hybridization show that six out of 20 capsule samples were tested positive for porcine DNA showing grey colour at the spots in the middle while negative results showed no colour formed in the middle. Results of the test is considered invalid when the internal control spots showed no presence of colour at all. However, when tested with conventional PCR, it failed to produce positive results. Contrast to the finding reported by Matsunaga et al. (1999) where they successfully detect porcine DNA, the possible reason for this problem might be due to the degradation of mitochondrial DNA but also due to the presence of only small fragments of DNA (Matsunaga et al., 1999; Teletchea, Maudet, & Hänni, 2005). Another reason is that gelatin is added in a very low concentration that further process causes the DNA to be completely vanished making the detection of gelatin more difficult (Eryılmaz et al., 2017). Table 2 shows molecular methods for gelatin differentiation.

Table 2. Molecular method for gelatin differentiation

Methods	Advantages	Limitations	LOD/LOQ*	References
Species-specific PCR assay	Aim: To identify animal source of origin in gelatin. Detection: Able to identify bovine and porcine in food and pharmaceutical products. Advantages: Inexpensive, sensitive for routine analysis and specific.	High-efficiency primers and suitable extraction methods would be useful to overcome short DNA fragments.	0.1% w/w of porcine and bovine DNA in gelatin-containing food products and capsule shells.	Shabani *et al*., 2015
Species specific PCR and LC/MS combined with PLS/DA and PCA	Aim: Identification of bovine, porcine and fish gelatin in commercial pure gelatin as well as drug and food products. Detection: Discriminate gelatin samples from its source of origin by pattern recognition. Advantages: LC/MS performs well due to its ability to discriminate animal source even when the DNA is denatured or removed.	The PCR method cannot be applied on denatured DNA or when DNA is removed from the products. Follow-up study using MS-based strategy will provide the information of the tissue origin (skin or bone) of bovine gelatin.	Not stated.	Jannat *et al*., 2018
Conventional PCR assay and Real-Time PCR assay using species-specific primer	Aim: Evaluate halal authentication in commercial gelatin and gelatin-containing food products. Detection: Confirms the presence of porcine DNA in 12 out of 36 samples by conventional PCR. 5 out of 12 positive samples were confirmed by cloning, sequencing and blasting. Advantages: Sensitive. Reliable. Real-time PCR is more reliable method compare to conventional PCR. Detects 27 positive samples compare to conventional PCR.	Primer have low specificity for porcine and slight cross-reactivity with bovine.	Not stated.	Amqizal *et al*., 2017
Species-specific PCR assay combined with WGA	Aim: Determining species origin of gelatin capsule. Detection: Successful in overcoming the problems regarding DNA denaturation in highly processed products. Advantages: Whole genome amplification techniques help in increasing the sensitivity of PCR assay. Sensitive.	Cross-contamination from positive samples is possible during DNA amplification.	Detection level of DNA for bovine-, porcine-, tilapia- and plant-derived in gelatin capsules as low as 0.01 ng/μL.	Lee *et al*., 2016
PCR combined with southern hybridization compared with conventional PCR	Aim: Detect porcine DNA by comparing between PCR-southern hybridization and conventional PCR. Detection: Detect low amount of porcine DNA amplicons in highly processed products. Advantage: Sensitive.	Conventional PCR is not sensitive and reliable enough in detecting low amount of porcine DNA. Failure of ATP6 primer in detecting porcine DNA due to degradation and low amount of mtDNA.	Not stated.	Mutalib *et al*., 2015

*LOD: Limit of Detection, LOQ: Limit of Quantitation

Electrophoresis method

There are several electrophoresis methods available for gelatin differentiation including SDS-PAGE (Nur Azira et al., 2014) and two-dimensional electrophoresis (2-DE) (Aina et al., 2013). Polyacrylamide gel electrophoresis (PAGE) is an example of one of the most applicable electrophoresis method due to its simplicity and rapid analysis (Eryılmaz et al. 2017). In a study by Nur Azira et al. (2014), they had successfully developed a new method in differentiating porcine and bovine adulterated samples by employing the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) technique combined together with principal component analysis (PCA). The limit of detection was up to 5% (v/v) of porcine gelatin in bovine gelatin. Protein were successfully extracted from food products using cold acetone method without affecting their electrophoretic profile of the polypeptides. They observed sixteen polypeptides with molecular weights ranging from 58 to 160 kDa which shows the adulteration of porcine to bovine gelatin and two polypeptides were detected for adulteration of bovine to porcine gelatin. This method, however, failed to detect bovine adulteration in porcine gelatine. Problems with this method will arise when counteracting complex commercial gelatin processed products as it will produce poor electrophoretic profiles.

The first study on gelatin using 2-DE was introduced by Aina et al. (2013) who studied about the determination of gelatin polypeptides biomarker based on the hydrolysis of collagen. Three samples of porcine skin gelatin were obtained from different producers and a total of 10 different biomarkers were obtained. They also mixed porcine and bovine to test the availability of the 10 biomarkers and the limit of detection is as low as 1.0% (w/w). Cold acetone method is applied in protein precipitation which helps in producing high quality images of gel. However, sample preparation is quite challenging due to the presence of minerals, salts and other compounds during gelatin processing and will affect the results by producing poor quality of gel images (Isola et al., 2011). Different methods of electrophoresis are tabulated in Table 3.

Table 3. Electrophoresis method

Methods	Advantages	Limitations	LOD/LOQ*	References
SDS-PAGE combined with PCA	Aim: To differentiate bovine and porcine gelatin in adulterated samples. Detection: Detect adulterated bovine gelatin with porcine gelatin successfully. Advantages: Protein can be successfully extracted from food products using cold acetone method without affecting their electrophoretic profile of the polypeptides.	Complex commercial gelatin processed product will produce poor electrophoretic profiles hence this method cannot be applied. Further studies for determining the purity of gelatin in the product is needed.	Detection of 5% (v/v) for porcine gelatin adulterant in bovine gelatin.	Nur Azira *et al*., 2014
	Aim: To identify the differentiation of gelatin origin in processed food. Detection: Detect gelatin origin of processed food based on the molecular weight region of electrophoretic profiles between bovine and porcine gelatins from range of 53 to 220 kDa. Advantages: Tolerant to contents impurity and simple sample preparation	Complex ingredients in commercial gelatins products require further examination using acetone precipitation method of sample extraction.	Not stated.	Nur Azira, Amin, & Che Man, 2012
2-DE	Aim: To determine gelatin polypeptides biomarker based in the hydrolysis of collagen. Detection: 10 biomarker gelatin polypeptides. Advantages: Able to spot proteins after post-translational modifications. Allows simultaneous resolutions of thousands of proteins.	Sample preparation is quite challenging due to the presence of minerals, salts and other compounds which comes from gelatin processing. Poor quality of gel images.	Detection of adulteration level of 1% w/w for porcine gelatin in bovine gelatin.	Aina *et al*., 2013

*LOD: Limit of Detection, LOQ: Limit of Quantitation

Immunochemical method

Immunochemical method refers to a process that utilize the highly specific affinity of an antibody for its antigen to determine the distribution of a given protein (antigen) in tissues or cells (Bergman, Bechtel, & Wiemann, 2006). One of the immunochemical methods available for gelatin differentiation is known as enzyme-linked immunosorbent assay (ELISA). This technique is a very sensitive for gelatin detection because not only the type and quality of a gelatin affect the results but the amount of concentration of gelatin will also contribute to either positive or negative results (Eryılmaz et al., 2017).

Protein based method such as ELISA is much more preferred as compared to DNA-based method as DNA is easily degraded by gelatin manufacturing process (Doi et al., 2009). In a study conducted by Nur Azira et al. (2018), they reported a technique which utilizes the use of ELISA technique using species-specific marker peptides in order to determine the mammalian gelatin in pharmaceutical capsule and two ELISAs have been developed based on antipeptide polyclonal antibodies (pAbs) namely pAb1 and pAb2. From the test, pAb1 able to cross-react with all samples, while pAb2 only shows small cross-reactivity to chicken and fish gelatin. Therefore, pAb2 are chosen to be tested on commercial pharmaceutical capsules. The results obtained were determined as positive if the value is greater than 0.25 µg/mL which will be considered as cut off value to reduce the false positive results. However, there might be some limitations in the protein extraction due to irreversible protein denaturation and protein insolubilization. Hence, further study on different types of capsules might be helpful in future research.

Nur Azira et al. (2016a), developed a technique in detecting porcine gelatin in edible bird's nest by employing ELISA method. They developed three polyclonal antibodies against porcine species-specific amino acid sequences of collagen, which is known as pAb1, pAb2 and pAb3 with limit of detection of 0.033, 0.082 and 0.052 mg/mL respectively. pAb3 shows no cross reaction with EBN hence we can conclude that the use for pAb3 in gelatin authentication to be quite a reliable method. Nur Azira et al. (2016b) also developed an ELISA method for determination of gelatin in confectionary products using polyclonal antibodies against peptide immunogen of collagen a2 (I) chain. They successfully detect mammals gelatin in commercially processed products e.g. gummy, marshmallow, jelly and premix powder. ELISA is a highly sensitive method which requires only simple sample preparation and proven to be an alternative to problems with DNA degradation in DNA-based methods. Several immunochemical methods used are presented in Table 4.

Table 4. Immunochemical methods

Methods	Advantages	Limitations	LOD/LOQ*	References
ELISA using species-specific marker peptides	Aim: Determination of mammalian gelatin in pharmaceutical capsules with the use of antipeptide polyclonal antibodies Detection: successful detection of gelatin in pharmaceutical capsules Advantages: Rapid, low-cost, high throughput	Drawbacks in protein extraction caused by irreversible protein denaturation and protein insolubilization Further study on different types of capsules will be helpful	0.03 and 0.08 µg/mL of mammalian gelatin for pAb1 and pAb2 respectively	Nur Azira *et al*., 2018
Competitive Indirect ELISA and SDS-PAGE	Aim: To determine the efficiency of polyclonal antibodies (pAbs) against peptide immunogens for detection of porcine gelatin in edible bird's nest Detection: All pAbs are able to recognise bovine and porcine gelatin Advantages: Sensitive, accurate, repeatable and specific	Cross-reactivity may occur	0.033, 0.082 and 0.052 µg/mL of porcine gelatin for pAb1, pAb2 and pAb3 respectively	Nur Azira *et al*., 2016a
Competitive Indirect ELISA	Aim: Determination of gelatin in confectionary products using polyclonal antibodies against peptide immunogen of collagen a2 (I) chain. Detection: Mammals gelatin in commercial processed products e.g. gummy, marshmallow, jelly and premix powder Advantages: Highly sensitive, requires only simple sample preparation, alternative to problems with DNA degradation in DNA-based methods	Repeatability is necessary	0.05 µg mL^{-1} of porcine gelatin	Nur Azira *et al*., 2016b
Indirect ELISA	Aim: To develop ELISAs for porcine gelatine adulteration using anti-peptide polyclonal antibodies Detection: Porcine gelatine in EBN Advantages: Highly sensitive and does not require highly sophisticated equipment or specialist trained staff	Circumstance was related to the high similarity of the amino acid sequences of collagen from different species	0.12, 0.10 and 0.11 µg g^{-1} in porcine gelatin for pAb1, pAb2 and pAb3 respectively	Nur Azira *et al*., 2015

*LOD: Limit of Detection, LOQ: Limit of Quantitation

Spectroscopic method

Spectroscopy method is the technique that use radiated energy to analyze properties or characteristics of materials (Dahman et al., 2017). Spectroscopic method such as ATR-FTIR is a practical method for gelatin differentiation and can provide us with the information on gelatin molecules and its structure (Sivakesava & Irudayaraj, 2002). Hence, with the help of combination of chemometrics detecting adulteration will be much easier because ATR-FTIR spectra does not provide us with a specific peak (Nimptsch et al., 2011; Boran et al., 2010). Spectrum from ATR-FTIR shows molecular fingerprint as a result from the radiation on the samples. Therefore, each sample will have its own unique fingerprint representing their structures (Raja Mohd Hafidz et al., 2012). Aloglu and Harrington (2018) in their work presented a method for differentiating bovine, porcine and fish gelatins. They employed the use of four different types of multivariate classifiers for the classification of bovine, porcine and fish gelatin such as fuzzy rule-building expert system (FuRES), two different support vector machine classification trees (SVMTreeG and SVMTreeH), super partial least squares discriminant analysis (sPLS-DA) associated with two data-preprocessing methods, i.e., standard normal variate (SNV) and principal component orthogonal signal correction (PC-OSC). They found out that ATR-DFTIR combined with the classifiers mentioned above as a reliable and accurate method for classifying of gelatin species.

Cebi et al. (2016) in their study presented that they successfully classify and discriminate bovine gelatin, porcine gelatin and fish gelatin by employing ATR-FTIR method coupled with chemometrics analysis. Two distinct spectral bands were used for comparison in PCA comprising of Amide-I (1700 - 1600 cm-1) and Amide-II (1565 - 1520 cm-1). Hashim et al. (2010) reported a technique in differentiating bovine and porcine gelatin using ATR-FTIR spectroscopic method. They found out that gelatin sources can be discriminate by observing two different peaks that is known as Amide-I and Amide II. The major differences between bovine and porcine gelatin can be observed by looking at the spectral range that is responsible for the deformation of N–H bonds which are 3290 to 3280 cm-1 and 1660 to 1200 cm-1. Analysis of PCA represented by Cooman's plot showed clear discrimination of gelatin according to their sources. As compared to the method used in Cebi et al. (2016), this method does not test for fish gelatin and the mixture of bovine and porcine gelatin. ATR-FTIR is a simple and low-cost method compares to other analytical methods available for gelatin differentiation. It provides rapid analysis and fast results can be obtained therefore it is a very suitable method to be used in routine analysis. Different spectroscopic methods used are summarized in Table 5.

Table 5. Spectroscopic method

Methods	Advantages	Limitations	LOD/LOQ*	References
ATR-FTIR with Pattern Recognition	Aim: To differentiate bovine, porcine and fish gelatin. Detection: Good separation of bovine, porcine and fish spectra. Advantages: Fast and reliable results.	Results can be inaccurate unless it is combined with other methods.	Not stated.	Aloglu, & Harrington, 2018
ATR-FTIR combined with chemometrics PCA	Aim: Differentiation and authentication of gelatin in food products. Detection: Clear discrimination of all types of gelatin. Advantages: Rapid, simple and economic.	Not reliable without PCA.	4% w/v of bovine, porcine and fish gelatins.	Cebi *et al*., 2016
ATR-FTIR combined with PCA	Aim: Discrimination of bovine and porcine gelatin Detection: Manage to classify gelatin into their sources Advantages: Fast and rapid method.	Same method can be employed for samples analysis.	2% w/v of porcine and bovine gelatins.	Hashim *et al*., 2010

*LOD: Limit of Detection, LOQ: Limit of Quantitation

Conclusion

Over the years, there have been thousands of research carried out to detect and differentiate gelatin and the sources of gelatin including electrophoresis methods, chromatographic methods, immunochemical methods, molecular methods and spectroscopic methods. However, with every method developed, there will always be advantages as well as some drawbacks. Studies on the types of methods available is compulsory when choosing the best method for gelatin differentiation before carrying out the research. Further studies still need to be done in searching for the most rapid, inexpensive, and accurate method in gelatin differentiation.

Conflict of Interest

The authors declare that there is no conflict of interest in this work.

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