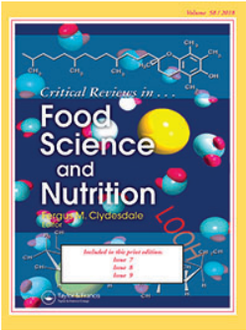

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Gelatin controversies in food, pharmaceuticals, and personal care products: Authentication methods, current status, and future challenges

Md. Eaqub Ali[a,b], Sharmin Sultana[a], Sharifah Bee Abd Hamid[a], M. A. Motalib Hossain[a], Wageeh A. Yehya[a], Md. Abdul Kader[c], and Suresh K. Bhargava[d]

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ABSTRACT

Gelatin is a highly purified animal protein of pig, cow, and fish origins and is extensively used in food, pharmaceuticals, and personal care products. However, the acceptability of gelatin products greatly depends on the animal sources of the gelatin. Porcine and bovine gelatins have attractive features but limited acceptance because of religious prohibitions and potential zoonotic threats, whereas fish gelatin is welcomed in all religions and cultures. Thus, source authentication is a must for gelatin products but it is greatly challenging due to the breakdown of both protein and DNA biomarkers in processed gelatins. Therefore, several methods have been proposed for gelatin identification, but a comprehensive and systematic document that includes all of the techniques does not exist. This up-to-date review addresses this research gap and presents, in an accessible format, the major gelatin source authentication techniques, which are primarily nucleic acid and protein based. Instead of presenting these methods in paragraph form which needs much attention in reading, the major methods are schematically depicted, and their comparative features are tabulated. Future technologies are forecasted, and challenges are outlined. Overall, this review paper has the merit to serve as a reference guide for the production and application of gelatin in academia and industry and will act as a platform for the development of improved methods for gelatin authentication.

KEYWORDS

Gelatin source authentication; protein- and nucleic acid-based techniques; biomarkers; multiplex platforms; pharmaceutical and personal care products

Introduction

The selective identification and quantification of animal materials are of enormous interest for food, pharmaceutical, and personal care products. Authentication of gelatin limits the spread of zoonotic threats by animal materials, prevents unfair competition in business settings, boosts consumer confidence and product sales, and brings long-term benefits to public health, social harmony, economic growth, and biological conservation of endangered species. Therefore, public awareness, regulatory laws, and authentication tools work side by side to achieve these overall objectives.

Gelatin is a highly purified protein derived from collagen, a connective tissue abundant in bones, skin, and animal hides (Liu et al., 2015). Gelatin is widely used as a coating, binding, gelling, and glazing agent in food, pharmaceuticals, and osmetic products, including confectionaries, creams, lotions, face-masks, capsule shells, and dietary supplements, because of its unique structural stability and excellent functional, nutritional, and other physio-chemical properties (Karim and Bhat, 2008; Zhang et al., 2009).

Gelatin is manufactured from the by-products of certain mammalian animals such as pigs and cows by acidic or alkaline treatment at high temperature and pressure (Karim and Bhat, 2008; Zhang et al., 2009). During acidic or alkaline treatment, the fibrous structure of collagen is broken down, cross-linkages between different polypeptide chains develop and gelatin is formed (Yang et al., 2007). Depending on the fibrous structure of collagen, there are two main processes of commercial gelatin production from raw collagen materials: (1) acidic treatment (type A), which is used to extract gelatin from collagen with fewer covalent cross linkages and from collagens with a high fat content (such as pig or fish skin) to avoid saponification or (2) alkali treatment (type B), which is employed in industries to extract gelatin from chopped spilt materials and bones (such as bovine and fish by-products; Schrieber and Gareis, 2007). The physicochemical characteristics of raw gelatin products are determined by the three-dimensional structure of the amino acid sequence, the molecular mass distribution, the ionic strength and the pH, because the two types of gelatin differ in terms of the isoelectric point (IEP). The IEP for type A gelatin is in the pH range of 8 to 9, whereas for type B gelatin, the IEP is only 4.8–5.5. The functional standards of gelatin products are associated with their surface behavior, such as the formation and stabilization of

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foams and emulsions, adhesive properties, dissolution properties, bloom strength, viscosity, and gelling properties (Schrieber and Gareis, 2007).

However, both the price and acceptability of gelatin products depend on the animal origin of the gelatin. For example, the Muslim halal and Jewish kosher dietary laws require gelatin or gelatin capsules to be free from porcine materials. A select denomination of the Christian community also does not like to use porcine products. Hindu religious customs also require gelatin to be free from bovine by-products. Furthermore, the religious and social acceptability of bovine gelatin depends on the slaughtering method. Additionally, bovine products are greatly avoided in Europe and the United States due to concerns with mad cow disease and bovine spongiform encephalopathy (BSE), which is a fatal neurodegenerative disease in cattle that causes spongy degeneration of the brain and spinal cord (Cai et al., 2012). Approximately 326,000 tons of gelatin is produced per year, and among the gelatin, 46% comes from pig skin, 29.4% comes from bovine hide, 23.1% comes from bones, and only 1.5% is produced from other sources (Karim and Bhat, 2008). Recent data indicates that gelatin could also be procured from fish, especially from the skin of scaly fish, using approaches similar to those used for other animals (Norziah et al., 2009; Liu et al., 2015). Fish gelatin has received wider acceptability because fish are permissible in most religions and cultures, and fish products also have superior health attributes (Liu et al., 2015).

Throughout the history of human civilization, religions, cultures, and health concerns have had a tremendous influence on the production and sale of consumers good. For example, in 2013, the turnover of the global halal market was $580–660 billion US dollars, and the market has continued to expand rapidly (Jahangir et al., 2016). In addition to the rapid increase in the Muslim population and halal awareness, one of the main driving forces enhancing the popularity of halal items is the increasing assurance of halal through authentication and market monitoring (Malik et al., 2016). Gelatin is one of the most studied items in halal-related research, and there is a need for a trustworthy and convenient technique for its authentication. Therefore, several verification methods, including spectroscopic (Hashim et al., 2010; Hermanto and Fatimah, 2013), immunochemical (Venien and Levieux, 2005; Tukiran et al., 2016b), chromatographic-chemometric (Nemati et al., 2004; Widyaninggar et al., 2012; Raraswati et al., 2013; Azilawati et al., 2015), electrophoretic (Hamdan and Righetti, 2005; Hermanto and Fatimah, 2013), and mass spectrometry coupled with liquid/gas chromatographic methods (LC/GC-MS; Ibáñez et al., 2013; Yilmaz et al., 2013), as well as chemisorption (Hidaka and Liu, 2003), lateral flow detection (LFD; Gendel, 2016), PCR and PCR-RFLP (Lin and Hwang, 2007), and PCR-Southern blotting (Mutalib et al., 2015) have been developed. These methods are mainly based on protein and DNA biomarkers and their spectral fingerprints. An overview of the major gelatin identification techniques is depicted in Fig. 1, and their comparative features are presented in Table 1.

Protein-based methods

The gelatin authentication methods based on protein or peptide biomarkers are vast and are briefly discussed as follows.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) has been extensively used to identify the specie origins of animal materials, including gelatin and gelatin products. This method is based on the combined effect of specific interactions between the raised antibody and target antigen (Ekins, 1991). During the ELISA technique, an unknown number of antigens is attached to an ELISA plate with several wells through specific or nonspecific interactions. Then, a specific chemically linked antibody is added to the surface of the wells to facilitate binding of the antibody with the target antigen to form an antigen–antibody complex. The antibody used in the assay is covalently linked to an enzyme, which at the final stage of the reaction produces a substrate that can produce a meaningful signal by changing the color of the reaction mixture, indicating the amount of antigen present in the unknown samples. Herein, after completing each step, the contents in the ELISA plate's wells is washed with a soft detergent solution to remove the unbound antibodies or proteins to avoid any false positive outcomes (Lequin, 2005). The operating principle of the ELISA method is depicted in Fig. 2. Among the ELISA methods, indirect and sandwich ELISAs are best suited for food product authentication (Chen et al., 1998). Indirect ELISA uses two antibodies, one of which binds to the specific antigen. The other is labeled with a reagent that can change the color of the final reaction mixture (Hsieh et al., 2002). With a sandwich ELISA, both antibodies bind to the antigen, but one of the two antibodies is coupled with an enzyme to produce a colored product (Berg and Otley, 2002).

An ELISA is an effective way to measure unknown antibodies or antigen concentrations. Recently, it has been used to detect gelatin sources in various food products (Tukiran et al., 2016b). Venien and Levieux (2005) identified animal-derived gelatin sources using polyclonal antibodies raised against tyrosylated porcine and bovine gelatin samples. The tyrosylation occurs using tyrosine to strengthen the immunogenicity of its parent collagen and converts it into a powerful antigen. In an indirect ELISA, the modified antibodies have large differences in their sensitivity during the manufacturing process. Some antibodies were able to detect the origin of gelatin in food, whereas other antibodies showed strong sensitivity to acidic or alkaline-processed gelatin. Herein, porcine-tyrosylated gelatin showed more sensitivity than bovine gelatin, except alkaline-treated gelatin (which comes from porcine bone). However, in this technique, some antibodies show low specificity due to the homology of the collagen sequence among different species.

To obtain greater specificity toward bovine gelatin with lower process sensitivity, Venien and Levieus (2005) established a new method of raising antibodies against the putative collagen α1-specific bovine sequence to show the changes in amino acid sequences between bovine and unrelated species. They selected one specific sequence from the central region of (N-terminal region) bovine α2 (I) telopeptide chain (Glu-Phe-Asp-Ala-Lys-Gly-Gly-Gly-Pro-Gly) as peptide 1 and (Gly-Pro-Ala-Gly-Ala-Pro-Gly-Pro-Pro-Gly) as peptide 2. Herein, antipeptide 2 antibodies have higher reactivity than antipeptide 1 when raised against gelatin and collagen. Between the two sources, antipeptide 2 showed greater sensitivity against bovine gelatin than porcine gelatin. Thus, the indirect

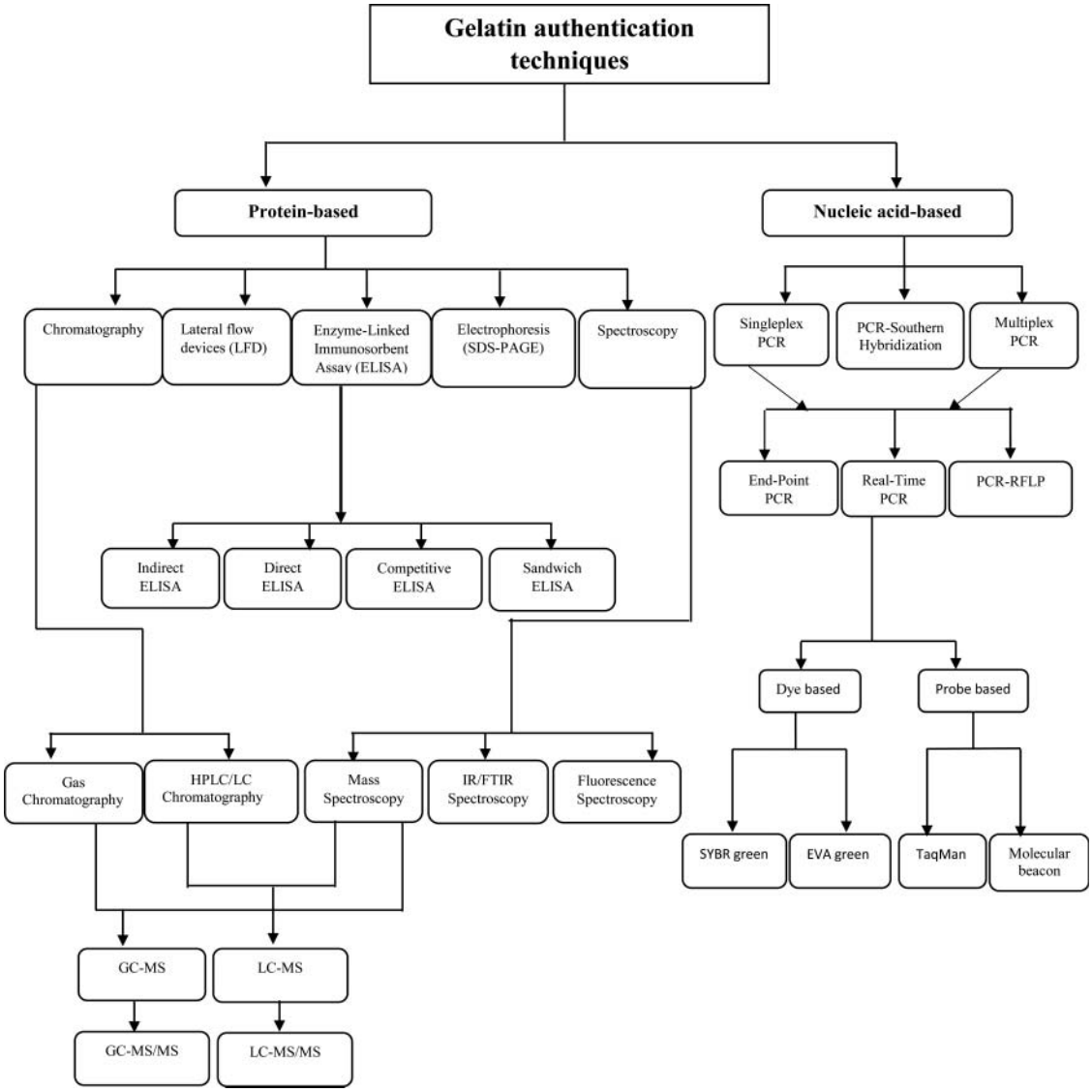

Figure 1. A summary of gelatin authentication techniques.

competitive ELISA method could be used as an effective analytical tool to discriminate porcine and bovine gelatin. However, the gelatin process treatment (such as acid or alkaline), species origin (bovine or porcine), and the source of by-products (bone, skin, and hide) can affect the sensitivity of the ELISA assay (Venien and Levieux, 2005; Nhari et al., 2012).

Therefore, to increase the sensitivity of the assay, Tukiran et al. (2016a) have developed another competitive indirect ELISA assay for the rapid detection of porcine gelatin in edible birds nest (EBN). They developed a polyclonal antibody using maleimide activated keyhole limpet hemocyanin (KLH) coupled with amino acid sequences from rabbit to raise antibodies against specific amino acid sequences of porcine collagen $\alpha 1$ (I) chain pAb3 (22-amino acid sequence) and $\alpha 2$ (I) chain pAb1 (14-amino acid peptides) and pAb2 (15-amino acid peptides). The pAb1 and pAb2 amino acid peptides showed cross reactivity with egg white and cave nest and blood cave nest, respectively, whereas no cross-reactivity was observed with pAb3 peptides. The authors concluded that the pAb3 amino acid peptides expressed greater sensitivity (with an LOD of 0.05 μg/mL), accuracy, specificity, and repeatability to recognize porcine and bovine gelatin. Thus, this proposed technique could be used to determine the quality of the gelatin.

A novel sandwich ELISA method was developed to trace the origin of bovine and porcine gelatin in commercial food products to reduce the risk of allergic reactions to gelatin in patients (Doi et al., 2009). The authors have developed two sandwich polyclonal antibodies: one from the immunization of gelatin in rabbits (pAb2- pAb1 ELISA; with pAb1 used for the capture reaction and pAb2 for the coating) and the other from goats (pAb3- pAb3 ELISA). The reactivity test for both ELISA methods showed strong reactivity against porcine and bovine gelatin, but they had very low reactivity for fish gelatin sources. The goat pAb3-pAb3 ELISA was reacted mostly against bovine and porcine gelatin that undergoes alkaline treatment, whereas the rabbit pAb2-pAb1 ELISA was reacted against alkaline-treated porcine gelatin. However, the goat pAb3-pAb3 ELISA showed no cross reactivity, whereas the rabbit pAb2-pAb1 ELISA showed cross reactivity with boiled squid. Finally, the authors successfully detected gelatin in all declared gelatin-containing commercial foods using the goat pAb3-pAb3 ELISA methods without showing any false positive or false negative results or

Table 1. Existing gelatin authentication techniques and their key features.

Method name	Major Features	Advantages	Limitations	Detection Limits	Products Detected	References
			Spectroscopic Techniques			
Spectroscopy (IR/FTIR)	Detection is performed based on IR fingerprints of various reference amino acids present in various gelatin preparations and the secondary structure of various gelatins	• Noninvasive and simple • Requires a small amount of sample	• Requires high-purity samples • Cannot detect gelatin under mixed matrices • Limited sensitivity	100 ng/mL	Bovine and porcine gelatin hydrolysates	(Ocaña et al., 2004; Hashim et al., 2010; Hermanto and Fatimah, 2013)
			Chromatography Techniques			
HPLC	Marker proteins and peptides are separated and detected based on known reference standards and post-HPLC PCA analysis	• Rapid and sensitive • Tolerable cost	• Labor intensive • Cannot provide quantitative data • Often require statistical analysis	0.05% (w/w)	Twelve commercial gelatins including bovine, porcine, and fish	(Aristoy and Toldrá, 2004; Azilawati et al., 2015)
HPLC-MS	Target proteins are digested with a suitable enzyme, and the fingerprint peptides are separated using a HPLC and then detected and mapped using a mass spectrometer	• Feasible • Sensitive and specific	• Analysis and laboratory set up and maintenance are highly expensive • Laborious • Requires high expertise skills	Not given	Commercial gelatin products (bovine and porcine)	(Zhang et al., 2008x)
NanoUPLC-ESI-Q-TOF-MS^{E}	NanoUPLC-ESI-Q-TOF-MS^{E} is a novel technique that can function in a data independent acquisition mode and alternate low and elevated collision energy applied to collect product ion and precursor information; thus, it is possible to generate accurate mass acquisition on the peptide level simultaneously with recognizing specific gelatin peptides	• Simple and specific method for authentication of commercial gelatin containing dairy food products • Moderate cost • Together with an exclusion list of common peptides, this method can also provide quantitative data	• Labor intensive • Obtaining quantitative data from complex food matrices is still immature and further studies are need	Not given	Porcine and bovine gelatin in commercial food products	(Yilmaz et al. 2013)
PEME	PEME uses a pulsed electric field for sample extraction, pre-concentration and analysis of derivatized amino acid compositions in gelatin. The amino acids of some selective species are derivatized using *o*-phthalaldehyde (OPA) to increase ultraviolet absorbance and lipophilicities. Then, the derivatized amino acids are detected and analyzed using the HPLC/UV system	• A novel, simple, and practical method for authentication of gelatin sources • Highly efficient for analysis of amino acids (with 43 and 79% extraction recoveries) • Highly sensitive	• Requires an electric field supply prior to extraction • Hydrophilic nature and low UV absorbance of target peptide sequence makes PEME difficult to differentiate gelatin origins • Cannot provide quantitative data	25–50 ng/mL	Animal sources of gelatin (bovine and porcine)	(Rezazadeh et al., 2015)
HPLC-PCA	HPLC-PCA is the unsupervised pattern recognition technique widely used for the differentiation and classification of objects based on the mathematical and statistical operations to distinguish the variables of analogous profiles	• Rapid and sensitive • Tolerable cost • Specific • Can be used for commercial gelatin source authentication	• Labor intensive • Cannot provide quantitative data • Often requires statistical analysis	Not given	Raw bovine and porcine gelatin; Pharmaceutical capsule shells	(Nemati et al. 2004; Widyaninggar et al., 2012)
			Enzyme linked immunosorbent assay (ELISA)			
ELISA	ELISA-based techniques are based on the principle of antigen-antibody interactions followed by color changes that can be detected visually or measured using a spectroscopic technique.	• Less costly • Simple and easy • Sensitive (0.05%) • Provides both qualitative and quantitative data	• Multiplexing not possible • Cannot detect gelatin under mixed matrices • Not suitable for extremely processed samples because of the denaturation of the protein based biomarkers	5 ng/mL	Bovine and porcine gelatin	(Tukiran et al., 2016x)

		Electrophoretic analysis (SDS-PAGE)				
SDS-PAGE	Separates proteins and peptides based on their molecular weight	• Simple and easy • Sensitive • Less costly	• Degradation profile of peptide marker • Need reference sample preparation • Difficult to detect gelatin from closely related animal species due to structural similarities • Nonquantitative	Not given	Bovine and porcine gelatin	(Amin et al., 2013; Hermanto and Fatimah, 2013; Malik et al,.2016)
SDS-PAGE PCA	A PCA score plot of the SDS-PAGE better discriminates closely related protein brands	• More sensitive than SDS-PAGE • Provide better resolution than SDS-PAGE	• Requires statistical analysis of SDS-PAGE products • Laborious and time-consuming • Non-quantitative	5% (w/w)	Bovine and porcine gelatins	Chen et al., 1998
		Lateral flow devices (LFD)				
LFD	LFD is a simple strip-like device that performs colorimetric and visual detection of the protein/peptide targets based on the movement and reaction of the molecules onto the strip and subsequent color changes. The detection reaction is similar to ELISA, which involves antigen-antibody reaction, but here it occurs on a latex film.	• Portable and hence offers onsite delivery of results • Easy to operate • No need for heavy instrumentation • Relatively sensitive • Fast (< 35 min)	• Non-quantitative • Multiplexing not possible • Sensitivity is less than PCR methods • Possibility of biomarker denaturation	2.5% (w/w)	Porcine gelatin	(Gendel, 2016)
		Polymerase chain reaction (PCR)				
End-Point PCR	Target biomarkers are selectively amplified from a pool of DNA using a pair of species-specific primers and detected following electrophoretic separation on an agarose gel, microfluidic, or capillary electrophoretic chips	• Highly stable DNA biomarkers • Both simplex and multiplex are possible • Highly sensitive and can be used for degraded samples • Products could be verified using RFLP and sequencing • Reasonable cost and time • Robust, reproducible, and efficient	▪ Lab-based methods ▪ Nonquantitative ▪ Difficult optimization	0.05–0.01% (w/w) or 0.0001 pg/mL	Bovine, porcine, tilapia fish, and plant gelatins	(Lee et al., 2016x)
		Real-time PCR (probe based)				
TaqMan Probe based real-time PCR	In addition to the species-specific primers, the TaqMan probe binds to the specific DNA target site during the denaturation phase of the PCR and then dissociates and undergoes a cleavage process to emit a fluorescent signal that provides quantitative data about the target in bulk mixtures.	• Highly stable DNA biomarkers • Both simplex and multiplex are possible • Highly sensitive and can be used with degraded samples • Targets could be selected by primers and probes • Products could be verified using RFLP and sequencing • Robust, reproducible, and efficient	▪ Complicated probe design and optimization ▪ Expensive and needs expertise	0.05–0.001% (w/w)	Bovine and porcine gelatin in capsule shells	(Tasara et al., 2005; Cai et al., 2012)
		PCR-southern hybridization on chips (PCR-SHC)				
PCR-SHC	PCR-SHC amplifies and detects PCR targets with very high sensitivity from gelatin formulations.	• High sensitivity • Rapid and specific	▪ Requires labor intensive Southern hybridization ▪ Costly	0.0001% (w/w)	Porcine gelatin	(Mutalib et al., 2015)

Table 2. Several techniques for gelatin authentication.

Method name	Major features	Advantages	Limitations	Detection limits	Species detected	References
		Multiplex PCR Techniques				
Multiplex endpoint PCR	Multiple target DNA markers are selectively amplified from a pool of DNA using several sets of species-specific primers and detected following electrophoretic separation on an agarose gel or microfluidic or capillary electrophoretic chips	• Reduced cost and time since many species are detected in a single assay • Possibility for total analysis	• Variable sensitivity for different species • Difficulties in primer developments for several species with closely spaced Tm • Cannot provide quantitative data	0.02–0.01 pg/mL	Monkey, dog, cat, rat, and pig DNA in food	(Ali et al., 2015)
Multiplex qPCR	The detection principle is similar to that of TaqMan probe simplex PCR, but multiple primers and probes are used	• Reduced cost and time because many species are detected in a single assay • Possibility of total analysis • More sensitive than sensitive multiplex endpoint PCR • Provides both qualitative and quantitative data	• Costlier than multiplex endpoint PCR • Limited detectors and reporter dyes • Complicated optimization • Requirement of statistical analysis	0.003% for bovine and 0.005% for caprine	Bovine and caprine DNA in feed products	(Safdar et al., 2014)
DNA barcoding	DNA barcoding involves amplification of small DNA fragments using a couple of primers followed by sequencing and comparison with reference sequences or barcodes to detect unknown species	• Detects many species in a single assay, saving time and money • Reliable technique	• Often encounter cross species detection • Uses long-length DNA biomarkers that often breakdown under processed states • Mitochondrial pseudo genes might result in false detection	Not given	Marine foods, edible birds, and animal species	(Wong and Hanner, 2008; Luo et al., 2011)
Microarray	Offers the possibility for the detection of hundreds of target DNA and proteins using real-time PCR and ELISA	• Possibility of detecting hundreds and even thousands of targets	• Cross reactivity gives false-positive results • Requirement of a specialized lab and skills	0.5% (w/w)	Fourteen animal species	(Iwobi et al., 2011; Ali et al., 2012)
		Nanotechnology approaches				
Nano Gold	Nano gold particles provide biocompatible surfaces for the immobilization and detection of DNA and protein markers using visual and spectroscopic methods	• Cheap and fast • Noninvasive • Portable • On field delivery of products	• Immature technology, mostly tested at the laboratory scale • Often provides confusing results • Less sensitive than PCR	4–6 μg/mL	Porcine DNA in mixed meats and commercial meat and chicken meatballs	(Ali et al., 2011, 2012)
Silica magnetic particles coupled multiplex PCR	Multiple targets are amplified using fluorescein, biotin, and digoxigenin tagged primers and detected electrochemically using silica magnetic particles	• Cheap and fast • Noninvasive • Portable • On field delivery of products	• Immature technology, mostly tested at the laboratory scale • Often provides confusing results • Less sensitive than PCR	0.0–1.04 ng/μL; 0.00–0.630 ng/μL; 0.0–0.258 ng/μL	*Salmonella, E. coli,* and *Listeria Monocytogenes* strains	(Brandao et al., 2015)
Graphene-Gold Platform	A capture probe is immobilized on a graphene/ Au nanorod/ polythionine surface, and two auxiliary probes are used to constitute the long-range self-assembled DNA nanostructure. The target DNA connects the DNA structure to the capture probe on the electrode surface and [Ru(phen)3]2^{+} is used as a redox indicator to amplify electrochemical signals	• Cheap and fast • Noninvasive • Portable • In-field delivery of products • Highly sensitive	• Immature technology, mostly tested on the laboratory scale • Often provides confusing results	4.03×10^{-14} mol/L	Human papilloma virus	(Huang et al., 2015x)

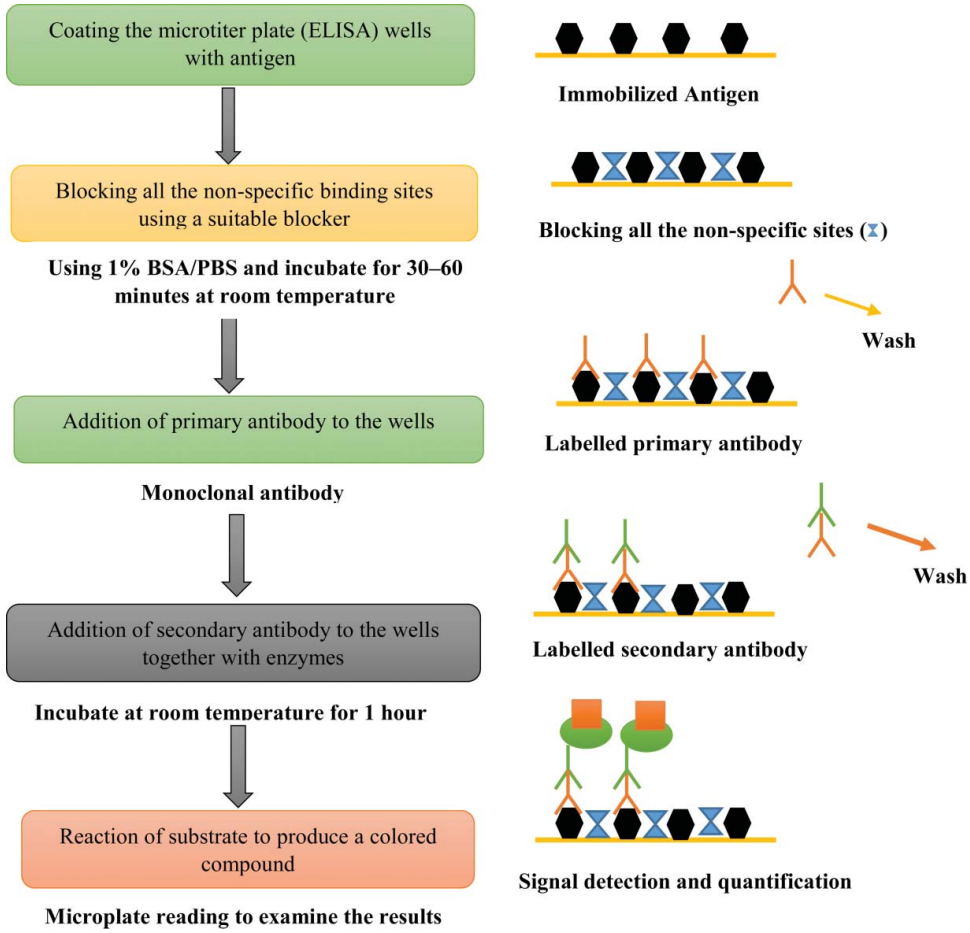

Figure 2. Standard procedure of an enzyme linked immunosorbent assay (ELISA).

cross reactivity. However, the main drawback of the proposed method was that it resulted in false positives or false negatives for gelatinized heated meats. The main factors that affect the sensitivity of the sandwich ELISA techniques include processing treatments in which the acidic treatment leads to less sensitive gelatin than the alkaline treatment. This analytical assay may be limited by cross-reactivity with meat from other species, such as chicken or seafood (squid, prawn, roe, and shellfish), and various commercial foods (Nhari et al., 2012).

Overall, ELISA techniques are able to detect gelatin products without the need of any expensive instrumentation or high-purity sample preparation, offering simplicity in operation and detection (Doi et al., 2009; Nhari et al., 2012; Tukiran et al., 2016b). In addition, ELISA methods provide rapid, low cost and high specificity to differentiate the various animal-derived gelatin sources (Asensio et al., 2008). However, none of them can detect fish gelatin, and because of the high degree of susceptibility of peptide biomarkers to denaturation under heat, chemical, and pressure treatments, these methods are not suitable for the routine analysis of highly processed gelatin products (Lin et al., 2015; Wolf and Lüthy, 2001). Previous studies on ELISA techniques for the differentiation of gelatin sources are depicted in Table 3.

Electrophoresis

Electrophoresis techniques have evolved as simple, rapid, and low-cost analytical tools to detect gelatin sources based on the molecular weight of the protein biomarkers (Hamdan and Righetti, 2005; Amin et al., 2013). Commercially available gelatins have different protein fractions, and the variation in molecular weight can be used as a basis for identifying gelatin origins. Gelatin contains α, β, and γ chains of the parent protein collagen with approximate molecular weights of 100, 200, and 300 kDa, respectively. However, there is significant variation in the lengths and molecular weight of these chains among the species, resulting in separate bands for different species upon electrophoretic separation (Zhang et al., 2006). The fragment profile of commercial gelatins of different species also varies considerably. For example, commercial bovine gelatin produced four fragments, but that of porcine origin resulted in 12 bands upon electrophoretic separation (Malik et al., 2016). Common polyacryl-amide gel (PAGE) that contains a gradient of denaturants is used to migrate protein fractions depending on their individual molecular weight (Hamdan and Righetti, 2005). The applications of different electrophoresis techniques, including sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), are well known for the identification of various animal species (Hamdan and Righetti, 2005). In fact, SDS-PAGE offers a simple and easy way to authenticate halal gelatin products (Hermanto and Fatimah, 2013; Azira et al., 2014). A combination of SDS-PAGE and FTIR spectroscopy was used to differentiate bovine and porcine gelatin products based on the two-dimensional conformation of the peptide or protein (Hermanto and Fatimah, 2013).

Table 3. Previous studies on ELISA methods for authentication of gelatin source.

Method name	Advantages	Disadvantages	References
Indirect ELISA	• Highly specific for bovine gelatin authentication • Can be used for pharmaceutical and commercial gelatin source authentication to ensure the security of the food supply chain • Highly depends on the processing treatments of gelatin (such as alkaline or acidic treatment) and sources (bovine and porcine)	• Can only identify bovine gelatin	(Venein et al., 2005a)
Indirect competitive ELISA	• Highly specific for porcine gelatin authentication • Sensitive and accurate assay compared with indirect ELISA • Can be applied to secure the quality of edible birds nest (EBN)	• Only pAb3 amino acid peptides give specific, sensitive, and accurate results, whereas other designed peptides such as pAb1 and pAb2 showed cross reactivity with egg white and cave nest in the case of porcine gelatin detection from edible birds nest (EBN)	(Venein et al., 2005a; Tukiran et al., 2016a)
Sandwich ELISA	• Can detect both bovine and porcine gelatin in commercially processed food • Accurate and specific • Goat pAb3-pAb3 ELISA has no cross reactivity with boiled squid and gives no false-positive or -negative results	• Gives false-positive results for highly heat-treated gelatin products • Unable to detect fish gelatin sources	(Doi et al., 2009; Nhari et al., 2012)

Currently, a combination of electrophoresis and the principle component analysis (PCA) technique provides better resolution and sensitivity for gelatin source authentication. A PCA score-plot distribution of the SDS-PAGE-densitometry was able to detect as low as 5% of gelatin under a mixed background (Chen et al., 1998). Gelatin was extracted from the reference porcine and bovine capsule shells using cold acetone, and the protein fractions were separated by SDS-PAGE; this results in 12 potential bands (239, 221, 200, 171, 158, 139, 122, 115, 108, 96, 90, and 83 kDa) for the porcine reference gelatin but only 4 (236, 222, 120, and 107 kDa) for the bovine.

Overall, electrophoresis is a simple and low-cost technique, and it effectively discriminates raw gelatin sources based on the molecular weight of the peptides. However, different sources of gelatin products contain various protein fractions with a variation in molecular weight that act as a basis for detecting gelatin origin under raw conditions using electrophoresis. However, in the case of highly processed gelatin products such as marshmallows, jellies, gummies as well as hard and soft capsule shells, the amino acid profile in the finished products might be more variable than that of the original gelatin products. Thus, electrophoresis techniques, which are based on the homogeneity of the gelatin structure and the composition, exhibits uncertainty in gelatin authentication under highly processed conditions.

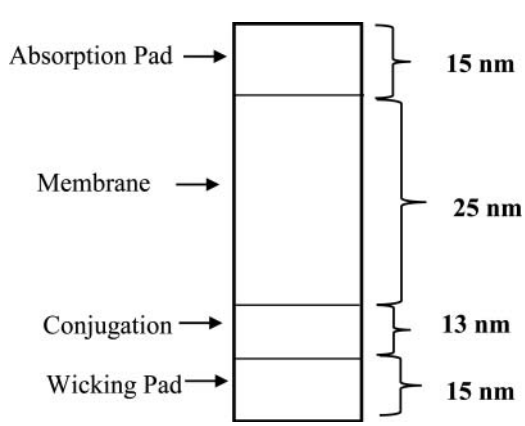

Figure 3. Lateral flow device consisting of an absorption pad, membrane, conjugation pad, and working pad.

Lateral flow devices

A lateral flow device (LFD), which is also referred to as a lateral flow immune-chromatographic assay, is a rapid and simple device used to detect the presence of a specific analyte in the mixed sample without the need for heavy instrumentation (Wong and Harley, 2009; Yetisen et al., 2013). Most LFD assays are noninstrumental, depend on the visual detection of a colored compound, and offer portability, which means testing can be performed any time and at any place (Wong and Harley, 2009).

LFD methods are mainly divided into two types: (1) a double antibody sandwich direct assay and (2) a competitive inhibitory immuno assay. LFD generally consists of different segments, including the absorption pad (15 nm), membrane (25 nm), conjugation pad (13 nm), and wicking pad (15 nm; Fig. 3), all of which are enclosed in a cassette commonly known as the housing, base or backing card that supports the entire system (Yetisen et al., 2013; Mazumder et al., 2010). Colloidal gold and monodisperse latex often perform the detecting function, wherein the antibody labeled gold particles that provide distinct extinction coefficients over the organic dyes are used. Currently, LFD assays are regarded as a time-saving, low-cost, portable, and easily handled operative tool for the speciation of meat, unknown adulterants, and gelatin (Gendel, 2016). Gendel (2016) developed a detection assay based on the LFD principle capable of detecting pig derivatives from raw, processed and gelatin samples within 35 minutes with 0.01%, 1.0%, and 2.5% sensitivity, respectively (Gendel, 2016). Although LFD is a low-cost method, saves time, is lightweight, and has minimum sample preparation, the growing demand for higher sensitivity is often a challenge, demanding the development of upgraded

formats (Wong and Harley, 2009). Moreover, LFD detection is merely qualitative and cannot be used for multiplexing or quantitative results. Furthermore, sample manipulation, manufacturing issues, operator errors, and cross reactivity can influence the results of an LFD assay (Wong and Harley, 2009).

Chromatographic techniques

Chromatographic techniques are effective in identifying chemical components in feed and food samples (Azira et al., 2014). They provide a rapid, cost-effective, and reliable means of identifying structurally similar components in mixed food matrices through unique chemical fingerprints that can easily differentiate various chemical compounds such as peptides, fatty acids, aldehyde, organic acids, nucleic acids, and various food additives (such as artificial color compounds, preservatives, and aromas) (Cserhati et al., 2005; Ibanez et al., 2013). Among the different chromatographic techniques, gas chromatography (GC), liquid chromatography (LC), and high-performance liquid chromatography (HPLC) have been used for separating various biomolecules with close structural similarities (Sander and Wise, 1987; Kupiec, 2004). Currently, reverse phase HPLC (RP-HPLC) has emerged as a useful authentication tool because it is easy to operate and capable of detecting a wide range of macromolecules (Sander and Wise, 1987), especially with the use of a fluorometer that offers additional detection sensitivity (Sander and Wise, 1987). LC separation is based on three primary chemical characteristics: electrical charge, molecular size, and polarity. It is widely used to identify various chemical components, such as carbohydrates, protein, amino acids, vitamins, phenolic compounds, chiral compounds, pigments and vitamins, whereas gas chromatography (GC) is based on the volatile or semivolatile characteristics of the molecules (Stefano et al., 2012). GC is a sensitive and powerful technique that can be used for simultaneous detection and separation of the specific analytes present in the head space volatiles of a liquid or solid sample (Ali et al., 2012). However, this method requires volatile organic components (VOCs) extraction and pre-concentration from the food sample before running the experiment, which might increase the probability of sample contamination and analyte loss; therefore, a large amount of sample is needed (Peterson and Cummings, 2006; Ali et al., 2012).

Recently, the combination of high-resolution chromatographic techniques with mass spectrometry, such as gas chromatography mass spectrometry (GC-MS/MS) or liquid chromatography-time-of-flight mass spectrometry (LC-TOF-MS), has been widely used for the authentication of food products, including adulterants in high-quality food products such as wines, olive oils, coffee, confectionary products, and saffron (Zhang et al., 2009; Stefano et al., 2012). Among these techniques, ultra-performance liquid chromatography and electrospray ionization quadrupole time-of-flight mass spectrometry (Nano-UPLC-ESI-q-TOF-MS^E) are novel techniques developed by Yilmaz et al. (2013) to detect the source of bovine and porcine gelatin in some dairy food products (ice cream, cheese, and yogurt). This assay is performed in two steps. First, gelatin is extracted from the above food products before preparing the MS sample. Second, tryptic gelatin peptide was separated and analyzed using nanoUPLCESI-q-TOF-MS^E. The originality of the developed assay is its ability to function in a data-independent acquisition mode and alternate low and elevated collision energy applied to collect product ion and precursor information; thus, it is possible to generate accurate mass acquisition on the peptide level to recognize specific gelatin peptides. Yilmaz et al. were able to detect the specific marker peptides for bovine and porcine gelatin that were added to the dairy food samples, revealing that their proposed assay could be an effective alternative for the detection and differentiation of gelatin derivatives in commercial dairy food products.

Nevertheless, the combinations of HPLC and mass spectroscopic methods reported by some researchers have the potential to detect some species' specific polypeptide chains after hydrolyzation with 3 mol/L HCL using HPLC/MS techniques (Ocana et al., 2004). The operating principle of the HPLC/MS method was based on the theory that gelatin contains degraded polypeptides from collagen type I, and the amino acid sequence from different animal-derived collagen is not identical. Thus, it can be detected after digestion with an appropriate enzyme using HPLC/MS (Zhang et al., 2008). Trypsin, 6-aminoquinolyl-N hydroxysuccinimidyl carbamate, and ortho-phthaldialdehyde enzymes are used to digest bovine and porcine gelatin to produce specific peptide sequences (Zhang et al., 2008; Widyaninggar et al., 2012; Raraswati et al., 2013). By comparing the MS/MS collagen data base, most of the peptide sequence in both type I and type II gelatin were found to be similar, but some partial sequences were specific. Some literature indicates that more marker peptides were identified in the $\alpha 2$ chain rather than the $\alpha 1$ chain because the difference of total amino acid residues in the $\alpha 1$ chain was 1.1% whereas the difference in the $\alpha 2$ chain was 2.3% (Zhang et al., 2009; Nhari et al., 2012). However, the accuracy and sensitivity of this assay might be influenced by the hydrolysis time and temperature (Ocana et al., 2004; Nhari et al., 2012).

Generally, porcine gelatin contains two amino acids (asparagine [ASN] and glutamine [GLN]), whereas bovine gelatin are deficient of these amino acids. Therefore, a selective assay for extraction, preconcentration, and analysis of GLN and ASN can be applied as a useful tool for gelatin source authentication. However, the hydrophilic nature and low absorbance upon UV-spectral phenomenon makes the identification of these two amino acids quite challenging. To overcome this challenge, Rezazadeh et al. (2015) established an efficient, simple, and practical method for the authentication of animal-derived gelatin based on pulsed electro membrane extraction (PEME) and HPLC techniques. They used pulsed electric field for sample extraction, preconcentration, and analysis of derivatized amino acid compositions. They derivatized amino acids of some selective species with the use of *o*-phthalaldehyde (OPA) to increase the ultraviolet absorbance and lipophilicities. Then, they applied a 137 V electric field for 20 min with 10 min^{-1} frequency to migrate the analytes through an organic liquid membrane of approximately 200 μm into an aqueous acceptor phrase. At the end of their experiment, the aqueous phase was analyzed by a HPLC-UV system. Their developed assay offered 43% and 79% extraction recoveries, whereas the LOD for asparagine and glutamine was 25 and 50 ng/mL, respectively (Rezazadeh et al., 2015). Overall, the outcomes of the developed

assay confirmed that OPA derivatized amino acids analyzed by the PEME and HPLC-UV assays were able to detect the origin of animal-derived gelatin.

Furthermore, the combination of chromatographic techniques with chemo metric tools such as PCA has been used to detect the exact source of gelatin. This authentication is capable of differentiating bovine and porcine gelatin under raw conditions (Nemati et al., 2004). The principle of this detection techniques relies upon an individual amino acid profile analysis using RP-HPLC. Amino acid analysis can be used to detect and quantify proteins and peptides of target species, as well as some unusual amino acids sequences. The RP-HPLC chromatographic results of bovine and porcine gelatin are almost the same, whereas bovine gelatin contains an extra amino acid sequences compared to porcine gelatins. However, this result cannot be used alone to differentiate bovine and porcine gelatins, as they have similar chemical properties. Therefore, a PCA score plot was applied because it gives the overall peaks of amino acids (such as the height, width, areas, and area percent) to obtain significant variables and differentiate bovine and porcine gelatins (Nemati et al., 2004). In this respect, Widyaninggar et al. (2012) developed an assay and showed that combinations of PCA and HPLC techniques can differentiate species-specific gelatin products. They obtained amino acid profiles of gelatin containing capsule shells using HCL-hydrolysis protocols, and the variations in the amino acid profiles of two gelatin sources were sorted out by a PCA score plot, wherein the PC1 and PC2 demonstrated 64.4% and 15.7% variations within the bovine and porcine gelatin capsules, respectively (Hermanto and Fatimah, 2013; Widyaninggar et al., 2012). Although, chromatographic techniques offers higher specificity and sensitivity of the reaction assay and are effective at discriminating raw gelatin sources. Moreover, the hydroxylation of some amino acids during enzyme treatments could increase the complexities in peptide differentiation, and degradation of marker peptides during manufacturing conditions makes chromatographic techniques less suitable for mixed gelatin source authentication (Zhang et al., 2009). The recent applications of chromatographic techniques to detect and differentiate gelatin sources are presented in Table 1.

Spectroscopic techniques

Spectroscopic techniques produce remarkable, reliable, and precise results from small sample volumes. They measure the absorption of high energy light at 200–800 nm that causes excitation of the pi-electron bonds that are ubiquitous in a wide range of biomolecules (Schmid, 2001). Different types of biomacromolecules, such as lipids, proteins, and carbohydrates, contain delocalized electrons in the aromatic systems that can absorb UV light at 150–400 nm (Aitken and Learmonth, 2002). For example, peptide bonds give absorbance at a range of approximately 180–230 nm, and the aromatic site chains of some amino acids, such as tyrosine, trypsin, and phenyl-alanine, give absorbance at 240–230 nm. These spectral fingerprints provide the basis for identifying gelatin products under complex matrices (Schmid, 2001; Aitken and Learmonth, 2002).

Among the numerous spectroscopic tools, infrared spectroscopy (IR) is very dominant and is broadly used to generate IR fingerprints of a large domain of functional groups in biomolecules (Barth, 2007; Hashim et al., 2010; Hermanto and Fatimah, 2013). IR measures the molecular vibrations caused by absorbed light, and it has successfully recognized the special secondary structure of various gelatins, the thermal self-assembly of macromolecules, and the crosslinking patterns of collagens from different species (Hashim et al., 2010). During IR radiation, each sample passes through a separate pathway; thus, each sample from different origins gives a different IR fingerprint that represents unique molecular structures. This makes IR a useful analytical approach for gelatin source authentication because its results represent actual information on various functional groups of marker peptides (Nhari et al., 2012). Fourier transform infra-red spectroscopy (FTIR) is an advancement of IR spectroscopy, and it is used to identify organic molecules in polymer or polymer blends, providing special sensitivity towards inorganic adulterant detections (Barth, 2007). Currently, the FTIR technique has been used to authenticate halal products such as meat and gelatin species. Hashim et al. (2010) conducted a useful study based on FTIR and attenuated total reflectance (ATR) to discriminate bovine and porcine gelatin. ATR is an advanced technique whereby the analytical sample is placed in an ATR element, and spectrum phenomenon of the sample is recorded based on the ATR element. The spectral results for both types of gelatin are very similar within the range of 650 to 4,000 cm^{-1}, whereas the major differences were found between 3,280–3,290 cm^{-1} and 1,200–1,660 cm^{-1}. The 3,280–3,290 cm^{-1} area is donated by a hydrogen bonded amide group (N–H). The discriminant analysis for the amide bond of gelatin at this region can detect unknown gelatin origins. These two areas show specific characteristics through analysis of major spectrum differences in bovine and porcine gelatin. These comparisons were confirmed based on the intensity of spectra that varies from one gelatin to another. Cooman's plot analysis was used to show the discriminant analysis that is designed from the Mahalanobis distance (the distance between the clusters). Their developed techniques can detect and discriminate gelatin origins; however, this technique requires high purity samples, which makes the assay quite difficult when subjected to mixed gelatin sample detection from different sources. Additionally, this assay was unable to detect gelatin from fish. In this respect, Cebi et al. (2016) developed a similar method to overcome the drawbacks of Hashim et al. (2010) and successfully classified and discriminated fish gelatin in addition to bovine and porcine gelatin using ATR-FTIR along with hierarchical cluster and PCA. Additionally, they were able to detect bovine and porcine gelatins from mixed samples.

However, the sensitivity of this technique is often limiting, and the method also has the potential of quantitative measurement using multivariate analysis methods such as partial least squares (PLS). Recently, some authors have used spectroscopic techniques (such as FTIR) coupled with PLS regression analysis to authenticate the presence or absence of pig substances in halal food products (Rohman and Che Man, 2012). Basically, PLS is a chemo metric technique used along with spectroscopic techniques (such as NMR, FTIR, or IR) for the quantification of specific analytes present in the food sample (Wise et al., 2006). In the conventional spectroscopic techniques, the target sample can only be quantified if the intensity values are directly equal to the concentrations of the components of interest, but

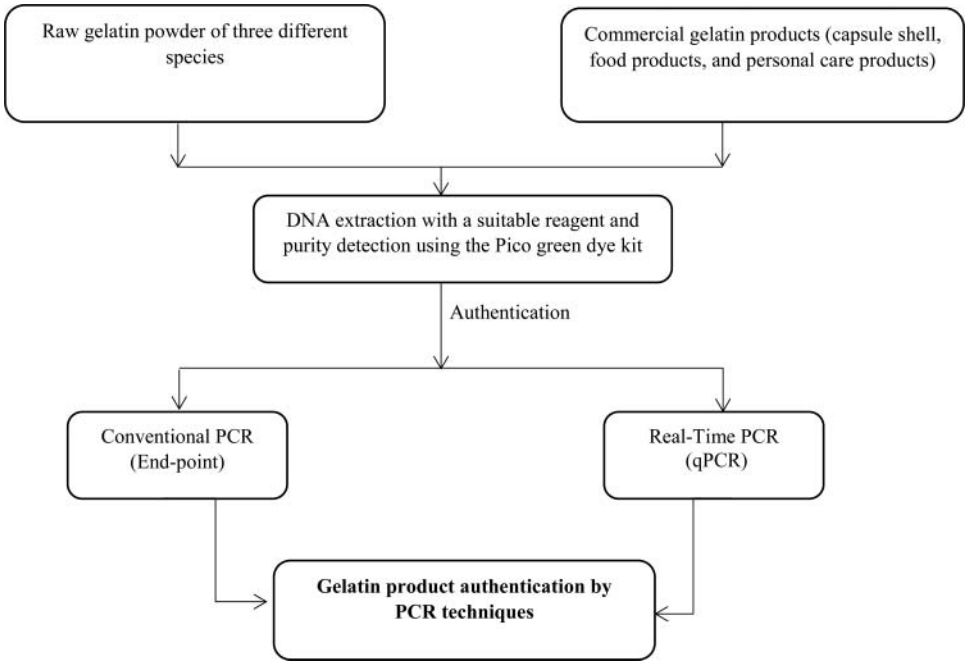

Figure 4. Major analytical steps for gelatin and collagen product authentication using PCR techniques.

PLS regression analysis can also quantify the target sample if there is a certain amount of nonlinearity within the spectral data set and their previous concentrations (Wise et al., 2006; Kumar and Mishra, 2015). PLS implicates the instantaneous analysis of all the spectral variables; therefore, one can create a better calibration model to analyze unknown samples (Wise et al., 2006), although different spectroscopic methods are useful to detect and differentiate bovine and porcine gelatin. Moreover, the sensitivity of spectroscopic techniques is not equivalent to DNA-based methods.

DNA-based methods

Spectroscopy is an advance method for authenticating gelatin sources based on the spectral fingerprints, but it demands a highly pure sample; and hence, it is not suitable for detecting gelatin under complex matrices (Zhang et al., 2009; Hashim et al., 2010). HPLC-coupled PCA can differentiate bovine and porcine gelatins under raw conditions, but it fails in highly treated gelatin products because of the denaturation or degradation of the species authenticating protein or peptide biomarkers (Aristoy and Toldrá, 2004). In this regard, LC-MS is quite successful to differentiate gelatin sources by peptide mapping, but it also fails because of the instability of the peptide biomarkers under extreme thermal and chemical treatments (Zhang et al., 2009). However, ELISA is a highly sensitive, quantitative, and rapid method to authenticate gelatin sources, but it is also unfit for the repetitive analyses and highly processed gelatin products due to the denaturation of the biomarker epitopes (Asensio et al., 2008). Thus, the numerous disadvantages of the protein-based methods have initiated a paradigm shift toward the DNA-based techniques because of the exceptional stability of DNA biomarkers, which can survive extreme denaturing conditions such as the heat, pressure, and chemical treatments involved in gelatin and gelatin product processing (Tasara et al., 2005; Cai et al., 2012). The main advantages of using DNA-based techniques include greater biomarker stability and the extraordinary sensitivity, which is provided by significant amplification of the biomarker targets from a single copy or a few copies into easily detectable quantities (Sun et al., 2014). A uniform information content, the ubiquitous presence of certain nucleic acid materials in multiple copies, such as mitochondrial DNA, and wider availability of polymorphic features throughout the genome further strengthens the sensitivity of this method. Herein, the major challenge for gelatin source authentication using DNA-based techniques is the extraction of DNA from gelatin and its derivatives. This is because of its very low abundance in the finished products and the possibility of degradation under extreme heat and other processing treatments. The development of an improved DNA extraction protocol and shortening the amplicon targets with superior stability might greatly overcome the limitation. Recently, Mohamad et al. (2015) established a procedure that significantly eased the extraction of DNA from gelatin. The authors established that quality DNA extraction entirely depends on the successful lysis of the gelatin product using proteinase K and loosening of protein-DNA interactions using a pretreatment that involves pH modifications; wherein the use of an approximate pH of 8.5 prior to the precipitation of DNA into isopropanol can yield quality DNA (Mohamad et al., 2015). However, the low content of DNA and the strong interaction between the DNA and non-lysed gelatin residues made it impossible to measure the concentration of the extracted DNA using spectrophotometric approaches. This was overcome by using a pico Green dye kit (Molecular Probes, Eugene, OR, USA) that successfully measured both the concentration and purity of the extracted DNA (Mohamad et al., 2015). Among the different DNA-based methods, the PCR assay occupies the central position, and the various PCR-based approaches to authenticate gelatin products are briefly discussed as follows and are schematically shown in Fig. 4.

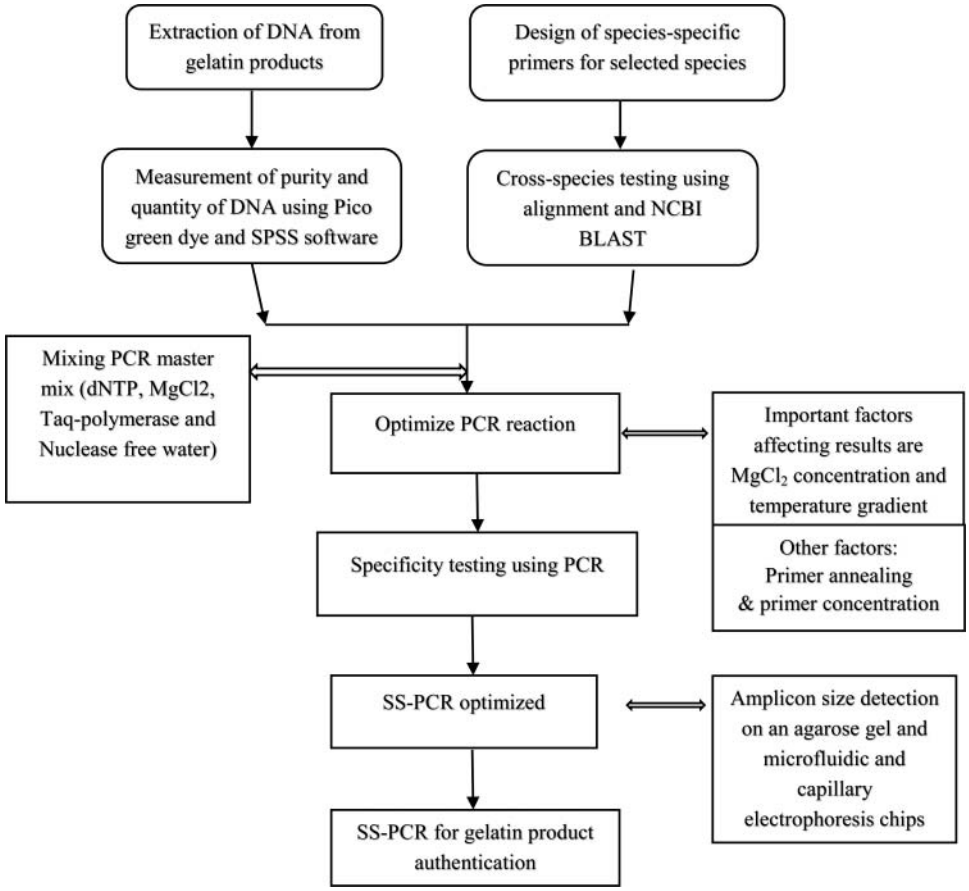

Figure 5. Schematic presentation of various steps in the development of species-specific (SS-PCR) for gelatin product authentication.

Species-specific PCR assay (endpoint PCR)

Recently, species-specific PCR (SS-PCR) has received major attention to authenticate gelatin sources (Tasara et al., 2005). The technique usually involves amplifying a target segment of a mitochondrial gene using a couple of species-specific primer pairs together with a buffer, enzymes, and magnesium chloride, followed by identification of the amplified product on an agarose gel using ethidium bromide and other staining agents (Fig. 5). SS-PCR is a simple, accurate, and low-cost technique, and it has been used to authenticate gelatin from various sources (Tasara et al., 2005; Lee et al., 2016). Tasara et al. (2005) documented a conventional SS-PCR and a qPCR method to detect and differentiate bovine, porcine, and fish gelatin, wherein the sensitivity of the developed qPCR assay was 0.1–0.001%.

Recently, Shabani et al. (2015) developed a species-specific conventional PCR method for halal gelatin source authentication. They designed primers from the conserved regions of mitochondrial DNA (cytochrome b gene) with an amplicon size of 212 bp (porcine) and 271 bp (bovine) to evaluate the halal authenticity of gelatin-containing products. The sensitivity of the proposed method was 0.1% (w/w) for both types of gelatin under binary mixture conditions. For product analysis, they chose eight food products and eight pharmaceutical capsule shells labeled as containing bovine gelatin. PCR amplification results of these products showed that all samples give positive results for bovine gelatin. Thus, this method could be used to authenticate gelatin and gelatin-containing food products to ensure its suitability for halal markets. Lee et al. (2016) also established an SS-PCR assay to discriminate bovine-, porcine-, fish-, and plant-based gelatin in pharmaceutical capsule shells. Briefly, they designed species specific primer pairs for bovine, porcine, and tilapia fish and a universal primer pair for fish species targeting the mitochondrial 16S rRNA gene to amplify specific targets from the gelatin capsules. The limit of detection for tilapia, bovine, and porcine was 0.1, 0.001, and 0.01 ng/μl, respectively, whereas the limit of detection was 0.01 and 0.0001 ng/μl for the universal targets of the fish and plant species, respectively (Lee et al., 2016).

In another experiment, Mutalib et al. (2015) reported a PCR-southern hybridization and endpoint PCR assay for the identification of porcine derivatives in pharmaceutical capsule shells. Although six out of 20 capsule brands demonstrated positive results in PCR-southern hybridization, the endpoint PCR did not detect porcine in any of the capsules, indicating that PCR-southern hybridization is more sensitive than endpoint PCR. The authors further showed that the sensitivity of the developed assay varies with target gene sequences, wherein the LOD was 0.25 ng for cytb, 0.1 ng for cytochrome oxidase subunit II (COII), and 0.0001 ng for the ATP6 gene sequences. Finally, the authors concluded that on-chip-PCR-southern hybridization is a reliable and sensitive method for authenticating porcine DNA in pharmaceutical capsules.

However, the lack of specificity, low and unequal amplification efficiency, and inherent complexity of the method made it

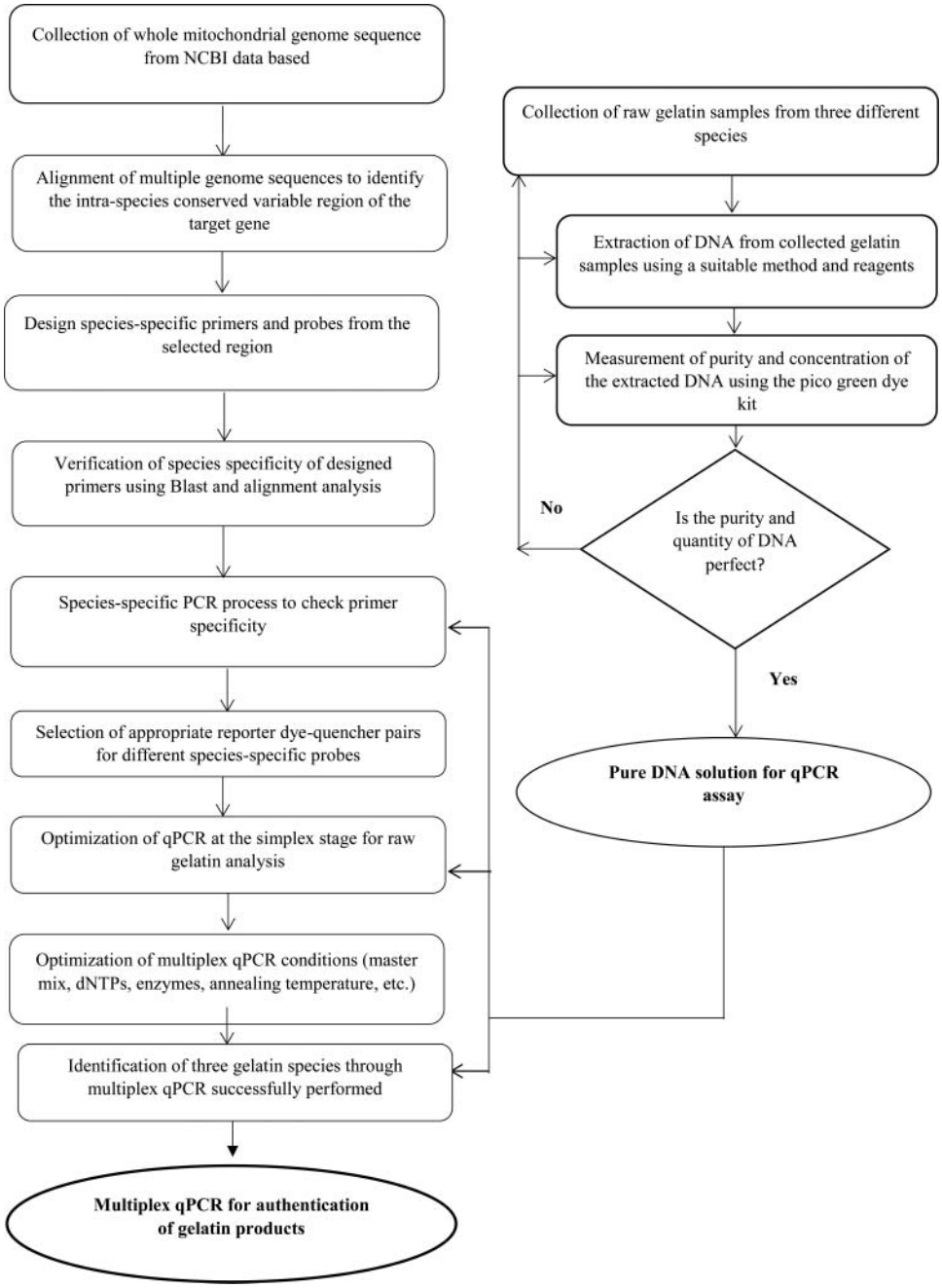

Figure 6. Steps in the development of real-time PCR (qPCR) techniques for the authentication of gelatin products.

unsuitable for quantitative application (Ali et al., 2014). Moreover, singleplex endpoint PCR assays can detect only one species at a time, incurring analytical cost and indicating the need for further development (Ali et al., 2014).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) is a modern technique and has recently become an essential analytical tool in biochemistry, molecular biology, food analysis, and other molecular diagnostic research areas in both the academic and industrial settings. Precision, analytical speed, automation, and sensitivity are all incorporated in qPCR (Rojas et al., 2011). In contrast to conventional PCR, which relies primarily on endpoint analysis of the amplified PCR product, qPCR detects and reports the progress of the amplified DNA in real-time through the assistance of a reporter dye (Ali et al., 2011, 2014). Two types of dyes are adapted for a qPCR system: (1) nonspecific fluorescent dye that intercalates with double-stranded DNA in a blind fashion and (2) DNA probes that consist of an oligonucleotide labeled with a fluorescent reporter dye at one end and a quencher at the other; the reporter dye fluoresces while the probe hybridizes to the target DNA, signaling the amplification of specific targets (Ali et al., 2012). The various steps in the development of qPCR are presented in Fig. 6.

In addition to identification, qPCR also offers quantification of the DNA targets by plotting the fluorescent signal of the exponential phase of the PCR reaction against the cycle number in a logarithmic scale that provides a straight line for the amplicon quantities that exponentially increase with the reaction

progress. The cycle at which fluorescence overshoots the base line fluorescence is called the threshold cycle (Ct) or cycle number, wherein the amount of the amplified DNA doubles at each cycle during the exponential phase and is calculated based on the relative Ct values. Relative Ct values can be calculated from a standard curve that is constructed with several known concentrations of DNA; then, the concentration of the unknown samples is determined by extrapolating the Ct values of the unknown onto the standard curve (Ali et al., 2011; Cai et al., 2012; Rahman et al., 2016). qPCR techniques have been used for the detection and quantification of specific gelatin origins in pharmaceutical capsule shells (Tasara et al., 2005; Cai et al., 2012) and in processed food products, such as marshmallows (Demirhan et al., 2012). Cai et al. (2012) detected 1.0% bovine and porcine gelatin under a mixed background using qPCR. The qPCR system was further used to authenticate the gelatin origin in marshmallows, Turkish delights and gum drops (Demirhan et al., 2012). Overall, qPCR assays provide quantitative data but involve a high cost and difficult steps for optimization and probe design.

Future prospects and challenges

The growing demand for functional foods has pressured manufacturers to produce foods and supplements with added nutritive value by adding raw ingredients that contain better nutrients. This has increased the need to disclose product information that would comply with both the religious and safety standards of various countries, continents, religions, and cultures. A high content of essential amino acids and attractive physiochemical properties have made gelatin an attractive candidate to use in food, pharmaceuticals, and personal care products to improve the quality, texture, and appearance of the final product (Liu et al., 2015). The outcomes include the recent explosion of interest in the development of analytical techniques for gelatin source authentication, giving rise to widespread speculation about the future availability of less expensive, faster, and more accurate means for the quantitative determination of source materials in raw and finished gelatin products. A myriad of methods along with their key features are listed in Table 1; these are just a sample of the endeavors made in this area.

As stated earlier, protein-based verification tools involve inaccuracies, high costs, and inconveniences that make them cumbersome for performing gelatin source authentication (Ali et al., 2014). However, the universal abundance of DNA in all cells and tissues, codon degeneracy, polymorphic, and duplicative features, as well as the greater stability under the state of decomposition of the carefully designed DNA biomarkers have made DNA-based methods a superior fit for future technologies (Sahilah et al., 2012; Ali et al., 2014). Because the first report on the authentication of gelatin from three different species by Tasara et al. (2005) using both the conventional and real-time PCR assays, many other studies have used singleplex PCR techniques for the authentication of gelatin (Ali et al., 2012; Sahilah et al., 2012; Mohamad et al., 2015; Rahman et al., 2016). Thus, the singleplex PCR method is a mature technology for gelatin identification, but it is a costlier approach in terms of analytical time and absolute cost, indicating the need for innovative multiplex platforms that are capable of identifying multiple target species in a single assay format, saving both cost and time. Although several multiplex PCR assays have documented the analysis of animal, plant and microbial species in food and feed products (Ali et al., 2014), none of them have been applied to gelatin source identification. We believe several sets of primers could be designed targeting bovine, porcine and fish species and could be optimized, along with an internal universal control for plants and animals, for the devolvement of a multiplex PCR system for the discriminatory detection of bovine, porcine and fish gelatin in a single assay platform. Both the conventional and real-time multiplex PCR assays could be used for gelatin source authentication, and an outline of the assay development schemes could be extracted from our recently published papers (Aida et al., 2007; Ali et al., 2014; Ali et al., 2015; Hossain et al., 2016). The major challenges of this multiplex scheme are not limited but include critical steps in the optimization and design of the primer sets, wherein all primers must have the same or a very closely spaced melting temperature (T_m) so that all of them can anneal to their respective targets at the same temperature, which is challenging when multiple species are involved under complex matrices. A second challenge may be the difficulties in maintaining all the amplicon-lengths within 200 bp or less since longer targets are broken down under extreme food processing treatments (Ali et al., 2014). A third problem may be the availability of appropriate dyes and detectors when real-time PCR is involved. Currently, available PCR machines can detect a maximum of 0.001 ng/μL targets in a multiplex platform (Safdar et al., 2014). Finally, the LOD of different targets might be different in the same multiplex PCR (Ali et al., 2015; Razzak et al., 2015).

Recently, Hossain et al. (2016) developed a new method for detecting animal species using double gene targeted multiplex PCR-RFLP analysis. This novel multiplex PCR-RFLP approach designed biomarkers with a very short length (73, 90, 106, 120, 138, and 146 bp) by targeting two different sites of mitochondrial conserved regions (cytochrome *b* and ND5 genes). All of the biomarkers were stable under high heat and autoclaving treatments. To perform PCR-RFLP analysis, they digested the PCR products with AluI, EciI, FatI, and CviKI-1 restriction enzymes. Finally, they evaluated their developed assay with commercial frankfurter foods and found that most of the frankfurters labeled as beef showed positive results for buffalo, but all frankfurters were negative for pork. Thus, this new idea of targeting double genes instead of one can be a new pathway for detecting animal and gelatin sources in commercially available food products.

Recently, nanotechnology-aided biosensor and microarray approaches for DNA detection have received enormous support from various sources, including funding agencies, researchers, and industries. Nanostructured materials with unique optical and electrochemical properties greatly facilitate biorecognition of specific DNA targets that often act as a fingerprint marker for a particular disease or species and thus contribute significantly to point-of-care biodiagnostics. A higher stability, polymorphic flexibility, and stringently protected regions of DNA molecules are amazing for identifying specific target sequences for disease diagnostics, paternity testing, and other forensic and biotechnological applications. Various tailor-made engineered nanomaterials with cutting-edge

useful and attractive features have made it possible to develop nano platforms with in-built DNA recognition sites that could be detected using spectroscopic, electrochemistry, magnetic, and other analytical tools upon target hybridization. In nanoscale sensing, the synergistic effects of functionalized nanomaterials and immobilized DNA probes provide effective recognition of specific DNA targets. The fusion of nanomaterials with specific probe DNA or single-stranded DNA (ssDNA) is key for the development of DNA biosensors. The DNA hybridization detection depends on the physio-chemical properties of the material or their hybrids and on base pairing with the complimentary single-stranded DNA to form a double helix. Nucleic acids are linear and directional polyanions that exhibit molecular recognition and self-assembly. Single-stranded DNA shows exquisite affinity and specificity for its complementary strand, and double-stranded (ds) DNA can be designed to self-assemble into topologically diverse three dimensional structures. The phenomenon of complimentary DNA sequence binding allows researchers to develop probes of known DNA sequences with identification footprints. It has made possible the recognition of unknown samples of specific DNA targets either for species authentication or for mutant gene analysis for disease diagnosis or gene therapy treatment. At nanoscale, materials exhibit unique properties that greatly facilitate the fabrication of sensors with high sensitivity and analyte detection. A wide range of nanomaterials has been used as an entrapped bioreceptor probe that is specific to an analyte of interest. Among the diverse range of nanomaterials, nanogold, silica, metal oxides, quantum dots, nanowires, dendrimers, graphenes, and carbon nanotubes have shown great potential in nucleic acid sensing. Sensing involves probe DNA immobilization on the sensor platforms. An exciting outline of the fusion or integration patterns of a variety of nanomaterials and DNA molecules along with the different analytical platforms, such as optical and electrochemical approaches for the detection of target nucleic acid sequences, are thoroughly discussed in our recently published book chapter (Ali et al., 2016).

The biosensor applications of nanomaterials are very wide, ranging from nanometric devices in computer chips to the coatings of giant industrial components. Both the synthetic oligos and real DNA targets for porcine (Ali et al., 2011) and microbial species (Brandão et al., 2015) have been detected both in single (Ali et al., 2011) and multiplex platforms (Brandão et al., 2015), but no attempts have been made for gelatin authentication.

There are several major challenges in identifying DNA using sensor devices. First, these devices are in the development stage; therefore, most of these sensors are still at the laboratory scale and are being tested with purified DNA, which must be extracted from a biological specimen. Thus, the most significant challenge is the on-site delivery of results, which must be addressed in future research. Second, the devices use surface-tethered probe DNA that greatly limits the flexibility in surface reaction in target hybridization. The substrate, conjugation chemistry, and grafting density seriously affect the nucleic acid conformation and freedom of movement on the surfaces. Moreover, DNA attached to the surfaces by electrostatic or hydrophobic interactions tends to adopt a flat conformation, allowing the phosphate backbone or the hydrophobic bases to interact strongly with the substrate surface, limiting the base-to-base interaction that performs the specific recognition of the incoming targets. However, covalent attachment by end grafting or affinity coupling allows greater control over the orientation, but the nucleic acid chains tend to adopt an extended conformation. To overcome these limitations, diamond quantum dots have recently been developed to detect DNA targets in a solution-like environment (Švorc et al., 2015). The third challenge might be the crowding or steric hindrance that is frequently encountered in probe target interactions onto the surface-tethered immobilized probes. Using a suitable linker in the immobilized probe might overcome this limitation. Fourth, nonspecific interactions frequently occur due to the cross-hybridization of the probe with the target, as well as nonspecific adsorption of the target onto the surface. The use of suitable blockers, a specifically designed probe, optimization of the salt concentration and hybridization definitely ameliorate nonspecific interactions.

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Authors' contribution and declaration

Md. Eaqub Ali declares that he generated the idea, managed the funds, and guided Sharmin Sultana to draft the manuscript, and he has no competing interests in the publication of this paper.

Sharmin Sultana declares that she drafted the manuscript, and she has no competing interests in the publication of this paper.

Sharifah Bee Abd Hamid declares that she supervised Sharmin Sultana and edited the manuscript, and she has no competing interests in the publication of this paper.

M.A. Motalib Hossain declares that he helped Sharmin Sultana with drafting the manuscript, and he has no competing interests in the publication of this paper.

Wageeh A. Yehya declares that he edited the manuscript, and he has no competing interests in the publication of this paper.

Md. Abdul Kader declares that he edited the manuscript, and he has no competing interests in the publication of this paper.

Suresh K. Bhargava declares that he edited the manuscript, and he has no competing interests in the publication of this paper.

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