Review article

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Gelatin: overview of identification methods

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Abstract

Background and objective: Gelatin is mostly produced from porcine and bovine collagens (skin/hide and bone) and less from poultry and fish. Acceptance of gelatin resource is based on religious and cultural beliefs, health and dietary aspects. In fact, high sensitivities to the resource of gelatin are reported in various societies. Therefore, conformity of gelatin with customer needs should essentially be ensured. In this review, summarized information on gelatin extraction and identification methods, structure, uses and possible substitutes from 1960 to recent years are presented.

Results and conclusion: Several methods have been used for identification of halal gelatin origins. These methods are majorly based on sedimentation, high performance liquid chromatography, enzyme linked immunosorbent assay, DNA identification using polymerase chain reaction, and electrophoresis. Efficiency of these methods should be assessed based on the method ability to accurate and sensitive distinguish gelatin sources in mixtures. In recent years, high performance liquid chromatography coupled with mass spectroscopy has been used frequently with high sensitivity and accuracy to detect gelatin origins. In comparison, DNA identification include several advantages such as use of small quantities of target materials, high sensitivity and accuracy, low dependency on destructive process factors, and good functional capability. Although, if long times and high temperatures are used during gelatin extraction, DNA may be degraded. Other than identification methods, study of diverse gelatin substitutes is recommended for further studies due to the current concerns about source of gelatin.

Keywords: Collagen, extraction, gelatin, identification, substitutes

1. Introduction

Gelatin is a soluble protein compound obtained by partial hydrolysis of collagen, the main fibrous protein constituent in bones, cartilages and skins; therefore, the source, age of the animal, and type of collagen, are all intrinsic factors influencing

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the properties of the gelatins [1]. Collagen is the most abundant structural protein in vertebrate and non-vertebrate (approximately 30%) animals. Up-to-date, 29 types of collagens have been identified in animals. Gelatin, a translucent, colorless, brittle (when dry) flavorless protein, is soluble in water and polyhydric alcohols (glycerol and polyethylene glycol) and is driven from collagen [2,3]. Global production of gelatin was nearly 32,600 tons in 2007, majorly from pork and bovine skins (46 and 23.1%, respectively) and their bones (23%). Fish (lower than 1.5%) and other sources have included small proportions (Figure 1). However, gelatin extraction from other sources has increased in recent years [4].

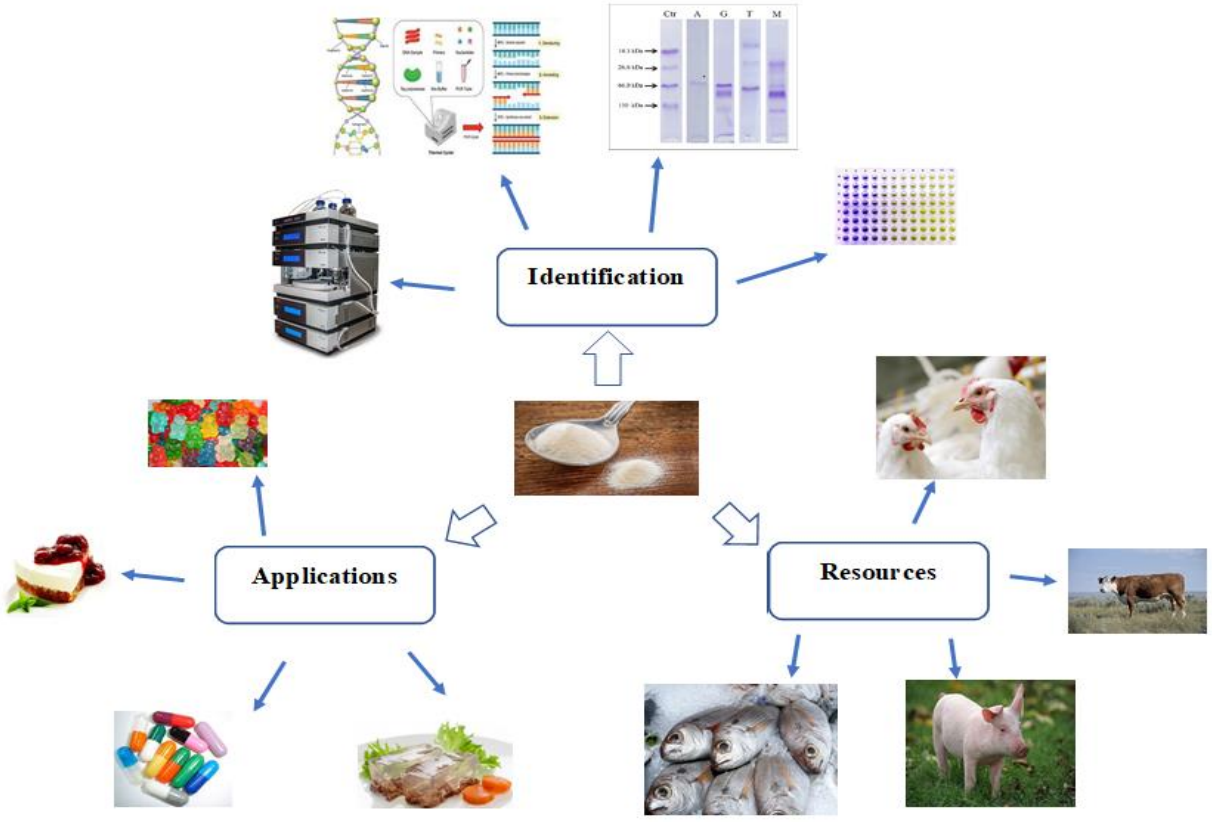

Figure 1- Different resources, applications, and identification methods of gelatin

Gelatin includes several functionalities such as a foaming, stabilizing, emulsifying, clearing, coating and wetting. Therefore, it includes various industrial uses, especially in food (meat and dairy products, sweets, desserts) and drug (pills, ointments, and capsules) industries [4,5]. Gelatin includes superior advantages such as being a structural material of various organs and tissues and an excellent surface-active agent through lipid free interfaces and having high biodegradability, low antigenicity, and high biocompatibility rates, compared to other polymer sources. These advantages make gelatin a great source for food, biomedical, and pharmaceutical uses [6]. Based on factors such as consumer cultures, dietary regimes, health concerns, and religious beliefs, the gelatin origin usually varies. For example, porcine gelatin is forbidden in Muslim and Jewish populations and cattle gelatin is forbidden in Hindu populations. Another example includes gelatin refusal from animal sources by vegetarians. Furthermore, safety concerns became important after outbreak of special

diseases such as bovine spongiform encephalopathy (BSE) and swine flu [7,8]. Nowadays, Muslim beliefs play important role in acceptance of these products. Total annual trade of halal food products was estimated as eighty billion dollars or 12% of agri-food products in the world in 2007, which may easily reach 20% by 2025 [7]. In this study, we review identification and detection methods of halal bovine gelatin.

2. Identification methods

Since 1960, different identification methods have been used for gelatin. The most important methods studied in 2010–2020 are summarized in Table 1.

2.1. pH precipitation (dropping method)

Conversion of hydroxyapatite (HAP) into amorphous calcium phosphate (ACP) is pH dependent. At low pH, ACP is dominant. Gelatin (as collagen) is able to convert ACP into HAP. As pH is lowered, transformation of HAP into ACP can be delayed in presence of gelatin [9]. Therefore, effects of specific types of gelatin may be assessed by measuring the consumed calcium and induction time in presence of gelatin. Hidaka and Liu differentiated porcine skin and bovine bone gelatins by assessing the induction time. Their results showed that differences between the two gelatins were significant at concentrations of 0.5 and 2 mg/ml. In fact, HAP transformation increased at lower gelatin concentrations but decreased after reaching the peak values. Peak values varied based on the gelation types as 0.5 mg/ml for bovine bone gelatin and 4 mg/ml for porcine skin gelatin [10].

2.2. Fourier transform infrared spectroscopy (FTIR)

It is an appropriate method for food fraud assessments. In this method, the infrared (IR) spectrum passes through the substance and the molecular absorption and transfer rate create spectra, presenting molecular fingerprints of the substance. Based on specific groups, IR spectra of bovine and porcine gelatins can be classified. FTIR in combination with attenuated total reflectance (ATR) and discriminant analysis was used by Hashim et al. to compare bovine and porcine gelatin spectra. Spectra of the two gelatins were similar at 4000–650 cm^{-1}. The major differences in gelatin spectra were observed at 3290–3280 (due to NH bond-stretching mode of hydrogen bonded amide group) and 1660–1200 cm^{-1}. However, this method includes advantages such as simple sample preparing and rapid analysis but needs high purity of samples [2,10].

2.3. High performance liquid chromatography (HPLC) coupled with principal component analysis (PCA)

The amino acid (AA) analysis can be used to differentiate protein sources. For this, Nemati et al. hydrolyzed bovine and porcine gelatins using reversed phase-high performance liquid chromatography (RP-HPLC). Of 20 peaks in chromatographs, one was quite typical in bovine gelatin. Using PCA on matrix of height and width to achieve significant variables and classifications, good results were reported in differentiation of bovine and porcine gelatins. However, this method was not efficient when samples contained several types of gelatins because of AA similarities, interfering effect of various concentrations, and non-consistencies in each analysis [9,11]. In another study, Widyaninggar et al. developed a HPLC method equipped with fluorescence detector for AA profiling and detecting porcine gelatin in capsule shades. The AA profiling was followed by PCA. Results indicated capability of the method for differentiating bovine and porcine gelatins [12].

2.4. HPLC coupled with mass spectroscopy (MS)

This method is based on identification of marker peptides, which were produced after hydrolysis of various gelatin sources. Using HPLC-MS, Zhang et al. investigated hydrolysates of bovine and porcine gelatins after digestion with trypsin.

Based on data with collagen database, most of the peptide sequences of bovine and porcine collagens type I were similar; however, sequences were partially specific. More marker peptides were detected in α2 chain than α1 chain (1.1 and 2.3% of total AA residues, respectively). In α2 chain, marker peptides for bovine and porcine collagens included TGPPGPSGISGPPGPPGPAGK and IGPPGPSGISGPPGPPGPAGK, respectively [13]. Other scientists used HPLC/tandem mass spectrometry. Results showed that more marker peptides with higher molecular weight could be detected using this method. However, they succeeded in differentiating bovine and porcine gelatins. Variation in hydroxylation of proline and lysine and also different process sensitivity to peptides are two difficulties in differentiation of marker peptides [14].

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA is an immunological method based on enzymatic detection of antibodies or antigens in samples. This method is sensitive and specific and most commonly used in indirect and sandwiched forms. In general, ELISA can be used for detecting origins of animal tissues in food and pharmaceutical products. Bovine and porcine gelatin tyrosylation can produce high-titer antibodies. Venien and Levieux developed two ELISA methods of indirect and competitive ELISAs for porcine and bovine gelatin identification. Specific antibodies were achieved using rabbit immunological response against tyrosylated gelatin. Some antisera were not sensitive to gelation sources and could be used as gelatin quantification agents while others were 10 to 30-fold more sensitive to porcine acidic gelatin than bovine gelatin. Other antisera were developed that were able to detect subtle conformation changes in gelatin structures such as those with a 1000-fold higher sensitivity to bovine acid hide, compared to other limited antibodies. The overall results of their study showed that the ELISA method was more process sensitive for gelatin differentiation, compared to the methods that were species sensitive [15]. Then, authors collected species-sensitive antibodies by immunizing rabbits against species-specific sequences of the bovine collagen alpha 1 (I) chain. Results showed that competitive indirect ELISA could be used as a reliable sensitive method, capable of detecting 2-4 parts per 1000 parts of bovine gelatins in porcine gelatins from laboratory chemical suppliers [16].

People may show allergic reactions to foods containing gelatin. Doi et al. prepared two sandwich ELISAs using polyclonal antibodies from immunization of rabbits (pAb2, pAb1 for coating and capture reactions, respectively) and goat (pAb3, pAb3 for both coating and capture reactions) with bovine gelatin. Based on the reactive assays with various gelatins, the two competitive indirect ELISAs showed strong reactions to bovine and porcine gelatins but not to fish gelatin. The two ELISAs were able to detect gelatin in commercial foods containing collagen. However, the goat pAb3-pAb3 ELISA reacted strongly with bovine and porcine gelatins while the rabbit pAb2-pAb1 ELISA mostly reacted to porcine gelatin (alkaline process). The ELISA sandwich methods did not include false positives results, except for heated meat products analyzed in various commercial foods. However, the pAb2-pAb1 ELISA of rabbit cross-reacted with that of boiled squid. Therefore, the pAb3-pAb3 ELISA of goat was preferred to detect gelatin contamination in processed foods due to its poorer activities with cooked meats, less interactive reactions with cooked squids, and lack of false-positive and false-negative responses. However, limitations of the ELISA method included false-positive results from gelatinous heat-treated meats. Indeed, production conditions of gelatin affected its identification, as goat antibodies reacted more strongly with alkaline-treated bovine and porcine gelatins than acid-treated ones [17].

2.6. Electrophoresis

Electrophoresis is a powerful isolation technique that can simultaneously isolate thousands of proteins. Two-dimensional (2-D) electrophoresis includes high-resolution performances. This method is based on separation of polypeptides duo to their negative or positive charges and molecular weights in electric fields. Composition of gelatin peptides varies based on their original source. Moreover, electrophoretic patterns of gelatin vary from one source to another one and even in similar sources of raw materials, depending on the conditions of preparation. Aina el al. used 2-D electrophoresis to detect porcine gelatin of various manufacturers and its adulteration with bovine gelatin. Based on their findings, total number of peptides in the porcine samples was specific for each manufacturer with a small variation. Of the isolated peptides, ten peptides were identified as marker proteins in porcine gelatin of various manufacturers. In mixtures of porcine and bovine gelatins prepared in alkaline (five mixtures of bovine and porcine gelatins with quantity of the porcine gelatin in the prepared solution of 1-5%), presence of porcine gelatin was detectable at 1% w/w [18]. In another study, Azira et al. used electrophoresis with PCA to detect porcine and bovine gelatin in mixtures (5% of porcine gelatin to 50% of bovine gelatin and vice versa). Based on their results, gelatin extraction of homemade jelly samples with cold acetone did not affect electrophoretic profile of the gelatin polypeptides. Furthermore, poor electrophoretic profiles were seen in heterogenous commercial processed products. The minimum detection of porcine gelatin in bovine gelatin was achieved as nearly 5% of porcine gelatin. However, they reported that using conditions of their study, PCA was not able to differentiate between the blended samples when porcine gelatin was adulterated with bovine gelatin [19]. In another study, Hermanto et al. identified differences between porcine and bovine gelatins (types A and B) before and after pepsin hydrolysis. Digestion was done up to 20% preferentially after the N-terminal of aromatic amino acids. After that, FTIR and UV-Vis were used to identify functional groups of each gelatin resource and SDS-PAGE was used to identify molecular weights. Results showed that the two gelatin sources included various UV-Vis absorptions before and after hydrolysis at 229 and 240 nm (due to C=O bonds of amides) and differences in conformations of 2-D peptides. FT-IR spectra were similar before and after gelatin hydrolysis except for region of 2800-3000 cm^{-1}. The two gelatins included peaks at wavelengths of 3300-3400 cm^{-1} (NH stretching), 1600 cm^{-1} (C=O stretching, amide), 1500 cm^{-1} (CN stretching), and 767-620 cm^{-1} (bending OCN). Due to different AAs composition of the two sources, differences in region of 1300-1450 cm^{-1} were observed. These findings demonstrated relative differences in AA sequences of the two sources of gelatin. The SDS-PAGE results were similar for the two sources at molecular weights of 100-200 kDa. At molecular weights below 50 kDa, three various bonds were seen; of which, two were seen at 36.2 and 28.6 kDa for porcine gelatin hydrolyzed for 1 h. However, no bonds were seen for bovine gelatin at this molecular weight, seen for porcine gelatin hydrolyzed for 2 and 3 h at molecular weights below 28.6 kDa [20].

2.7. DNA-based methods

Protein identification methods (HPLC, ELISA, and protein profiling) are highly effective in unprocessed foods due to sensitivity of proteins to thermal process; however, they include disadvantages such as inability to discriminate between gelatin sources sometimes. In addition, their use is difficult and includes low abilities to quantify the materials in short times. In contrast, DNA-based methods include more efficiency in identifying alternatives in foods. One of these characteristics includes higher stability of DNA against process conditions (compared to proteins). In addition, DNA-based methods able to amplify genetic materials extracted from foods at

low quantities by polymerase chain reaction (PCR) [21]. The major limitations of standard PCR include their low sensitivity and need for endpoint product analysis by agarose gel electrophoresis. Real-time PCR uses sensitive reliable techniques of continuous monitoring of PCR products by fluorescence markers. Zang et al. used TaqMan real-time PCR to assess quantities of bovine DNAs in meat, milk, and cheese samples. They succeeded to detect and quantify up to 35 pg of bovine DNA with no interactions with DNAs of sheep, goats, and pigs using mitochondrial cytochrome b gene [22]. In agreement, Demirhan et al. conducted real-time PCR by specific porcine primers to detect quantities of gelatin in marshmallows and candies. In their study, the minimum detectable quantity of cheating was 1% w/w in marshmallows and candies [23].

Table 1- Common identification methods used for gelatin authentication during 2010–2020

Method	Study design and result	Ref.
HPLC-MS/MS	Effects of one stage extraction temperature on porcine gelatin identification were studied. A sample digestion process was carried out after digestion. HPLC-MS/MS identified 64, 74, and 71 tryptic porcine peptides in gelatin extracted at 55, 65, and 75 °C, respectively. Between theses peptide fractions, 47 common peptides were identified for porcine gelatin.	24
HPLC-MS/MS	Effects of three extraction stages of gelatin processing on tryptic peptide characteristics were studied. Porcine gelatin was hydrolyzed using trypsin digestion and then analyzed using HPLC-MS/MS. Researchers compared these findings with those from one-stage purification process and hydrolyzed using trypsin. Using HPLC-MS/MS, 97, 88, and 58 characteristic tryptic peptides were identified in first, second, and third stages of processing, respectively. Of these peptides, 46 common peptides were identified. During comparison of the two methods, eight unmodified peptides were identified used as marker for porcine gelatin identification in industrial scales.	25
HPLC-MS/MS	This study was carried out to differentiate three types of mammalian gelatins from bovine, porcine, and donkey hides. Hemoglobin only was detected in donkey hide gelatin. A digestion stage was used on the extracted gelatins before sample identification by HPLC-MS/MS. Using NCBI database and other databases, sequences from researches, unique peptides from bovine and porcine and donkey mixtures were identified. It was shown that lower target gelatin contents decreased detectable marker peptides.	26
HPLC-MS/MS	A public database for identifying Equidae hide gelatin in a combination of horse, donkey, and their hybrid (Equine family) gelatins was developed. Three peptides were used as markers to distinguish origins of gelatins from Equine family. They reported sensitivity of the method as 0.05 and 0.1% for horse and hybrid hide gelatins, respectively.	27
HPLC-MS/MS	In this study, a label free technique in combination with HPLC-MS/MS in SIM was developed to quantify bovine gelatin as representative of mammalian gelatins. First, tandem mass spectroscopy was used to assess bovine gelatin characteristic peptides and then indices such as linearity, accuracy, and reproducibility were achieved through various sample concentrations. Results showed that from 17 identified marker peptides for pure bovine gelatin, seven were able to quantify it with higher linearity, reproducibility, and specificity.	28

UPLC/Q-TOF-MS coupled with PCA analysis	The study was carried out to identify five gelatin types, including those from donkey hide, bovine hide, pig hide, tortoise shell glue, and deerhorn glue. Gelatin samples were hydrolyzed using trypsin treatment, separated using chromatography, detected using mass spectroscopy, and analyzed using PCA. UPLC/Q-TOF-MS with PCA classified gelatins of various origins. Marker peptides were achieved based on PCA loading plot and comparison with LC/MS data and previous database from associated gelatin types.	29
Conventional PCR and real-time PCR	Various food samples (36) from various countries were analyzed, some included gelatin in ingredients and others were gelatin free. Conventional PCR using seven pairs of species-specific primers were used to investigate halal authenticity of the samples. Conventional PCR showed presence of 12 positive porcine gelatin from 36 samples. Using cloning and sequencing, presence of porcine DNA was verified in five of 12 samples. Frequency of porcine positive DNA was higher when real-time PCR was used (27 positive samples instead of 12 positive samples).	30
UPLC-MS/MS	Gelatin of various sources (porcine, bovine, chicken, and fish) was hydrolyzed using trypsin to identify biomarker peptides. Produced data from UPLC-MS/MS were analyzed to find biomarker peptides specific to each species using Chemometric Software (Mass Profiler Professional) and untargeted workflow. This software is matched with data set from GC/MS, LC/MS, CE/MS, and ICP/MS. Combining UPLC-MS/MS with MPP Software allowed identification of 21 unknown gelatin samples with 100% accuracy.	31
Conventional PCR	Conventional PCR was used to investigate halal authenticity of gelatin products. Using species-specific primers, amplified products with 212 and 271 bp were produced for porcine and bovine gelatins, respectively. Despite DNA degradation during gelatin extraction process, the method was able to detect 0.1% w/w of porcine and bovine gelatins in binary mixtures.	32
UPLC-MS/MS	Deer-hide gelatin is a precious substance with medicinal use and hence it includes high probability to adulterated with other animal gelatins such as pig or horse gelatins. In this study, untargeted mass spectroscopy was used to analyze peptide profile of trypsin hydrolyzed gelatins. Using a set of mathematical theories, various peptides were linked to special species and using targeted MS based method, biomarkers were verified. Using this method, two peptides were achieved that could be used to identify deer-hide gelatin in a mixture of other gelatins.	33
Gel electrophoresis coupled with PCA and RFLP	Two protein-based and DNA-based methods have been used to investigate gelatin type in capsule shells. Gelatin achieved using acetone precipitation was used for gel electrophoresis and DNA extraction. PCA was used to classify gelatin samples. Porcine gelatin showed 12 major bonds while bovine gelatin showed eight major bonds on 8% tris-glycine gels. Cytochrome b gene was used to differentiate one species from other species in DNA based method.	34
Sensor	QCM sensor was developed to investigate frequency change in bovine and porcine gelatin solutions at pH 9. The QCM sensor was treated using layer-by-layer deposition of nickel compound on the surface of gold electrodes. Differentiation of the two gelatin types was carried out using shifts in direction of frequency response. Sensor activity was assessed in real foods. Results showed that QCM sensor could be used as a vehicle to distinguish halal (bovine) and non-halal (porcine) gelatins.	35

MALDI-TOF-MS	MALDI-TOF-MS was used to differentiate bovine and porcine gelatins. First, mixtures of bovine and porcine gelatins were prepared, and porcine trypsin was added to the mixture and co-crystalized with matrix solution onto the MALDI target. Mass spectra were analyzed using cluster analysis and Bruker Biotyper Software. Findings showed that MALDI-TOF-MS methods do not include sensitivity of other described methods but include advantages such as use by untrained people, relative reliability, and no needs for high performance equipment.	36

*Ref., reference; HPLC-MS/MS, high performance liquid chromatography/tandem mass spectrometry; SIM, ion monitoring mode; PCA, principal component analysis; UPLC/Q-TOF-MS, ultra-performance liquid chromatography-quadrupole-time off light mass spectrometry; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; QCM, quartz crystal microbalance; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry

3. Conclusion

Gelatin is a protein compound with important functional characteristics and hence used in a wide range of food and pharmaceutical products. These characteristics majorly include gelling, emulsifying, foaming, and process aiding. In recent years, need of this product has increased steadily. For various reasons such as religious beliefs, dietary regiments, and health concerns, identification of gelatin source is important. Therefore, it is necessary to introduce methods with the ability of detecting the source of gelatin in non-processed and processed states. Identification methods are used based on their cost, sensitivity, applicability, and also type of food. For example, DNA-based methods are useful in identification of processed foods and mass spectroscopy methods are preferred when the highest sensitivity is required. Due to the concerns about the source of gelatin and its safety, creating diversity in gelatin substitutes without any adverse effect to health is essential. Solutions include those which are basically originated from gelatinous sources such as fish and poultry wastes and those which are originated from non-gelatinous sources as well as carbohyd-rate-based alternatives. However, it is noteworthy that most of these substitutes cannot compete with gelatin to display its characteristics and hence modifications are necessary.

4. Conflict of interest

The authors declare that there is no conflict of interest.

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