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Evaluating PCR and ELISA for porcine detection in collagen-based products for halal authentication

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Abstract

Collagen is a widely used protein in various highly processed products across the food, cosmetic, pharmaceutical, and biomedical industries due to its versatility and unique properties. Its primary sources include pigs, cows, and marine animals, with industrial extraction typically performed from hides, bones, tendons, and skin. Given the importance of halal authentication, especially in Muslim-majority markets, a key challenge lies in reliably detecting porcine-derived collagen in highly processed products due to DNA degradation, protein denaturation, and matrix interference. These issues often result in detection failures and false negatives, underscoring the need for a comparative evaluation of available analytical methods. This study compares two analytical approaches for porcine detection: the DNA-based Polymerase Chain Reaction (PCR) and the protein-based Enzyme-Linked Immunosorbent Assay (ELISA). A total of nine collagen-based samples were analysed. PCR successfully detected porcine DNA in three samples, while ELISA detected porcine antigen in two samples, including one not detected by PCR. However, two porcine-labelled samples were missed, leading to a false negative rate of 66.7%. Four samples, specifically samples 5, 6, 8, and 9, resulted in an Overall Agreement Rate (OAR) of 44.4%. The combination of real-time PCR and ELISA offers complementary advantages. Real-time PCR is particularly effective for detecting low-level porcine DNA in undenatured type II collagen. At the same time, ELISA helps mitigate false negatives that may arise from DNA degradation or PCR inhibition caused by the presence of the collagen matrix. These findings suggest that integrating real-time PCR for detecting trace DNA in less processed matrices with ELISA for identifying degraded proteins in hydrolysed products enhances the overall reliability of porcine detection and strengthens halal authentication protocols across diverse product types.

1. Introduction

Detecting porcine contamination in processed collagen-based products is a significant challenge in halal authentication. As collagen undergoes extensive hydrolysis and thermal processing, its DNA may become highly fragmented and denatured, reducing the effectiveness of DNA-based detection methods like PCR (Othman *et al.*, 2023). Similarly, protein denaturation can limit the performance of immunoassays such as ELISA. This technical barrier poses a critical risk in halal compliance for processed foods, pharmaceuticals, and cosmetics (Nawwaruddin *et al.*, 2024). In Islam, halal (permissible) and *tayyib* (pure, wholesome) are essential principles derived from the *Qur'an*, which commands: "O people, eat from the earth what is *Halalan Tayyiban*" (*Qur'an* 2:168). *Halalan Tayyiban* not only denotes permissibility under *Shari'ah* law but also assures that the product is safe, clean, and beneficial for health (Hayat *et al.*, 2023). In Islamic jurisprudence, halal refers to that permitted under *Shari'ah* law, while *tayyib* implies that the product is lawful but also safe, clean, nutritious, and beneficial. According to Aghwan (2021), the *Halalan Tayyiban* concept encompasses products permissible under *Shari'ah* and free from elements harmful to health, reflecting the broader interpretation promoted by institutions such as the Halal Industry Development Corporation (HDC) Malaysia.

Food processing has become increasingly complex with the rapid advancement of science and technology. This complexity makes it more difficult to trace the origins of ingredients and increases the risk of unintentionally including non-halal substances (Jaswir & Guntarti, 2021). Highly processed foods (HPFs) have experienced rapid global growth, particularly in Asia, with the highest increase in sales from 2009 to 2019 observed in India, Pakistan, and Indonesia (Baker *et al.*, 2020). The HPFs, also known as ultra-processed foods, often consist of industrially reformulated mixtures containing ingredients from questionable or untraceable sources, such as additives, stabilisers, and emulsifiers (Shinozaki *et al.*, 2023). This complexity increases the difficulty in identifying specific components like collagen, which is frequently used in processed formulations and poses challenges for halal

authentication.

Collagen is widely used in food, cosmetic, and pharmaceutical products, but its sourcing from porcine materials presents a serious halal concern. While pig-derived collagen is cost-effective and structurally similar to human collagen, it is strictly prohibited in Islam. Ensuring collagen-based products are free from porcine derivatives is essential for halal compliance. While high-end analytical platforms such as Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) and Nuclear Magnetic Resonance (NMR) offer highly sensitive detection of animal-derived compounds, they are often costly and less accessible (Sani *et al.*, 2023). As such, molecular-based (e.g., PCR) and immunoassay-based (e.g., ELISA) techniques remain widely used for routine halal authentication due to their cost-effectiveness, specificity, and applicability to various matrices (Abdullah Sani *et al.*, 2021). However, the authentication process is complicated by the effects of hydrolysis, heating, and chemical treatment, which degrade DNA and denature proteins. DNA-based methods like PCR may fail due to fragmented or low-quality DNA (Muflihah *et al.*, 2023). Protein-based methods like ELISA can suffer from reduced antigenicity or matrix interference. These limitations highlight the need for a comparative evaluation of both methods to identify a more robust approach to detecting porcine residues in highly processed products. Identifying the animal origin of ingredients, such as collagen, is essential to ensure compliance with halal standards. Thus, Food authenticity is a critical issue that concerns consumers and industry stakeholders.

Polymerase Chain Reaction (PCR) is currently the most widely used DNA-based method for detecting species-specific genetic material in processed food products. Its sensitivity and specificity allow it to amplify even trace amounts of porcine DNA (Rosyid *et al.*, 2023). However, applying PCR to highly processed samples, such as collagen, may face challenges, including DNA degradation, low DNA yield, and inhibition caused by proteinaceous matrices (Chen *et al.*, 2025). These limitations may lead to false negatives or inconclusive results.

Protein-based detection methods, such as the Enzyme-Linked Immunosorbent Assay (ELISA), have been employed as complementary or alternative approaches to address these limitations. ELISA targets antigenic proteins and is highly specific and sensitive, even for heat-treated samples (Nhari *et al.*, 2019). Commercial ELISA kits, such as sandwich-type ELISAs, can detect porcine collagen proteins even after prolonged heating. Halal regulatory frameworks, such as the Malaysian Standard MS 1500:2019, the Manual Procedure for Malaysia Halal Certification (MPPHM) 2020, and the Malaysian Halal Management System (MHMS) 2020, emphasise the importance of ensuring products are free from non-halal ingredients, including porcine derivatives. In practice, both PCR and ELISA methods are widely used in halal testing laboratories and are recognised for their specificity and sensitivity in detecting animal-derived substances. However, the standards do not prescribe a definitive method for all matrices, which presents challenges when verifying highly processed products, such as collagen (Yörük, 2021). This gap underscores the importance of evaluating and optimising both methods for halal authentication.

While PCR is a widely used method for porcine detection due to its high sensitivity and specificity, its effectiveness significantly decreases in hydrolysed or extensively processed collagen due to DNA fragmentation and matrix inhibitors (Muflihah *et al.*, 2023). ELISA, which targets antigenic proteins, may offer complementary advantages in such cases (Liu *et al.*, 2024). However, a direct performance comparison between PCR and ELISA for porcine detection in highly processed collagen-based products remains limited, particularly in halal authentication.

This study aims to compare the efficacy of PCR and ELISA in detecting porcine content in highly processed collagen samples. Specifically, it assesses each method's ability to classify samples as porcine-positive or porcine-negative accurately and evaluates the false negative rate for both techniques. Real-time PCR offers advantages in low-level DNA detection, while ELISA may overcome false negatives caused by poor DNA extraction or PCR inhibition. Ultimately, the findings support the integration of both DNA- and protein-based methods to enhance the robustness and reliability of halal authentication in complex processed products.

2. Methodology

2.1 Materials and equipment

The materials used in this experiment consisted of nine highly processed collagen-based products, purchased from local retail stores in Malaysia—product categories included powdered supplements, jelly, gummies, cream masks, and liquid supplements. Information from product labels, including stated ingredients, collagen source, and halal certification status, was recorded to support sample classification and the interpretation of results. Also, chloroform, nuclease-free water, Milli-Q® water, and deionised distilled water were of analytical grade. A DNA Extraction Using In-House CTAB method, Porcine DNA RealTime PCR, Halkit II, and a commercial kit of ELISA Porcine Gelatine Detection Halkit were employed from Global Haltech Sdn. Bhd., Malaysia. Powdered porcine type I collagen (YO Proteins AB) and bovine type I collagen (Merck KGaA, Darmstadt, Germany) were purchased and employed as positive and negative controls, respectively.

The equipment utilised in this experiment included an analytical balance, vortex mixer, incubator, centrifuge, thermal cycler with Fluorescein Amidite (FAM) detection capability for real-time PCR (qPCR), and a microplate reader set to 450 nm for Enzyme-linked Immunosorbent Assay (ELISA) analysis.

2.2 Experimental design and data analysis

The experimental workflow is illustrated in Figure 1. Each of the nine collagen-based product samples underwent DNA- and protein-based analysis, following parallel workflows. A total of nine samples were purposively selected based on product diversity, including various forms (powder, cream, gummy, jelly, and liquid), different collagen sources (porcine, fish, marine, plant-based), and a mix of halal-certified and non-halal-certified products. This sample size was considered sufficient for an exploratory comparative study to observe trends in detection capability across different matrices and halal claims. In addition to the market samples, two reference materials were included: pure porcine collagen (positive control) and pure bovine collagen (negative control), both in powder form and obtained from certified suppliers. These were used as external validation controls in both qPCR and ELISA assays.

For DNA analysis, samples were subjected to CTAB-based

extraction followed by real-time PCR using a porcine-specific acetone and buffer extraction for ELISA-based detection of porcine antigens. Each test was performed in duplicate, and the results were compared to determine concordance and false-negative rates.

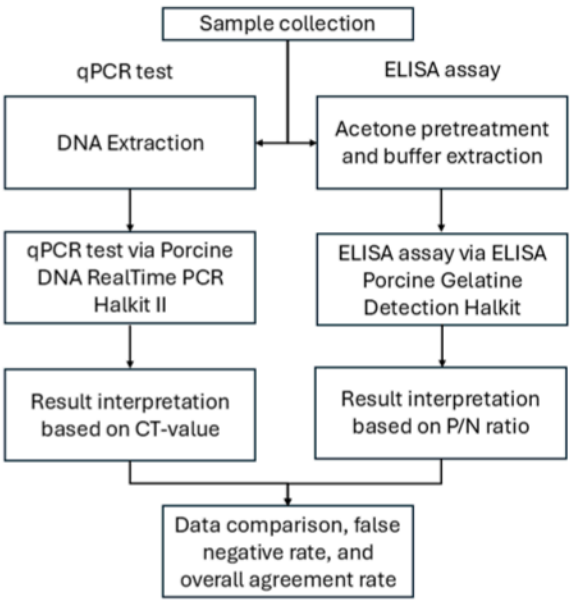

Figure 1: Overview of experimental workflow for porcine detection in highly processed collagen-based products.

Agreement between qPCR and ELISA results was assessed qualitatively based on matched positive/negative outcomes, and an overall agreement rate (%) was calculated across all nine samples.

2.3 DNA extraction

Before being subjected to the real-time PCR analysis, the samples must undergo the following extraction steps outlined in the Porcine DNA RealTime PCR Halkit II (Global Haltech Sdn. Bhd., 2021b)

2.3.1 Sample preparation and cell lysis

Samples were weighed for 1 g. Other than powder, solid samples were cut into smaller pieces and weighed to 1 g for each sample. A volume of 10 mL of Elution Buffer (EB) and 10 µL of Proteinase K were added to the sample. However, the liquid samples used 5 mL EB and 5 µL of Proteinase K. It was then vortexed to obtain a homogeneous solution. Next, the sample was incubated at 65°C for 1 hr in a shaking water bath to ensure thorough digestion. Then, it was centrifuged at 4000 x g for 20 mins. Then, 1000 µL of supernatant was transferred into a new 2 mL tube. Then, 1000 µL of chloroform was transferred, vortexed, and centrifuged for 15 min at 10,000 × g. The upper layer of the aqueous supernatant was subjected to DNA precipitation.

2.3.2 DNA precipitation

A volume of 1000 µL of the upper layer aqueous supernatant was transferred into a new 2 mL tube. A volume of 700 µL of Isopropanol (IPA) and 5µL of Glycogen were added and mixed by vortexing. The tubes were left at -20°C for an hour or more for precipitation. Lastly, the sample was centrifuged for 15 mins at 10,000 × g to precipitate the DNA. All the supernatant was then carefully discarded, without disturbing the pellet formed at the bottom of the tube. The pallet was subjected to DNA washing.

2.3.3 DNA washing

A volume of 700 µL of 75% ethanol was added to the pellet and mixed by vortexing. It was then centrifuged for 5 mins at 10000 x g. The supernatant was discarded without disturbing the pellet. This series of steps was performed twice. Afterwards, the tube with only the pellet was centrifuged again at 10000 x g for a minute. The remaining supernatant was carefully removed to eliminate residual waste. The tube was left at room temperature for 2 mins to dry the residual waste completely. The washed pellet was subjected to DNA elution.

2.3.4 DNA elution

100 µL of preheated Milli-Q water at 65°C was added directly to the DNA pellet and gently mixed to dissolve the pellet. The DNA sample was stored at -20°C before proceeding with the qPCR test.

2.3.5 Assessment of DNA purity

The purity of extracted DNA was assessed using the A260/A280 ratio, with average values ranging from 1.70 to 2.00, indicating acceptable purity for PCR amplification (Sani *et al.*, 2023).

2.4 Real-time polymerase chain reaction (qPCR)

Reagent preparation was done by spinning down all tubes in the Porcine DNA Real-Time PCR Halkit II (Global Haltech Sdn. Bhd., 2021b). For each DNA sample, a reaction mix was prepared using 10 µL of 2X qMastermix and 2 µL of 10X porcine-specific probe/primer mix provided in the kit, which targets a conserved region of the porcine mitochondrial genome. The remaining volume was completed with DNA template (≥ 50 ng) and nuclease-free PCR water, to a final reaction volume of 20 µL. Next, sample DNA templates were prepared for each of the samples. A volume of 10 µL containing at least 50 ng of DNA template was pipetted into each tube. For a negative control tube, 8 µL of nuclease-free PCR water was used. The final volume in each tube should be 20 µL. It was then spun down briefly to collect the reaction mix at the bottom of the tubes.

A TaqMan®-based probe was employed to target a conserved region of the porcine mitochondrial cytochrome b (cyt b) gene for species-specific identification due to its high copy number and interspecies variability. The primers and probe sequences were provided within the Porcine DNA Real-Time PCR Halkit II (Global Haltech Sdn. Bhd., Malaysia); however, the specific sequences are proprietary. Fluorescence detection was performed using the Fluorescein Amidite (FAM) channel.

A Real-time PCR (qPCR) test was performed according to the protocol outlined in Table 1. A standard curve was generated using serial dilutions of the positive control provided in the kit to validate assay efficiency and linearity. The qPCR machine determined the CT value. This calibration ensured consistent amplification performance and CT-value interpretation, as in Figure 2. Reaction efficiency was considered acceptable between 90% and 110%.

The results were presented and interpreted as described in Table 2. Each qPCR run included internal kit controls and was accompanied by a pure porcine collagen sample as an external positive control and a bovine collagen sample as an external negative control. These controls were used to verify method accuracy and the absence of cross-reactivity.

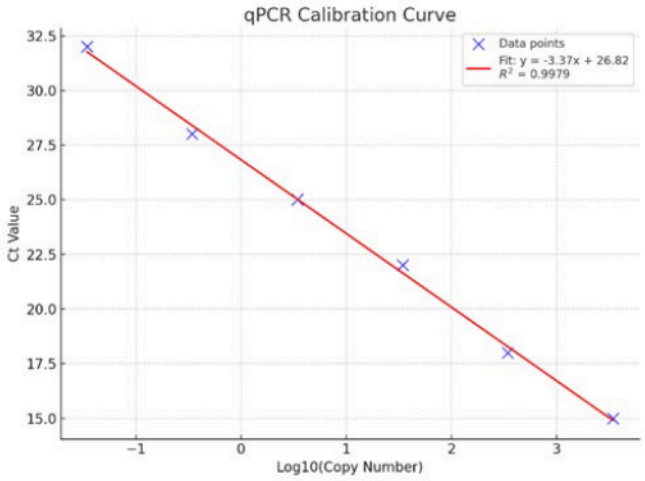

Figure 2: Calibration curve for porcine DNA real-time PCR Halkit II.

Table 1: Amplification protocol of real-time PCR

Cycle	Step	Time	Temperature
1	Initial Denaturation	2 mins	95°C
40	Denaturation	15 sec	95°C
	Annealing	1 min	60°C

2.5 Pretreatment of collagen samples for enzyme-linked immunosorbent assay (ELISA)

Before conducting the ELISA assay, all collagen samples underwent a pretreatment procedure. The sample was weighed at 1 g in the collection tube, washed with 2 mL of cold acetone (-20°C) and the acetone was discarded. An additional 2 mL of cold acetone was added, and the sample was then centrifuged directly at 300 × g for 5 mins. After discarding the remaining acetone, the sample was resuspended in 10 mL of buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM Ethylenediaminetetraacetic Acid (EDTA), and 1% Sodium Dodecyl Sulfate (SDS), then cooled at -20°C for 30 mins. Afterwards, the solution was sonicated at 10000 rpm for 2 × 10 seconds and left to cool at -20°C for 5 mins. It was then centrifuged at 10,000 x g for 20 mins. The supernatant was transferred to a new tube and labelled. A volume of 2 mL of the liquid portion of this pretreated mixture was transferred and stored at -20°C for ELISA analysis.

2.6 Enzyme-linked immunosorbent assay (ELISA)

500 µL of the pretreated sample and 1 mL of 0.5 M NaCl were added to the collection tube. The tube was closed and vortexed vigorously for 30 seconds until the sample dissolved. The tubes were then centrifuged at 3710 rpm for 15 mins, and they were ready for the ELISA assay.

The assay procedure was performed using the ELISA Porcine Gelatine Detection Halkit (Global Haltech Sdn. Bhd., 2021a), a sandwich-type ELISA specific for porcine collagen peptides, with a modification of loading 100 µL of 0.5 M NaCl into the blank well. A 100 µL NaCl solution was added to the blank well instead of the sample buffer to serve as a matrix-matched control and maintain ionic strength consistency across all wells, minimising background signal variation during absorbance reading. The absorbance (OD) of the wells was read at 450 nm with a Thermo Fisher Scientific Multiskan SkyHigh Microplate Spectrophotometer (Waltham, Massachusetts, USA). The manufacturer-specified cut-off for positive detection was a P/N ratio ≥ 1.3, where P is the sample's optical density (OD) and N is the OD of the negative control. The kit's reported limit of detection (LOD) is 0.1% porcine gelatin (w/w) in a food matrix. According to the manufacturer's specifications, the kit exhibits no cross-reactivity with bovine, fish, or chicken collagen proteins. (Global Haltech Sdn. Bhd., 2021a). These performance criteria were used to interpret the ELISA results obtained in this study.

The results were interpreted using the porcine collagen P/N ratio, where P refers to the mean optical density (OD) of the test sample, and N refers to the mean OD of the negative control. This ratio standardises the absorbance values by comparing the test sample signal against the background or baseline signal. The formula is shown below:

$$Porcine\ collagen\ \frac{P}{N}\ ratio = \frac{Mean\ OD\ of\ test\ sample}{Mean\ OD\ of\ negative\ control}$$

Table 2: Result interpretation of real-time PCR

Case	CT-value			Interpretation
	Sample	Positive Control	Negative control	
1	CT < 38	CT < 38	No CT or CT > 38	A porcine-specific gene was detected in the sample.
2	No CT or CT > 38	CT < 38	No CT or CT > 38	A porcine-specific gene was not detected in the sample.
3	No CT or CT < 38 or CT > 38	No CT or CT > 38	No CT or CT > 38	Invalid, rerun PCR.
4	No CT or CT < 38 or CT > 38	CT < 38	CT < 38	Invalid, rerun PCR.

A P/N ratio of 1.3 or greater indicates positive detection of porcine collagen, while a value of less than 1.3 indicates a negative result, as summarised in Table 3.

Table 3: Result interpretation of ELISA

Case	Porcine contamination	P/N value
1	Positive	≥ 1.3
2	Negative	< 1.3

ELISA analysis included external validation with pure porcine collagen as a positive control and pure bovine collagen as a negative control. Both were processed using the same pretreatment steps as the test samples.

2.7 Statistical analysis

The results for duplicate samples of the qPCR and ELISA were calculated as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Tukey's test was performed to determine the significant difference between the means at a 95% confidence level ($p < 0.05$) using XLSTAT 2025 statistical software (Lumivero, USA).

2.8 Determination of false negative rate

The qualitative data obtained from the qPCR and ELISA methods were gathered, plotted, and compared, and the False Negative Rate (FNR) of each method was calculated with the following formula:

$$False\ Negative\ Rate\ (FNR) = \frac{FN}{(TP + FN)}$$

Where FN is a false negative for porcine labelling, and TP is a true positive for porcine labelling.

2.9 Overall agreement rate (OAR)

An overall agreement rate (OAR) was determined to measure the general concordance between the two methods. The OAR is the proportion of samples for which both methods, qPCR and ELISA, give the same positive or negative result. The formula to calculate OAR is as follows:

$$Overall\ Agreement\ Rate\ (OAR), \% = \frac{Number\ of\ samples\ with\ matching\ qPCR\ and\ ELISA\ results}{Total\ number\ of\ samples}\ x\ 100$$

3. Results and discussion

3.1 Porcine detection by real-time polymerase chain reaction

Polymerase Chain Reaction (PCR) is an oligonucleotide-based method (DNA approach) widely used to detect the authenticity of food components, particularly in complex matrices such as highly processed products. The sensitivity of the real-time PCR method using porcine collagen was previously validated at 0.001% (Global Haltech Sdn. Bhd., 2021b). This study interpreted samples as porcine-positive when their cycle threshold (CT) values were below 38 for both test duplicates and the spiked positive control (Global Haltech Sdn. Bhd., 2021b). A lower CT value indicates a higher concentration of target porcine DNA, and vice versa. This result was in line with the Malaysian Standard MS 2627: 2017 Detection of porcine DNA - Test method - Food and food product, which states that a CT-value of 32 to 40 indicates the positive presence of porcine DNA (Department of Standards Malaysia, 2017).

Out of nine highly processed collagen samples tested, real-time PCR detected porcine DNA in three samples, namely samples 2, 3, and 4, corresponding to fish peptide, undenatured type II porcine peptide, and marine collagens, respectively, as shown in Table 4. The CT-values of these samples were not significantly different ($p < 0.05$), indicating a similar level of porcine DNA presence. These samples included one porcine-labelled sample and two unexpectedly positive samples despite being labelled as fish- and marine-based products. This result highlighted both the method's sensitivity and the challenges posed by the complexity of the sample matrix. (Al-Shaibany *et al.*, 2022), where DNA degradation and cross-contamination can occur (Kim *et al.*, 2023).

Sample 3, which contains undenatured Type II porcine collagen, has been successfully detected with CT values of 30.62 ± 0.01. This supports findings by Chen *et al.* (2023), who reported that DNA-based methods, such as PCR, are highly effective for detecting biological origins, especially in minimally processed collagen types. Undenatured collagen, less subjected to extreme processing, retains enough DNA to be detected, reinforcing the importance of preserving molecular integrity for accurate halal authentication.

However, the two other porcine-based samples (Samples 1 and 6) containing hydrolysed collagen peptides yielded no CT values (ND), resulting in non-detection. According to Woo *et al.* (2017). Hydrolysed or denatured collagen undergoes enzymatic, thermal, or pH treatment, which breaks it down into peptides and degrades the DNA, making detection via PCR difficult. This is supported by Erwanto *et al.* (2011), who highlighted that shortened DNA fragments and potential inhibitors within protein-rich matrices pose significant challenges in PCR-based detection, especially when laboratory staff are required to perform complex extraction procedures. Similarly, Septiani (2024) found that DNA purity values in collagen supplement products were frequently below 1.8, indicating contamination by proteins or degradation, which compromises PCR amplification efficiency.

The same issue was observed in Sample 6, a cream-based product, which aligns with findings by Hanum *et al.* (2018) that DNA yields from processed or emulsified products are typically lower than those of raw food matrices. This observation was further supported by Zabidi *et al.* (2020), who confirmed that over-processing and low DNA recovery can significantly limit PCR application for halal authentication.

Interestingly, Samples 2 and 4, labelled fish and marine collagen, respectively, tested positive for porcine DNA. The detection of porcine markers in products labelled as non-porcine or halal was unexpected. While this may indicate the presence of trace-level porcine DNA, the result should be interpreted with caution, as other factors, such as analytical cross-reactivity, matrix effects, or incidental contamination during production, cannot be ruled out (Schröder *et al.*, 2023). No traceability or confirmatory testing was conducted in this study to determine the source of the detected markers. As such, no definitive conclusions regarding mislabelling or halal non-compliance can be drawn solely from these findings.

Table 4: Cycle threshold (CT) value of real-time PCR for porcine DNA detection

Sample no.	Product type	Collagen type[1]	Source	Halal certification status[2]	CT-value[3,4,5,6]		Result[8]
					Result	Spiked sample[7]	
1	Powder	Hydrolysed peptide	Porcine	Non-halal-certified	ND	N/A	-/-
2	Powder	Peptide	Fish	Non-halal-certified	**31.70 ± 0.21[a]**	**21.27**	+/+
3	Powder	Undenatured type II peptide	Porcine	Non-halal-certified	**30.62 ± 0.01[a]**	**19.89**	+/+
4	Powder	Not available	Marine	Non-halal-certified	**30.40 ± 1.26[a]**	**20.25**	+/+
5	Powder	Not available	Fish	Non-halal-certified	ND	N/A	-/-
6	Cream mask	Hydrolysed peptide	Porcine	Non-halal-certified	ND	N/A	-/-
7	Liquid supplement	Not available	Plants	Non-halal-certified	ND	N/A	-/-
8	Jelly	Type I & II peptide	Fish	Non-halal-certified	ND	N/A	-/-
9	Gummy	Type I & III peptide	Not available	Halal-certified	ND	N/A	-/-
10	Porcine (positive control)			NR	**27.54 ± 0.13[a]**	27.50	+/+
11	Bovine (negative control)			NR	ND	N/A	-/-

Note: [1]Not available, as data could not be obtained.
[2]NR = Not related
[3]ND = Not detected, i.e. no CT-value was determined, indicating no detectable amplification of the target DNA.
[4]Bold result indicates positive porcine presence. Values are presented as mean ± standard deviation of duplicate results. Values with different superscripts are statistically significantly different ($p < 0.05$).
[5]N/A = Not applicable, i.e. the result was not applicable since the porcine DNA was not detected in each replicate.
[6]qPCR kit sensitivity was reported as 0.001%, specificity was reported as 100%, and the limit of detection (LOD) was reported as 1 pg DNA, equivalent to less than 10 copies (Global Haltech Sdn. Bhd., 2021b).
[7]The sample was spiked with a positive control.
[8]+ = porcine DNA was detected; - = porcine DNA was not detected.

In contrast, the remaining samples (Samples 5, 7, 8, and 9), including the halal-certified gummy (Sample 9), tested negative for porcine DNA, confirming no cross-reactivity with other animal species and validating the specificity of the PCR assay. The result is consistent with the Porcine DNA Real-Time PCR Halkit II insert specification (Global Haltech Sdn. Bhd., 2021b).

To validate the performance of qPCR, 100% pure porcine and bovine collagen samples were analysed as independent controls. The porcine control produced strong amplification with CT values of 27.45 and 27.63 in duplicate, indicating precise positive detection. The bovine control yielded no detectable CT values, confirming the absence of porcine DNA and demonstrating high specificity. These results confirm that the qPCR assay discriminates between porcine and non-porcine collagen sources under the tested conditions.

Real-time PCR demonstrated strong sensitivity for detecting porcine DNA in undenatured collagen. However, its performance was limited in hydrolysed or heavily processed samples due to DNA degradation and matrix interference. Moreover, false positives in fish- and marine-labelled samples may raise concerns over supply chain traceability and the risk of contamination. These findings suggest that real-time PCR is a valuable tool for halal authentication. However, it may benefit from complementary methods, such as ELISA, to overcome its limitations in detecting porcine residues in complex or denatured matrices.

3.2 Porcine detection by enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a protein-based analytical approach commonly used to detect the presence of antigenic proteins in food and pharmaceutical products (El Sheikha *et al.*, 2017). This study employed a sandwich ELISA to detect porcine antigen in nine collagen-based samples of various sources and processing forms (Table 5).

Only Sample 1 was successfully detected among the three porcine-labelled samples, with P/N values of 1.34 ± 0.05, both above the threshold cut-off (≥ 1.3). This detection indicates the presence of porcine antigen, confirming the effectiveness of the applied pretreatment and extraction protocols (Global Haltech Sdn. Bhd., 2021a). Sample 1 consisted of hydrolysed porcine collagen peptides, despite their highly processed nature, which yielded a positive result due to optimised sample processing techniques. These steps included cold acetone precipitation, sonication with Radio-Immuno-Precipitation Assay (RIPA) lysis buffer, and replacement of the standard diluent with

Table 5: Porcine collagen P/N ratio for detection of porcine antigen with ELISA

Sample no.	Product type	Collagen type[1]	Source	Halal certification status[2]	P/N ratio[3,4,5]	Result[6]
1	Powder	Hydrolysed peptide	Porcine	Non-halal-certified	**1.34 ± 0.05^{e}**	+/+
2	Powder	Peptide	Fish	Non-halal-certified	1.11 ± 0.03^{cd}	-/-
3	Powder	Undenatured type II peptide	Porcine	Non-halal-certified	0.78 ± 0.02^{a}	-/-
4	Powder	Not available	Marine	Non-halal-certified	1.16 ± 0.01^{d}	-/-
5	Powder	Not available	Fish	Non-halal-certified	0.88 ± 0.05^{ab}	-/-
6	Cream mask	Hydrolysed peptide	Porcine	Non-halal-certified	0.87 ± 0.01^{ab}	-/-
7	Liquid supplement	Not available	Plants	Non-halal-certified	**1.86 ± 0.06^{f}**	+/+
8	Jelly	Type I & II peptide	Fish	Non-halal-certified	0.84 ± 0.03^{ab}	-/-
9	Gummy	Type I & III peptide	Not available	Halal-certified	0.98 ± 0.00^{bc}	-/-
10	Porcine collagen (positive control)			NR	**2.12 ± 0.05^{g}**	+/+
11	Bovine collagen (negative control)			NR	0.89 ± 0.03^{ab}	-/-

Note: [1]Not available, as data could not be obtained.
[2]NR = Not related
[3]A P/N ratio ≥ 1.3 indicates positive detection of porcine collagen, while a value of < 1.3 indicates a negative result.
[4]Bold result indicates positive porcine presence.
[5]ELISA kit sensitivity was reported as 0.5%, specificity was reported as 100%, while the limit of detection was reported as 0.5 mg pig gelatine in 1 mL test buffer (Global Haltech Sdn. Bhd., 2021a).
[6]+ = porcine antigen was detected; - = porcine antigen was not detected.

0.5 M NaCl, a stronger protein extractant. (Jain *et al.*, 2020). In prior trials without these optimisations, the same sample returned negative results, underscoring the crucial role of pretreatment in enhancing protein recovery from complex matrices**.**

The other two porcine-labelled samples (Samples 3 and 6) yielded negative results, likely due to limitations in antigen recovery or the presence of interfering compounds such as salts, lipids, or other coexisting substances. Nhari *et al.* (2019) reported that matrix effects in commercial processed foods can hinder antibody-antigen interactions, particularly when the antigen is below the assay's detection threshold. Although the current study employed pretreatment strategies to overcome this limitation, a lack of standardisation and optimisation for each sample type (solid vs. liquid) may have affected antigen extraction efficiency and assay reproducibility. Samples 3 and 6, despite being labelled as porcine-derived, returned negative results. This outcome is likely due to ELISA's reliance on intact antigenic protein structures**,** which can be significantly affected by extensive processing such as hydrolysis, high heat, or chemical treatment (Zhang *et al.*, 2025). Undenatured collagen in Sample 3 may still contain DNA (hence detectable by PCR) but could have lost conformational epitopes necessary for antibody recognition**,** as Nhari *et al.* (2019) suggested. Likewise, Sample 6, a cream-based formulation, may have yielded poor antigen recovery due to matrix complexity, surfactant interference, or limitations in protein solubility. Lipids and emulsifiers in cream-based formulation can form micelles or coat proteins, reducing their accessibility to the capture antibodies and compromising detection sensitivity (Henao-Ardila *et al.*, 2024). The low P/N values observed (< 0.9) support the interpretation that the detectable antigen was insufficient. This limitation highlights the importance of appropriate sample pretreatment protocols and the potential need for matrix-specific ELISA optimisation.

Other samples, including fish- and marine-derived (Samples 2, 4, 5, and 8) and the halal-certified gummy product (Sample 9), showed negative results**,** indicating no cross reactivity with non-porcine species**.** This agrees with the specificity statement of the Porcine ELISA kit used (Global Haltech Sdn. Bhd., Malaysia).

Similarly, the ELISA assay was validated using the same external controls. The porcine collagen yielded P/N ratios of 2.12 ± 0.05, above the positive threshold (≥ 1.3), confirming strong antigen detection. In contrast, the bovine collagen sample returned P/N ratios of 0.89 ± 0.03, indicating negative detection with no cross-reactivity. These findings support the assay's specificity for porcine collagen proteins and its suitability as a confirmatory tool when used with DNA-based methods.

In summary, ELISA was able to detect porcine antigen in one confirmed porcine-labelled product and one plant-labelled product, but failed to detect the antigen in other porcine-based samples, likely due to low antigen levels or insufficient extraction efficiency. The findings reinforce that while ELISA can serve as a complementary technique for porcine detection, especially in cases where DNA is degraded, its performance is highly dependent on sample processing and pretreatment protocols. This result further supports the manuscript's core proposition that an integrated approach using PCR and ELISA enhances the reliability of halal authentication for highly processed collagen products.

3.3 Comparison of porcine detection by real-time polymerase chain reaction and enzyme-linked immunosorbent assay

A comparative overview of qPCR and ELISA results is summarised in Table 6, highlighting detection discrepancies across different collagen-based samples. Out of nine tested products, agreement between the two methods was observed in only four samples, resulting in an overall agreement rate of 44.4%. qPCR demonstrated greater sensitivity in detecting porcine DNA in undenatured collagen samples (e.g., Sample 3), whereas ELISA was more effective in detecting antigenic proteins in hydrolysed forms (e.g., Sample 1). However, both

Table 6: Comparison of porcine detection results between Real-time Polymerase Chain Reaction (qPCR) and Enzyme-linked Immunosorbent Assay (ELISA), with corresponding false negative identification and method agreement

Sample no.	Product type	Collagen type[1]	Source	Halal certification status	Result[2]		False negative	Method agreement	Remark
					qPCR	ELISA			
1	Powder	Hydrolysed peptide	Porcine	Non-halal certified	-/-	+/+	Yes, missed by qPCR	No	DNA was likely degraded; the antigen was preserved.
2	Powder	Peptide	Fish	Non-halal-certified	+/+	-/-	No	No	DNA was detected; potential contamination or mislabeling.
3	Powder	Undenatured type II peptide	Porcine	Non-halal-certified	+/+	-/-	Yes, missed by ELISA	No	The protein epitope was likely denatured.
4	Powder	Not available	Marine	Non-halal-certified	+/+	-/-	No	No	Possible contamination; unverified label.
5	Powder	Not available	Fish	Non-halal-certified	-/-	-/-	No	Yes	Consistent negative.
6	Cream mask	Hydrolysed peptide	Porcine	Non-halal-certified	-/-	-/-	Yes, missed by qPCR and ELISA	Yes	Likelihood of matrix interference or degradation.
7	Liquid supplement	Not available	Plants	Non-halal-certified	-/-	+/+	No	No	Possible cross-reactivity or label issue.
8	Jelly	Type I & II peptide	Fish	Non-halal-certified	-/-	-/-	No	Yes	Consistent negative.
9	Gummy	Type I & III peptide	Not available	Halal-certified	-/-	-/-	No	Yes	Results were confirmed negative, consistent with the label.

Note: [1]Not available, as data could not be obtained.
[2]+ = porcine was detected; - = porcine was not detected.

methods failed to detect porcine content in Sample 6, likely due to the effects of matrix complexity and protein/DNA degradation. Conversely, mismatches in Samples 2, 4, and 7 suggest cross-contamination or limitations in one method, depending on the sample's processing stage.

These findings reinforce the strengths and limitations of each method: qPCR is highly sensitive to DNA presence but vulnerable to DNA degradation or inhibition. At the same time, ELISA relies on intact or recoverable proteins, which may be lost in highly processed or emulsified matrices. Thus, an integrated approach is recommended for more reliable halal authentication in collagen-based products.

While PCR and ELISA are widely used for porcine detection due to their relatively low cost, sensitivity, and ease of use, advanced techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) offer superior specificity. LC-MS/MS can precisely identify species-specific peptide markers, making it highly effective in detecting trace levels of porcine proteins even in extensively processed products (Yuswan *et al.*, 2025). However, its implementation in routine halal authentication is often limited by high equipment costs, technical complexity, and longer analysis time. In contrast, PCR and ELISA remain more accessible for high-throughput screening, especially in regions where rapid, cost-effective methods are prioritised. Therefore, although LC-MS/MS provides confirmatory strength, PCR and ELISA play a central role in first-line halal compliance testing, especially when complemented as a dual-method strategy.

3.4 False negative rate (FNR)

The performance of the PCR and ELISA methods was further evaluated by calculating the False Negative Rate (FNR), as shown in Table 6. In this study, the false negative rate (FNR) was calculated to evaluate the performance of both real-time PCR and ELISA in detecting porcine derivatives in highly processed collagen samples. FNR is defined as the proportion of actual positive samples that were incorrectly classified as negative, as given by the formula:

$$FNR = FN / (TP + FN)$$

Where FN is the number of false negatives and TP is the number of true positives.

Based on the confirmed porcine-labelled samples (Samples 1, 3, and 6), only one sample (Sample 3) was detected by real-time PCR, and one (Sample 1) by ELISA, while Sample 6 was not detected by either method. This resulted in 2 false negatives out of 3 total positives, yielding:

$$FNR = (2/(1 + 2)) \times 100 = 66.7\%$$

This value indicates that the detection methods missed 66.7% of known porcine-containing samples, even though they were expected to test positive based on their labelled contents.

Such a high false negative rate suggests that both methods, when used alone, may fail to reliably identify porcine contamination in specific collagen matrices, especially those that are extensively processed or hydrolysed.

Ferrer-Urbina *et al.* (2023) state that the FNR is a critical diagnostic performance metric. Cohen (1992) recommended that false negative rates should ideally not exceed 20% in applied research settings. Hence, the 66.7% FNR observed in this study exceeds acceptable thresholds, potentially compromising the reliability of halal authentication if these methods are used independently. This limitation may be attributed to the small sample size, the diverse physical forms of the samples (e.g., powder, cream, gel, liquid), and the complexity of collagen processing**,** which includes heat, enzymatic hydrolysis, and chemical treatment. Such factors are known to degrade DNA and denature proteins, complicating both PCR amplification and antigen detection**.** This study exceeds acceptable thresholds, potentially compromising the reliability of halal authentication if these methods are used independently. This limitation may be attributed to the small sample size, the diverse physical forms of the samples (e.g., powder, cream, gel, liquid), and the complexity of collagen processing**,** which includes heat, enzymatic hydrolysis, and chemical treatment. Such factors are known to degrade DNA and denature proteins, complicating both PCR amplification and antigen detection. These findings emphasise the need for combining DNA- and protein-based methods to minimise false negatives and improve confidence in porcine detection**.** Additionally, method optimisation, sample-specific pretreatment protocols, and validation using larger sample sets are recommended to enhance detection accuracy in future halal authentication work.

While the calculated false negative rate (FNR) provides valuable insights into the limitations of each method, it must be interpreted with caution due to the small sample size (n = 9) and the limited inclusion of externally verified control materials. The study relied primarily on commercial product claims and internal kit controls, which may not fully represent the complexity of porcine detection in diverse real-world matrices. Therefore, the observed FNR and overall agreement rates serve more as preliminary indicators than conclusive diagnostic metrics. Future research should aim to include larger sample sizes and use well-characterised, controlled matrices, both porcine-positive and porcine-free, to strengthen statistical confidence and validation across different product categories.

3.5 Agreement analysis between qPCR and ELISA

The overall agreement rate (OAR) was calculated to evaluate the consistency between the qPCR and ELISA methods across the nine tested collagen-based products (Table 6). OAR is defined as the proportion of total samples for which both methods yielded the same positive or negative result. In this study, agreement was observed in 4 out of 9 samples, specifically in Samples 5, 6, 8, and 9, resulting in an OAR of:

$$OAR = (4/9) \times 100 = 44.4\%$$

The remaining five samples (1, 2, 3, 4, and 7) exhibited mismatched results between the two methods, indicating only moderate alignment.

Several factors may account for this limited agreement. First, the differing analytical targets of the methods (DNA vs protein) inherently influence their detection capabilities. The qPCR targets species-specific DNA, which may degrade during hydrolysis, heating, or chemical processing, limiting its effectiveness in hydrolysed peptides or emulsions. In contrast, ELISA targets antigenic protein structures, which may be denatured or masked by matrix components, or present below the assay's detection threshold, especially in highly processed or diluted products.

Second, the complexity of the sample matrix likely contributed to detection discrepancies. For instance, emulsified or cream-based products (e.g., Sample 6) pose challenges for DNA and protein extraction due to low analyte concentration or interference from fats and surfactants. Third, label inconsistencies or sourcing issues, such as Sample 7 (labelled as plant-based yet ELISA-positive for porcine antigen), may point to cross-contamination, unverified ingredients, or incomplete halal verification in the supply chain.

Ultimately, the 44.4% agreement rate confirms that relying on a single detection method may be insufficient for halal authentication of highly processed collagen-based products. These findings underscore the need for a dual-method verification strategy, which combines the DNA sensitivity of qPCR with the protein-detection capability of ELISA, to reduce false negatives and enhance analytical robustness.

4. Conclusion

This study provides important insight into the performance of PCR- and ELISA-based methods for detecting porcine components in highly processed collagen-based products. While each method has limitations, their combined application can enhance halal authentication's reliability, particularly in products containing degraded DNA or denatured proteins. However, this dual-method strategy should be viewed as a promising approach rather than a definitive solution. Its broader application requires further validation through studies involving larger and more diverse sample sets, as well as harmonised testing protocols. The observed discrepancies between detection methods underscore the importance of matrix-specific validation and greater regulatory emphasis on multimodal screening strategies. These findings also highlight the need for transparent labelling, strict manufacturing controls, and the integration of both molecular and immunoassay tools to support halal integrity in complex consumer goods. Nonetheless, the findings should be interpreted with caution due to the small sample size (n = 9) and the absence of independent positive and negative control samples, which limit the statistical strength and generalisability of the results. These limitations call for more extensive validation studies using well-characterised reference materials and known control matrices. Future research should focus on refining detection thresholds, minimising false negatives, and aligning screening practices with established halal regulatory frameworks.

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6. Data availability

Data is available upon request.

7. Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that could be perceived to influence the work reported in this paper.

8. Author contributions statement

Camilla Dewanthy Putri Basuki - Writing original draft; Ruzanna Zainal - Conceptualisation, data curation, and reviewing the manuscript; Muhamad Shirwan Abdullah Sani - Methodology and reviewing the manuscript.

9. Declaration of generative AI in scientific writing

The authors declare that they have used a generative artificial intelligence tool (Grammarly) specifically to improve the readability and language of the manuscript. After using this tool, the author(s) reviewed and edited the content as needed and take full responsibility for the content of the published article.

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