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Research Paper

Assessment of Commercial DNA Extraction Kits for Porcine Gelatin Detection Using RT-PCR and ddPCR

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Abstract
Gelatin is extensively applied in various industries, including food, beverages, cosmetics, and pharmaceuticals. Although the determination of gelatin species is essential for religious, health, and consumer preference reasons, a standardized analysis method is absent. The challenge in identifying gelatin through DNA-based methods arises from the low DNA content and extensive DNA denaturation in the gelatin matrix. This study assessed the efficacy of two commercial DNA extraction kits, namely the Processed Food DNA Extraction (PF kit) and the DNeasy Mericon Food Kit (DM kit), for extracting DNA from porcine gelatin powder and commercial products derived from gelatin. Additionally, we evaluate the amplification of the extracted porcine DNA using real-time polymerase chain reaction (RT-PCR) and droplet digital polymerase chain reaction (ddPCR) techniques. The PF extraction kit demonstrated successful DNA extraction from porcine gelatin powder and commercial samples of porcine gelatin-based candies with a higher concentration (32.24-286.07 ng/μL) and purity (A260/A280 ratio of 1.82-2.33) compared to the DM kit (3.95-7.30 ng/μL and an A260/A280 ratio of 1.29-2.45). RT-PCR and ddPCR analyses yielded positive results for porcine DNA from gelatin powder for both PF and DM kits, albeit with differing Cq values and copy numbers. The choice of DNA extraction kit significantly impacted the amplification results when analyzing commercial samples of porcine gelatin-based candies. Using RT-PCR, all samples yielded negative results with the DM kit, while the PF kit detected one positive result for porcine DNA. Improved outcomes were observed with more sensitive analysis methods such as ddPCR, where the DM kit identified one positive result for porcine DNA while the PF kit detected positive results for all tested candies.

Keywords
ddPCR, DNA Extraction, Gelatin, RT-PCR

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1. INTRODUCTION

Gelatin, a polymer derived from the partial denaturation of collagen extracted from animal skin, bones, and connective tissue, serves multifaceted roles as a gelling, binding, coating, and stabilizing agent (Nikzad et al., 2017). Widely used in confectionery and dessert formulations including marshmallows, gummy candies, yogurt, pudding, ice cream, and cheese, its versatile physicochemical properties contribute significantly to the texture and stability of these products (Rohman et al., 2020; Yap and Gam, 2019). In the pharmaceutical industry, gelatin finds diverse applications such as in the production of soft and hard capsule shells, tablet coatings, encapsulation, and microencapsulation processes (Al-Nimry et al., 2021). Its functionalities extend to preventing oxidation, enhancing drug acceptability, and improving taste (Nurilmala et al., 2021). Moreover, gelatin plays a pivotal role in cosmetics and personal care products, functioning as a key ingredient in formulations such as hair gel, creams, lotions, shampoos, and various skincare products (Abdullah et al., 2018).

The origin of the gelatin source is essential for religious, health, and consumer lifestyle reasons. Gelatin on the market comes from 80% porcine skin, 15% cow skin, and 5% porcine bones (Sani et al., 2021). Muslim and Jewish consumers are prohibited from consuming products originating from porcine. In contrast, Hindus do not consume products originating from cows, and additionally, vegetarian consumers avoid animal-derived products altogether (Elyasi et al., 2020; Yayla and Ek-

inci Doğan, 2021). Protein and DNA-based analysis techniques are typically employed for the determination of the gelatin source species. Grundy et al. (2016) used the ELISA analysis method for gelatin and exhibited inconsistencies when applied to both porcine and bovine gelatin. Moreover, this method entails drawbacks such as an extended antibody production process, the possibility of false positive or negative outcomes due to non-specific antibody-antigen reactions, limited suitability for multiplex reactions, and reduced sensitivity for highly processed samples resulting in decreased protein stability (Uddin et al., 2021). Ismarti et al. (2022) conducted a study regarding the authentication of fish, beef, and porcine gelatin using the SPME-GCMS method based on volatile compounds. However, the results are still preliminary without quantification data and validation performance. In general, the SPME-GCMS method has shortcomings when used with complex matrices such as processed products (Medina et al., 2019). In contrast, DNA-based analysis methods like polymerase chain reaction (PCR) demonstrate higher sensitivity. Conventional PCR has been proven to detect the mixing of standard porcine gelatin and standard bovine gelatin with a detection limit of 0.1% (Shabani et al., 2015). Nikzad et al. (2017) also analyzed using the duplex PCR method, which detected the presence of 0.1% porcine DNA in the capsule shell. Additionally, analysis using the multiplex PCR-RFLP method Sultana et al. (2018a) successfully discriminates beef, porcine, and fish gelatin in capsule shells with a detection limit of 0.01 ng. While, Pratiwi et al. (2018), Yayla and Ekinci Doğan (2021), and Demirhan et al. (2012) reported the detection of capsule shell gelatin, standard gelatin, and commercial products using the real-time PCR (RT-PCR) method, obtaining detection limits ranging from 0.01% to 1%.

In general, the efficacy of PCR analysis is primarily determined on the purity and concentration of the extracted DNA template. Unfortunately, the DNA content within the gelatin matrix is distinctly low due to extensive denaturation during the gelatin production process, involving rigorous physical and chemical treatments such as heating, acidification, alkalization, washing, and drying (Demirhan et al., 2012; Khayyira et al., 2018). Furthermore, the complexity of the gelatin matrix is compounded by the presence of additional ingredients in processed food products (Sultana et al., 2018b). Sultana et al. (2018b) observed a relatively modest concentration of extracted DNA (4-12 ng/μL) using the FavorPrep TM Food DNA Extraction Kit (Favorgen Biotech Corp, Ping-Tung, Taiwan) for commercial products like gummy candy, marshmallows, soft candy, and pastilles. Given these constraints, an assessment of the efficacy of DNA extraction kits on gelatin matrices is warranted. While previous studies have compared the performance of commercial DNA extraction kits across various samples such as dairy products, cocoa, breast milk, bacteria, and genetically modified organisms (GMO) (Butler et al., 2022; Ha et al., 2015; Pacheco Coello et al., 2017), no studies to date have compared the performance of commercial DNA extraction kits specifically on gelatin matrices.

In this study, the performance of two commercial DNA extraction kits, namely the Processed Food DNA Extraction (PF kit) and DNeasy Mericon Food Kit (DM kit), will be assessed in extracting DNA from gelatin powder and gelatin-containing candy products. While the DM kit has been previously utilized for DNA extraction from gelatin powder matrices and drug capsules by Nikzad et al. (2017), Shabani et al. (2015), and Khayyira et al. (2018), the PF kit, although not specifically reported for gelatin DNA extraction, was designed for processed food products and may demonstrate efficacy in extracting DNA from gelatin matrices. Furthermore, this study will investigate the impact of the DNA extraction kit on the successful detection of porcine DNA in gelatin and commercial gelatin-based products using RT-PCR method and more sensitive method of droplet digital polymerase chain reaction (ddPCR).

2. EXPERIMENTAL SECTION

2.1 Materials

The materials used in the research were porcine gelatin powder (Sigma-Aldrich, St. Louis, USA), commercial porcine gelatin-based candies, DNeasy Mericon Food Kit (Qiagen, Hilden, Germany), and Processed Food DNA Extraction Kit (Tiangen, China). The instruments used for quantification of DNA extract result is NanoDrop™2000/2000c UV–VIS Spectrophotometer (NanoPhotometer Pearl, Implen GmbH, Munich, Germany), then droplet generation with Bio-Rad QX200 Droplet Generator (Bio-Rad, Hercules, CA, USA), PX1™PCR Plate Sealer (Bio-Rad, Hercules, CA, USA), ddPCR thermal cycling was conducted with T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA), reading the amplified ddPCR signal using QX200™Droplet Reader (Bio-Rad, Hercules, CA, USA), and RT-PCR amplification machine is CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

2.2 Methods

2.2.1 DNA Extraction

DNA was extracted from gelatin powder and candy (200 mg) using DNeasy® Mericon Food Kit and Processed Food DNA Extraction Kit following the protocol with modifications. The concentration and purity of the extracted DNA were measured using a NanoDrop™2000/2000c UV–VIS Spectrophotometer. The extracted DNA was stored at -20°C until it was used for PCR analysis. To ensure the reproducibility of the DNA extraction method, tests were conducted on various days and by different operators, with each test repeated twice. Subsequently, the DNA extraction results were validated using the RT-PCR and ddPCR methods.

2.2.2 Primer and Probe Design

The primer and probe design (Macrogen, Seoul, Korea) followed the ISO/TS 20224-3:2020(E) method. The target gene for detecting porcine is beta-actin (ACTB) with GenBank accession number DQ452569.1 (Table 1).

Table 1. Primer and Probe Design for ddPCR Analysis

Primer/Probe	Oligonucleotide DNA Sequences
Porcine-97bp-F	5'-CGTAGGTGCACAGTAGGTCTGAC-3'
Porcine-97bp-R	5'-GGCCAGACTGGGGACATG-3'
Porcine-97bp-P	5'-[FAM]-CCAGGTCGGGGAGTC-[NFQ-MGB]-3'

*F: Forward, R: Reverse, P: Probe, FAM: 6-carboxyfluorescein , MGB: minor groove binder (non-fluorescent chromophore)

2.2.3 RT-PCR Analysis

Each 20 μL PCR reaction mixture consisted of 10 μL iTaq Universal Probe Supermix (Biorad), 4 μL DNA template, 0.8 μL forward and reverse primer (400 nM final concentration), 0.4 μL TaqMan™MGB Probes (200 nM final concentration), and 4 μL nuclease free water (Tiangen). PCR amplification was carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) machine with conditions of initial denaturation cycle at 95°C for 10 minutes, then 45 cycles of denaturation at 94°C for 15 seconds and annealing-extension at 60°C for 60 seconds. Data were analyzed using CFX Maestro 2.0 software (Bio-Rad). The RT-PCR reaction was carried out in four replications. The validation of the RT-PCR method involved assessing its performance on commercial candy matrices.

2.2.4 ddPCR Analysis

Each 20 μL reaction mixture contains 10 μL ddPCR™Supermix Probes (No dUTP) (Bio-Rad), 4 μL DNA template, 1.8 μL forward and reverse primers (final concentration 900 nM), 0.5 μL TaqMan™MGB Probes (final concentration 250 nM), 1 μL HindIII, and 0.9 μL nuclease free water (Tiangen). Bio-Rad QX-100 droplet generator (Bio-Rad) was used to make 20 μL of the mixture into droplets. PCR amplification acquired place in a T100 thermal cycler with operating conditions of an initial denaturation cycle at 95°C for 10 minutes, then 40 cycles of denaturation at 94°C for 30 seconds, then an annealing stage at 55°C for 60 seconds, enzyme deactivation at 98°C for 10 minutes, and final incubation at 4°C. The entire protocol was performed at a ramp rate of 2°C/s. The signal amplification was read using a QX200™Droplet Reader (Bio-Rad), and then the absolute copy number was analyzed using QuantaSoft software (Bio-Rad) following the Poisson equation. The analysis was carried out in two replications and the ddPCR method was validated by testing the matrix effect on commercial candiess.

3. RESULTS AND DISCUSSION

3.1 DNA Quantification and Purity

It is generally well understood that the concentration and purity of the extracted DNA are important factors that determine the success of detecting target DNA in PCR analysis. In the case of DNA extraction from gelatin matrices, these factors pose challenges as the quantity and purity of DNA are typically lower compared to matrices like meat and its processed products. This disparity arises from the gelatin production process, which results in a significantly reduced and degraded DNA content (Demirhan et al., 2012; Mohamad et al., 2016; Piskata et al., 2019). In this study, we observed notable differences in DNA concentration extracted from gelatin powder using the Processed Food DNA Extraction (PF kit) compared to the DNeasy Mericon Food Kit (DM kit). The DNA concentration obtained with the PF kit was significantly higher than that with the DM kit ($p < 0.05$), with values of 34.03 ng/μL and 4.25 ng/μL, respectively (Figure 1a). The reproducibility of the gelatin extraction method was evaluated at various analysis times and by different operators, yielding standard deviation (SD) values of 0.38 for DNA concentration and 0.23 for purity (A260/A280). Even though there were no prior reports regarding DNA extraction from gelatin matrices using the PF kit however, nevertheless, the PF kit demonstrated superior efficacy compared to the DM kit. Previous studies have employed the DM kit for DNA isolation from gelatin powder, as reported by Nikzad et al. (2017) and Sultana et al. (2018b), achieving DNA concentrations of 34 and 96 ng/μL, respectively (Table 2). The variability in DNA concentration values across studies may stem from differences in the DNA content of gelatin powder samples and the type of samples analyzed. Furthermore, other DNA extraction kits such as the FavorPrep™ Food DNA Extraction Kit and the Biotecon DNA isolation kit have also been used for gelatin powder extraction, yielding DNA concentrations of 76, 86, and 126.3 ng/μL, respectively (Sultana et al., 2020; Sultana et al., 2018b; Yayla and Ekinci Doğan, 2021) (Table 2).

Following DNA extraction from gelatin powder, subsequent DNA extraction was conducted on porcine gelatin-based commercial products, specifically candy. These processed products are naturally more complicated due to additional production steps and the incorporation of diverse ingredients like emulsifiers, milk, sugar, and preservatives (Al-Kahtani et al., 2017; Baziwane and He, 2003; Khayyira et al., 2018) thus result in unpredictable outcomes for DNA extraction across different samples. The effectiveness of DNA extraction from candy products was evaluated based on the concentration of DNA extracted using both the PF kit and the DM kit, as detailed in Table 3. The results demonstrated that the PF kit extracted higher DNA concentrations from candy samples compared to the DM kit. Specifically, DNA extraction using the DM kit yielded concentrations of 3.95 ng/μL for candy 1, 5.30 ng/μL for candy 2, and 7.30 ng/μL for candy 3, while the PF kit achieved concentrations of 286.07 ng/μL, 32.24 ng/μL, and 64.86 ng/μL for the respective samples (see Table 3). Addi-

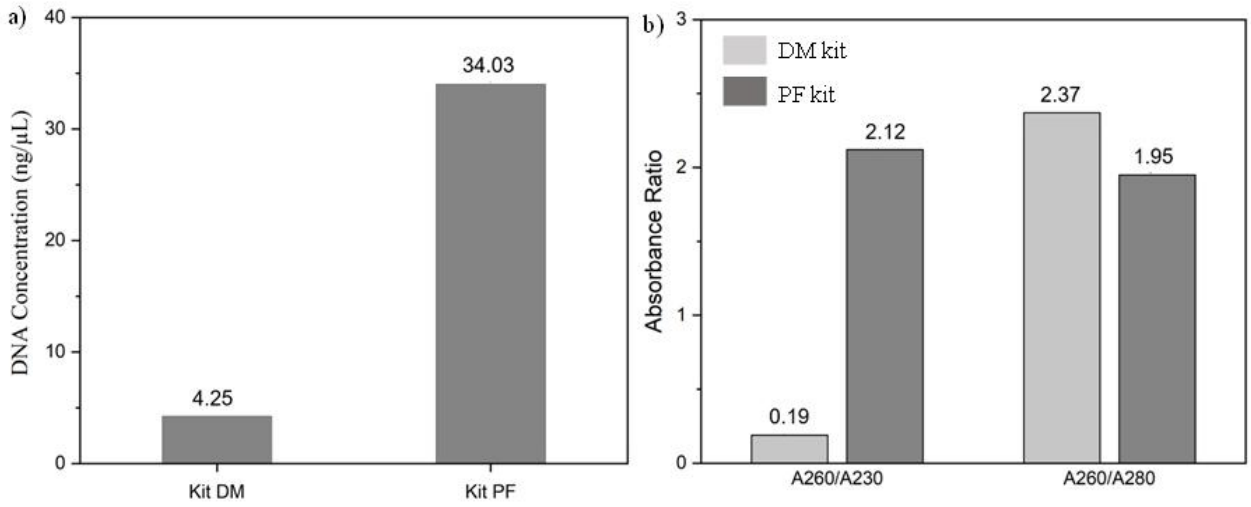

Figure 1. Comparison of DNA Concentration Values (a) and A260/A230, A260/A280 Ratios (b) in Gelatin Powder Samples Using Two Commercial Kits

tionally, Muñoz-Colmenero et al. (2016) previously reported lower DNA concentrations ranging from 0.0005 ng/μL to 0.328 ng/μL in candy products. In addition to candy, the ability of the DM kit in extracting DNA from other gelatin-containing commercial products has been previously reported. For instance, Nikzad et al. (2017) showed successful DNA extraction from piroxicam capsule shells at a concentration of 53 ng/μL. Furthermore, Sultana et al. (2018b) successfully extracted DNA from processed marshmallow products with an average concentration of 18.75 ng/μL.

Besides concentration, the purity of extracted DNA plays a crucial role in the success of DNA amplification. Nucleic acids exhibit maximum absorbance at 260 nm, and the ratio of absorbance at 280 nm to 260 nm (A260/280 ratio) is commonly used to assess purity in DNA and RNA extraction. A ratio close to 1.8 is generally indicative of high DNA purity, while ratios near 2.0 are considered suitable for RNA extraction. The DNA extracted from gelatin powder using the PF kit demonstrated superior purity, with an A260/280 ratio of 1.95, compared to the DM kit, which yielded a ratio of 2.37 (see Figure 1b). Previous studies by Nikzad et al. (2017) and Sultana et al. (2018b) reported A260/280 ratios of 1.7 and 1.8, respectively, for DNA extracted using the DM kit (refer to Table 2). However, in our study, a higher ratio of 2.37 was observed with the DM kit, suggesting contamination in the extracted DNA product (see Figure 1b). Furthermore, the purity of DNA extracted from commercial candy products using the DM kit ranged from 2.45 to 1.29, while the PF kit showed ratios of 2.33 to 1.91 (see Table 3). These findings suggest that the PF method is more effective in generating DNA extracts with higher purity levels.

In addition to assessing purity based on the A260/280 ratio, we also investigated the purity of extracted DNA using the A260/230 ratio to discern the presence or absence of chemical

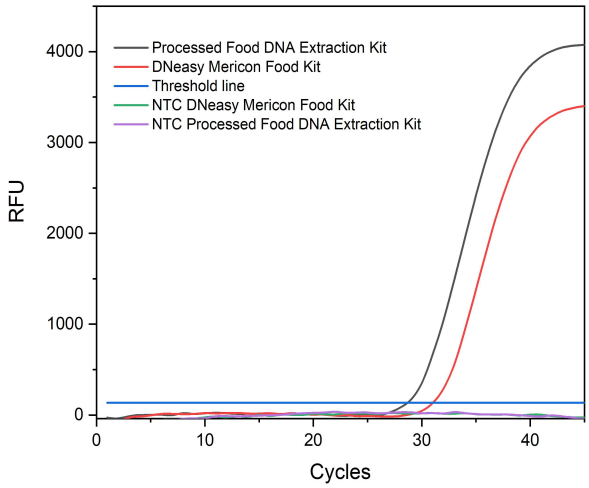

Figure 2. RT-PCR Amplification Curve of Gelatin Powder

contaminants such as residual chaotropic salts, which can impede PCR analysis. PCR inhibitors can disrupt various steps of PCR analysis, such as primer annealing to the DNA template, due to competitive inhibition of the inhibitor with the DNA template (Huggett et al., 2008; Opel et al., 2010). According to Yayla and Ekinci Doğan (2021), a desirable A260/230 purity ratio exceeds 1.5. The research findings depicted in Figure 1b reveal that the A260/A230 ratio for standard porcine gelatin DNA extraction using the PF kit is 2.12, whereas with the DM kit it is 0.19. Furthermore, for candy samples 1, 2, and 3, the DM kit yielded ratios of 0.27, 0.41, and 0.43, respectively, while the PF kit resulted in ratios of 0.85, 2.20, and 2.01 (refer to Table 3). The DNA purity based on the A260/A230 ratio is consistent with the purity determined by the A260/280 ratio, underscoring the capability of PF kit to produce DNA extracts

Table 2. Comparison of DNA Concentrations Extracted from Gelatin Powder Using Various Commercial Extraction kit

Extraction Method	Concentration (ng/μL)	A260/280	References
DNeasy Mericon Food Kit	34	1.7	(Nikzad et al., 2017)
DNeasy Mericon Food Kit	96	1.8	(Sultana et al., 2018b)
FavorPrep™Food DNA Extraction Kit	76	2.0	(Sultana et al., 2020)
FavorPrep™Food DNA Extraction Kit	86	1.9	(Sultana et al., 2018a)
Biotecon DNA Isolation Kit	126.3	1.8	(Yayla and Ekinci Doğan, 2021)
TübiGel DNA isolation	136	1.7	(Yayla and Ekinci Doğan, 2021)
Processed Food DNA Extraction	34.03	1.95	This study
DNeasy Mericon Food Kit	4.25	2.37	This study

Table 3. Concentration and Purity Values of Extracted DNA in Commercial Products

Commercial Product	Kit DM			Kit PF		
	Concentration (ng/μL)	260/280	260/230	Concentration (ng/μL)	260/280	260/230
Candy 1	3.95	2.45	0.27	286.07	2.33	0.85
Candy 2	5.30	2.22	0.41	32.24	1.82	2.20
Candy 3	7.30	1.29	0.43	64.86	1.91	2.01

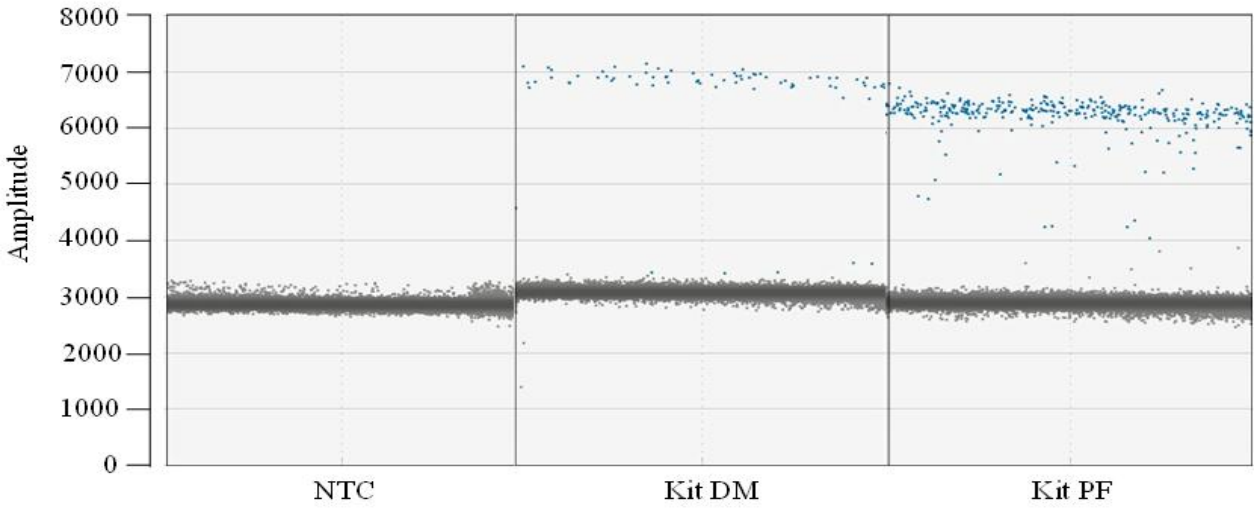

Figure 3. Copy Number Amplitude in Gelatin Powder Samples

Table 4. Comparison of RT-PCR and ddPCR Results

Commercial Product	RT-PCR		ddPCR	
	Kit DM	Kit PF	Kit DM	Kit PF
Candy 1	Negative	Negative	Positive	Positive
Candy 2	Negative	Positive	Negative	Positive
Candy 3	Negative	Negative	Negative	Positive

with higher DNA purity compared to the DM method within the tested samples.

3.2 DNA Amplification Results using RT-PCR and ddPCR

Further validation using RT-PCR was conducted to confirm that the DNA extracted using the DM and PF kits corresponds to porcine DNA. The RT-PCR method offers sensitivity and specificity adequate for detecting trace amounts of target DNA, such as those found in food products containing gelatin (Shabani et al., 2015). For this analysis, primers targeting the beta-actin gene (ACTB) with gene bank accession number DQ452569.1 were utilized to detect porcine DNA, in accordance with ISO/TS 20224-3:2020(E). The RT-PCR protocol involved denaturation at 94°C for 15 seconds, followed by annealing-extension at 60°C for 60 seconds, across 45 reaction cycles. Figure 2 illustrates a comparison of Cq (quantification cycle) values obtained from the amplification of porcine target DNA extracted from gelatin powder samples. The Cq analysis revealed positive outcomes for porcine DNA with both commercial extraction kits. However, the PF kit demonstrated a lower Cq value of 28.61 compared to the DM kit, which yielded a Cq value of 31.03. This discrepancy suggests a higher

concentration of DNA extracted by the PF kit relative to the DM kit, as corroborated by DNA concentration analysis. The choice of DNA extraction kit was notably impactful, particularly when analyzing commercial samples. Through RT-PCR analysis, DNA extracted using the DM kit yielded negative results for all candy samples tested, whereas the PF kit yielded one positive result with a Cq value of 34.92. The limited DNA concentration and lower DNA purity may contribute to amplification failure in RT-PCR, highlighting the significance of DNA extraction methods in the analysis of porcine DNA in gelatin matrices.

To ascertain the DNA extract quality from both kits in commercial products, we conducted subsequent analysis using a more sensitive molecular method, namely droplet digital polymerase chain reaction (ddPCR). The ddPCR mechanism involves partitioning the PCR reaction mixture into tens of thousands of droplets, amplifying the target DNA within each droplet, and generating a fluorescence signal indicating a positive or negative result (Hindson et al., 2011; Shehata et al., 2017). One of the advantages of ddPCR is its ability to provide more accurate and absolute quantification of target DNA, eliminating the need for a standard curve as required in RT-PCR and enhancing precision in measuring target DNA at low concentrations (Shehata et al., 2017). The ddPCR primers utilized were identical to those used in the previous RT-PCR analysis. The ddPCR protocol involved an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, an annealing stage at 55°C for 60 seconds, enzyme deactivation at 98°C for 10 minutes, and final incubation at 4°C.

Figure 3 illustrates the amplitude of ddPCR analysis results, indicating positive porcine DNA detection in all gelatin powder samples extracted using the DM and PF DNA extraction kits, consistent with the RT-PCR analysis outcomes. Fluorescence amplitude data displayed the absence of porcine DNA copy numbers in the negative control (NTC). Notably, the cluster pattern was distinct between positive and negative droplets in both commercial kits, with concentration values of 84.40 copies/reaction for the DM kit and 424 copies/reaction for the PF kit. Compared to RT-PCR, the ddPCR method yielded improved analysis results for candy samples. Specifically, the DM kit amplified porcine DNA from one candy sample with approximately 1.4 copies/reaction, while the PF kit yielded positive results for all three candies, with values of 6.1, 3.1, and 2.3 copies/reaction, respectively (see Table 4). These findings underscore the higher sensitivity of the ddPCR method over RT-PCR. This increased sensitivity was previously demonstrated by Nuraeni et al. (2023), who reported a ddPCR detection limit of 1 copy/reaction for porcine DNA plasmid samples, whereas the RT-PCR method had a detection limit of 5 copies/reaction with a Cq value of 37. In conclusion, the comparative analysis of DNA extraction performance between the DM and PF kits revealed that the efficiency of the PF kit is higher compared to the DM kit in extracting porcine DNA from the tested commercial gelatin products.

4. CONCLUSIONS

This study successfully evaluated the efficacy of two commercial DNA extraction kits for isolating DNA from gelatin powder and porcine gelatin-based candies. Based on the results of the concentration and purity of the DNA extract, the PF kit was proven to be more effective in extracting DNA from gelatin powder and commercial products than the DM kit. The RT-PCR technique effectively amplified porcine DNA from the extraction results obtained using both kits on gelatin powder samples. In contrast, disparate outcomes were observed in candy samples, with the DNA extract from the DM kit failing to amplify porcine DNA in any of the candies, whereas the PF kit successfully amplified porcine DNA in one out of the three candies tested. Better outcomes were observed using a more sensitive technique, specifically ddPCR, with one candy demonstrating the presence of porcine DNA in the DNA extracted from the DM kit. In contrast, the PF kit showed successful amplification of porcine DNA in all candies.

5. ACKNOWLEDGMENT

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