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Detection and Quantification of Genetically Modified Organisms (GMOs) in Halal Food Products by qPCR Method— Utilization of GMO-Positive Cabbage Seeds

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ABSTRACT

Use of genetically modified organisms (GMOs) in international food production has resulted in religious and health issues, especially among Muslim consumers. There is a need to have good analyses that will guarantee the authenticity and safety of the halal food products. **Objectives:** To identify and determine the concentration of GM contamination in both raw and processed foodstuff samples using a sensitive quantitative polymerase chain reaction (qPCR) method. **Methods:** Certified Reference Material (CRM-BF410ep Soya Bean) was used to construct a qPCR standard curve by 10-fold serial dilution (0.1% 0.01% 0.001% 0.0001%). GMO-positive cabbage seeds were added to processed food (China noodles, mixed spices, rice protein) and unprocessed food (brown rice, basmati rice, IRRI-6 rice). The NOS terminator sequence has been measured using qPCR to amplify DNA by extracting it with a commercial kit, quantifying it, and subsequently analyzing the DNA sample. Efficiency and limits of detection. **Results:** NOS terminator sequence was easily identified in a concentration of 0.001% and the mean Ct values were similar to the CRM standard. The statistical analysis (p.05) showed that there is no significant differentiation between the CRM and spiked samples, which proves the accuracy and reproducibility of the method at low concentrations of DNA. **Conclusions:** The tested qPCR technique proved to be very sensitive in the detection of GM contamination in various food matrices at 0.001 percent. Conclusions: This method will aid in halal food authentication, and a reliable molecular tool will help in avoiding accidental intake of GM haram ingredients.

INTRODUCTION

Food laws and safety must be reviewed regularly due to their global significance and influence on public health. The massive cross-border commerce in food commodities presents serious issues for the global food business [1]. Due to related ethical and health issues, some religions forbid genetically modifying food. In order to create gene combinations that do not exist naturally, genetically modified organisms (GMOs) are living things that have had their genetic makeup changed by biotechnological methods that involve the insertion of genetic material from other species, such as bacteria, viruses, plants, or animals [2]. Products made from haram (prohibited) sources, such as those that have been genetically modified using non-halal ingredients, are not acceptable for ingestion from an Islamic standpoint [3]. Muslims view following the halal diet as a way to preserve their spirituality as well as a religious obligation. It is crucial to make sure that genetically modified (GM) products adhere to Islamic principles, especially when the genetic material comes from animals that are forbidden or unclean materials [4]. A key component of Islamic dietary regulation is avoiding haram foods, which are expressly prohibited in the Quran and

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Hadith [3]. "Food and beverages comprising results and/or by-products of GMOs or ingredients derived from non-halal sources are not halal," according to the Standards and Metrology Institute for Islamic Countries (SMIIC) [5]. Because of their high nutritional content, vegetables—especially those in the Brassicaceae family are vital dietary components. China, India, and Pakistan are the world's top producers of cauliflower and cabbage, and Poland is in the top 10 worldwide [6]. China and India accounted for roughly 72% of the world's 25.5 million tonnes of cabbage production in 2020. The growing frequency of GM crops, including soybean (47%), maize (32%), cotton (15%), and canola (5%), highlights the growing need for efficient detection technologies, even if genetically modified cabbage is not yet commercially available [7]. Although the majority of genetically modified meals now come from plants, goods made from genetically modified animals (GMAs) and microorganisms (GMMs) should soon be available on the market [8]. Global adoption of genetically modified crops for food and feed uses has been made easier by the quick development of recombinant DNA technology [9]. Due to its sensitivity and repeatability, Polymerase Chain Reaction (PCR) continues to be the most dependable analytical technique for detecting GMOs in both raw and processed foods [10]. Even in complicated food matrices, the precise determination of GM content is made possible by quantitative PCR (qPCR), a refined form of PCR [11]. Because of its exceptional sensitivity and accuracy in identifying even the smallest DNA amounts, qPCR was used for this investigation. Compared to qualitative approaches, the technique provides a more detailed assessment by quantifying the quantity of GM DNA in a sample. Genetic components like the nopaline synthase terminator (NOS terminator), which is derived from Agrobacterium tumefaciens, are frequently detected in order to identify GMOs. Assays based on nucleic acids are essential for verifying the existence of transgenic material [12]. In various dietary matrices, prior studies have shown LODs of 0.1% [13], 0.01% [14], 0.9% [15], and 5.0% [16]. By establishing detection at an unusually low threshold of 0.001%, the current study expands the analytical limitations of qPCR for halal food surveillance, building upon earlier discoveries.

Despite the global rise in genetically modified crops, reliable detection of GMOs in halal food products remains a challenge, particularly at very low concentrations. While previous studies have demonstrated qPCR detection of GMOs, most methods report limits of detection ranging from 0.01% to 0.1%, which may not be sufficient for strict halal certification. Moreover, there is limited research in Pakistan on validating sensitive qPCR methods for detecting GM contamination in complex processed and unprocessed food matrices. This study addresses this gap by evaluating an advanced qPCR approach to detect GMOs at unprecedentedly low levels in diverse food products. The study aims to enhance GMO detection reliability and sensitivity, achieving a limit of detection (LOD) as low as 0.001% and to establish the precision, sensitivity, and reproducibility of a qPCR assay for GMO detection in complex food matrices.

METHODS

Sample mixtures (SM) of China noodles, mixed spices, rice protein, brown rice, basmati rice, and IRRI-6 rice were tested in this study. The samples utilized in this study were obtained from the commercial market and were subjected to GMO testing at the Halal Lab of the Industrial Analytical Center, Hussain Ebrahim Jamal (HEJ) Research Institute of Chemistry, from March 2023 to September 2023. For the GM-positive sample, cabbage seeds were used, which were the F1 hybrid obtained from the Department of Plant Protection, Karachi, Pakistan. An available positive sample for reference is GM cabbage seeds, which are positive for the target gene. The certified reference material (CRM-BF410ep Soya Bean) that contains 10% GMO-positive Soya matrix was utilized, likewise positive for standard curve preparation by diluting it to 0.1%,0.01%,0.001%, and 0.0001% and was purchased from Fapas65 Gresham St, London. The samples utilized in this study were obtained from the commercial market using a convenience sampling approach. This sampling strategy is appropriate for the method validation purpose of this study, which prioritizes assessing analytical performance across diverse matrices over achieving statistical representation of the broader food market. Written informed consent was taken. A total of six commercially available food products were selected to represent a diverse range of matrix complexities relevant to halal food testing. This included processed foods with potential PCR inhibitors like China noodles (high starch, potentially high oil), mixed spices (high polyphenols, polysaccharides), and rice protein (high protein), as well as unprocessed grains like brown rice, basmati rice, and IRRI-6 rice. The choice was specifically meant to test the DNA extraction and qPCR assay in different compositions, and in doing so, give a strong preliminary confirmation of the suitability of the method. The samples were homogenized individually and contaminated with GMO-positive cabbage seed and mixed with the 10% GM-positive sample that consisted of taking around 180 mg of China noodles and mixed spices, and contaminated with 20 mg of GMO-positive cabbage seed. SM 2 includes 10% of the GM-positive sample, which was prepared by taking approximately 180 mg of processed rice protein and raw brown rice, which was contaminated with 20 mg of GMO-positive cabbage seeds. SM 3 includes 10% of the GM-positive sample, which was prepared by taking

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approximately 180 mg of unprocessed IRRI 6 rice and basmati rice with the contamination of 20 mg of GMO-positive cabbage seeds. SM 4 includes a GM-negative sample, which was prepared by taking approximately 180 mg of processed China noodles and mixed spices with contamination of 20 mg of GMO-negative. As this study focuses on analytical method development and validation, a formal a priori sample size calculation was not performed. Instead, the experimental design and replication strategy were based on internationally accepted guidelines for the validation of qualitative and quantitative PCR methods, a relevant guideline here if desired, e.g., from ISO or MIQE. The robustness of the method was demonstrated through technical triplicates for each qPCR reaction to assess precision and biological replicates (independently prepared sample mixtures, SM1-SM5) to assess reproducibility across different matrices. The validation parameters were determined experimentally, which were the linearity (R 2), the amplification efficiency, coefficient of variation (CV%), limit of quantification (LOQ), and limit of detection (LOD). To statistically confirm the ability of the method to discriminate between the concentrations of GMOs, a one-way Analysis of Variance (ANOVA) was performed on the Ct values of CRM on its serial dilutions (0.1, 0.01, and 0.001%). This will ensure that the performance characteristics of the method are evaluated with a sufficient level of statistical rigor to implement the method. The findings showed that the qPCR test is able to differentiate accurately between these concentration levels by showing a significant difference between the mean Ct values of these dilutions ($F(2,6) = (\text{insert F-value})$, $p<0.001$). Approximately 180 mg of uncooked basmati rice and IRRI 6 rice were sprayed with 20mg of GMO-positive cabbage seeds to form the 10 percent GM-positive sample that is represented in SM 5. The extracted genomic DNA was obtained using the Kogenebiotech GMO Extraction Kit (Kogenebiotech, Geumcheon, Korea). In order to ensure that the accuracy is maintained, approximately 180 mg of each of the samples was carefully weighed and then used to prepare the 10% GM-positive sample. Controlled contamination was done using 20 mg of GMO-positive cabbage seeds introduced using an analytical balance of Mettler Toledo, Switzerland. A high-sensitivity analytical balance is necessary to determine the level of reliable results and obtain high levels of precision in weighing the samples. The sample homogenization was thereafter done using a Tissue Lyser II (QIAGEN, Germantown, MD, US). Besides, the samples were supplemented with 3mL of lysis buffer A,300 µL of lysis buffer B,10 µL of proteinase K, and 10 µL of RNAse. The extraction of the genomic DNA (gDNA) was performed according to the instructions of the manufacturer. Upon extraction, to achieve the same proportion, the DNA samples were diluted to a 10-fold serial dilution (lowest concentration being 0.0001%). The CRM was also extracted using the same protocol. The robustness of the experimental design is shown by the meticulous attention to detail in both the DNA extraction and sample preparation steps. The quality of the extracted gDNA was evaluated by 1% agarose gel electrophoresis. These procedures are essential for guaranteeing the precision and repeatability of the data, which eventually strengthens the validity of study conclusions. Using GMO CRM, 4 dilutions of 10-fold were prepared, i.e.0.1%,0.01%,0.001%, and 0.0001%. Sample mixtures SM1, SM2, SM3, SM4, and SM5were diluted at 10-folds, i.e. 0.1%, 0.01%,0.001%, and 0.0001%. Among all the dilutions, the 4th dilution (0.001%) was taken as a DNA template (sample) for further analysis, as the present research work aimed to quantify this concentration of DNA. Qubit Fluorometer 3.0 (Invitrogen Life Technologies, US) was used to evaluate the quality and concentration of DNA. QubitTM ds DNA HS standard 1 and QubitTM ds DNA HS standard S2 were produced using 189 µL of buffer, 1 µL of fluorescent dye, and 10 µL of each standard supplied with the Kit. To prepare the sample, 198 µL FOf buffer was dispensed into a tube containing 1 µL of dye (Qubit Assay Tubes, Thermo Fisher SCIENTIFIC). After adding 1 µL of the extracted DNA sample, the tubes were incubated at room temperature (25°C) for 1 minute before being placed into a Qubit fluorometer for observation. The amplification process using the PCR method was conducted in a final volume of 25 µL. 5 µL of template DNA, 12.5 µL of basic mix, and 7.5 µL of oligo mix made up this volume. The following approaches were used in a Thermal Cycler system (Rotor Gene Q, Qiagen Germany) to carry out the reactions: 45 cycles at 95°C for 10 minutes, 95°C for 10 minutes, and 60°C for 90 seconds. The data were assessed with the help of the QIAGEN rotor gene Q series program (version 2.3.1). Every sample mixture (SM1-SM5) was an independent biological replication that was generated independently in the same conditions, and each qPCR reaction was performed in technical triplicate to ensure analytical repeatability. In order to determine intra- and inter-assay precision, mean cycle threshold (Ct) and the corresponding standard deviations (SD) were calculated. Controls were employed in order to exclude any false positive or negative outcomes; these were borrowed from the PCR assay Kit. The 4 dilutions of 0.1 per cent,0.01 per cent, 0.001 per cent, and 0.0001 per cent of the GMO CRM (reference material) were amplified as standards. In addition to the 5 sample mixtures that had been diluted to 0.001% and 0.0001% to observe the GM event NOS terminator. Varying dilutions are employed with the aim of ensuring that this assay is sensitive enough to indicate the presence of GMO at an assortment of concentrations. All the data from qPCR were acquired in triplicate and evaluated as the mean and SD. The coefficient of variation

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(CV%) was computed in each dilution and was not more than 2, which proved a high degree of reproducibility. The results of CRM and spiked sample Ct values were compared using independent-sample t-tests, and no significant differences were detected ($p>0.05$). The fact that there was low variation between the replicates speaks in favor of both the precision and reliability of the assay.

RESULTS

To analyze genetically modified organisms (GMOs) in raw and processed food matrices at the lowest detectable concentration, this study applied quantitative polymerase chain reaction, or qPCR. This is the first formal effort that we have ever known in Pakistan to recognize GMO-positive cabbage seedlings. The control design of the study was based on the principles of analytical method validation. All analyses were performed in technical triplicate to ensure the accuracy of the results. The data are indicated in the form of mean and standard deviation (SD). The concentration and purity of DNA in the CRM and the samples of the test were determined using the Qubit Fluorometer 3.0 (Invitrogen, USA) at 430-495nm (blue) and 510-580nm (green). All samples had A260/280 absorbance ratios between 1.8 and 2.0, indicating little protein contamination and good DNA purity. The DNA yield derived from each sample combination was compiled in the findings. Quantifiable DNA was obtained from all extracted DNA samples at the desired 0.001% concentration, with quantities varying between 0.052 and 0.073 ng/µL. The DNA extraction and quantification procedures are very reproducible, as evidenced by the low standard deviations across triplicate assays (e.g., ± 0.004 to ± 0.008 ng/µL). This steady recovery from matrices that have been treated and those that have not shows how reliable the extraction procedure was (Table 1).

Table 1: DNA Concentration (ng/µl) Extracted from Sample Mixtures and CRM (Mean ± SD, n=3)

Dilution	SM1	SM2	SM3	SM4	SM5	CRM
Stock DNA	–	–	–	–	–	73.20 ± 0.15
0.1%	–	–	–	–	–	7.30 ± 0.12
0.01%	–	–	–	–	–	0.77 ± 0.09
0.001%	0.052 ± 0.006	± 0.004	0.064 ± 0.005	0.055 ± 0.007	0.073 ± 0.008	0.070 ± 0.005
0.0011%	Below Detection	Below Detection	Below Detection	Below Detection	Below Detection	Below Detection

SM1: China Noodles and Mixed Spices + Cabbage seeds (Positive) SM2: Rice protein and Brown Rice + Cabbage seeds (Positive) SM3: IRRI 6 Rice and Basmati rice + Cabbage seeds (Positive) SM4: China Noodles and Mixed Spices + Cabbage seeds (Negative) SM5: Basmati Rice and IRRI 6 Rice + Cabbage seeds (Negative) Certified Reference Material: ERM-BF410ep Soya Bean 10% GMO.

To improve detection reliability, qPCR was carried out employing both Cauliflower Mosaic Virus 35S (CaMV 35S) promoter targets and NOS terminator (T-NOS). To assess assay repeatability, each amplification was carried out in triplicate. Excellent linearity between concentration and cycle threshold (Ct) was confirmed by regression analysis of the CRM standard curve, which yielded a correlation coefficient (R2) of 0.992. Results are shown as mean Ct ± SD, and amplification efficiency was constant across replicates. Dual-marker reliability was confirmed by the amplification plots, which showed effective detection for both T-NOS and CaMV 35S targets in three of the five sample combinations (SM1–SM3) at the 0.001% level. Due to either matrix inhibitory effects or the lack of GM-positive material, two combinations (SM4 and SM5) did not exhibit any amplification (Table 2).

Table 2: Mean Ct (± SD, n=3) for CRM and Sample Mixtures at Different Dilutions

Dilutions	SM1 (Mean CT ± SD**)	SM2	SM3 (Mean CT ± SD)	SM4 (Mean CT ± SD)	SM5 (Mean CT ± SD)	CRM (Mean CT ± SD)
Stock DNA	–	–	–	–	–	21.69 ± 0.06
0.1%	–	–	–	–	–	26.71 ± 0.07
0.01%	–	–	–	–	–	30.67 ± 0.07
0.001%	33.72 ± 0.04	33.96 ± 0.67	33.69 ± 0.18	Not Detected	Not Detected	33.44 ± 0.13
0.0011%	No Amplification	No Amplification	No Amplification	No Amplification	No Amplification	No Amplification

SM1: China Noodles and Mixed Spices + Cabbage seeds (Positive) SM2: Rice protein and Brown Rice + Cabbage seeds (Positive) SM3: IRRI 6 Rice and Basmati rice + Cabbage seeds (Positive) SM4: China Noodles and Mixed Spices + Cabbage seeds (Negative) SM5: Basmati Rice and IRRI 6 Rice + Cabbage seeds (Negative) Certified Reference Material: ERM-BF410ep Soya Bean 10% GMO **SD = Standard Deviation.

Sm1 represents China noodles and mixed spices spiked with GMO-positive cabbage seeds; SM2 comprises rice protein and brown rice with GMO-positive contamination; SM3 includes IRRI-6 and basmati rice with GMO-positive cabbage seeds; SM4 depicts China noodles and mixed spices containing GMO-negative cabbage seeds; SM5 corresponds to basmati rice and IRRI-6 rice with GMO-negative cabbage seeds. The CRM (ERM-BF410ep Soya Bean, 10% GMO) served as the positive control for generating the standard curve (Figure 1).

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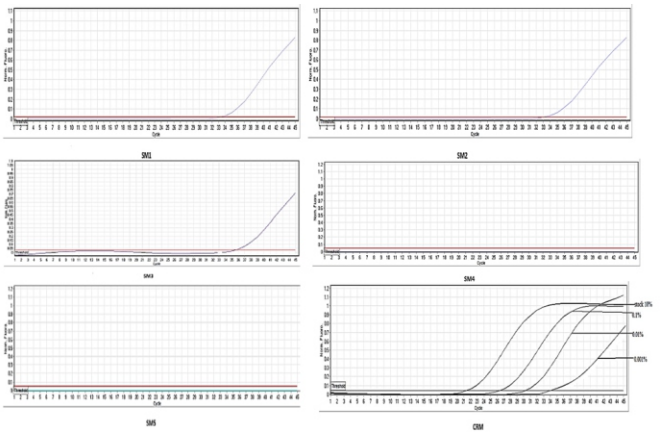

Figure 1: Amplification plots of sample mixtures and Certified Reference Material (CRM) analyzed by qPCR for GMO detection.

All CT data were analyzed using SPSS v25 (IBM, USA). Independent-sample *t*-tests were applied to compare mean Ct values between CRM and spiked samples. The *t*-test revealed no significant difference (*p* > 0.05), validating the reproducibility. Validating the reproducibility of detection across sample matrices. Mean ± SD and coefficient of variation (CV%) were within acceptable limits (<2%). The experimentally determined limit of detection (LOD) for both markers was 0.001%, while the limit of quantification (LOQ) was established at 0.01%, as verified through standard curve regression. The precision of the assay was statistically supported by the high R^2 value and low variability among triplicates. The precision of these limits was statistically verified by the high linearity of the standard curve (R^2 = 0.992), which confirms a precise and reliable relationship between the log of the DNA concentration and the Ct value across the tested range, including the LOD."

DISCUSSION

There are two important steps in the process of detecting the presence of genetically modified organisms (GMOs) in food products: the DNA should be extracted and purified, and the inserted genetic material should be amplified with the help of the polymerase chain reaction (PCR). The fact that food preparation can destroy DNA and break it down is irrelevant because even little pieces of DNA can be sufficient to conduct an accurate detection [17-19]. This necessitates the use of strong molecular tests to ensure consumer safety and to enforce the halal dietary laws, since this has been demonstrated by the increasing consumption of GMOs in food production. Other molecular techniques, such as quantitative PCR (qPCR), have proven to be a sensitive and accurate method of GM content detection and measurement. This study was able to identify the Agrobacterium tumefaciens nopaline synthase terminator (T-NOS) sequence in real-time qPCR with a concentration as low as 0.001% which illustrates the reliability of the method used in processed and unprocessed foods. The NOS terminator is a typical aspect of most transgenic plants, and an ideal target to be regularly screened by GMOs [20, 21]. Increased LODs occur typically in thermally processed foods due to DNA destruction, and similar studies done in other international countries have identified LOD of 0.001 -0.005 per cent in maize and soy-based matrices [22]. The fact that our results agree with those of past publications proves that, despite the differences in the food matrix structure, the achieved analytical sensitivity of our results is competitive on an international level. The low detection threshold that was achieved in this work and supports the quality of increased analytical sensitivity is facilitated by the use of Certified Reference Material (CRM) as a calibration reference. Statistical analysis showed that CRM and spiked samples were not different (p>0.05), which proves the accuracy and repeatability of the assay. Two out of five mixes of the samples (SM4 and SM5) failed to amplify at 0.001%, and this is likely to be caused by reduced DNA extraction efficiency or the effects of the matrix. Substances such as insecticides or polysaccharides, or residual lipids, may have inhibited the PCR amplification process. To minimize false negatives and enhance the test strength, these putative inhibitors stand to bring to notice the importance of the best extraction methods, purification steps, and internal amplification controls in future studies. The proper molecular detection is particularly important when it concerns the checking of halal food. Identification of GMOs is relevant to Muslim buyers because it ensures that they follow the Islamic regulation of diet, besides food safety. The clear labeling is achievable through the reliable screening techniques, such as qPCR, that enhance the confidence of consumers in food certification systems [23]. One of the most important requirements is to attain reproducible amplification results, which hinges on the quality of DNA. The A260/280 values in the 1.8-2.0 range corresponded to a decent DNA quality; however, the yield in this experiment could have been affected by the slight contamination or pesticides left in cabbage seeds. A260/280 ratios (1.8 to 2.0) and evident and intact agarose gel bands verified the purity of the DNA used to guarantee the integrity of the samples to be analyzed by trusted qPCR. Despite these challenges, the strength of the assay in various food matrices is evidenced by the fact that it was able to identify the assay in extremely low concentrations. Interrogation of assays with several additional transgenic components, including the CaMV 35S promoter or event-specific sequences, would add greater specificity and reduce the chances of false negatives, although this study focused on a single detection target (T-NOS) [24]. The sensitivity limits of different types of foods might be further optimized in the future with respect to other validation studies by comparing with either multiplex or digital PCR procedures and incorporating matrix-

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specific controls. GMOs are also being updated because newer versions might not have the classic marker genes, so the molecular testing methods should also be updated in accordance with the emergence of advanced gene-editing technologies, such as CRISPR-Cas9 [25]. The present research provides a reliable and reproducible paradigm to detect the presence of very low GMO contamination, even though the methodology has certain limitations, including the small size of the sample and the convenience-based sampling method. However, it must be said that the work contributes to another growing body of evidence that qPCR remains a reliable technique of GMO screening when used in culturally sensitive food systems such as halal certification. It can be seen that future research involving a greater sample size and detection targets will increase the accuracy and usefulness of qPCR in halal food safety monitoring and authentication. Since the samples were picked from conveniently available commercial sources, it is important to note that the sampling strategy applied was convenience-based. Thus, although the results on the sensitivity of detection were strong with the tested matrices, they cannot be directly translated to reflect the contamination levels or the effects of the matrices in the whole food market. Yet, this is in line with the main objective of this paper, which was to validate the method of analysis and not to conduct a wholesale survey of the market. It was aimed at showing the accuracy, sensitivity, and reproducibility of the method under controlled conditions of spiking.

A limitation of the current study is the use of a limited number of food matrices and controlled spiking with GMO-positive cabbage seeds, which may not fully represent real-world contamination scenarios. Future research could expand the range of processed and raw foods tested and include naturally occurring GM samples to further validate the method. Additionally, integrating multiplex qPCR or digital PCR could enhance detection sensitivity and allow simultaneous screening for multiple GMO events, supporting more comprehensive halal food authentication.

CONCLUSION

Considering food fraud and adulteration, the problem of consuming contaminated and dangerous food items was considered in the current study. It provides a reliable qPCR-based method of GM contamination quantification by placing GM-positive cabbage seeds in a range of food matrices. Nevertheless, it may be possible to measure even below the given limit by increasing the amount of DNA.

Authors' Contribution

Conceptualization: SAN, SA
Methodology: SAN, AA, IAK
Formal analysis: SAN
Writing and Drafting: NF, DM
Review and Editing: NF, DM, SAN, AA, IAK, SA

All authors approved the final manuscript and take responsibility for the integrity of the work.

Conflicts of Interest

All the authors declare no conflict of interest.

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