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Performance Evaluation of a *Jug r 2*-Targeted Real-time Polymerase Chain Reaction Assay in Processed and Commercial Food Matrices: Implications for Allergen Risk Management

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Abstract

Background and Aim: Although several polymerase chain reaction (PCR)-based methods have been developed for walnut identification, information regarding their performance in complex food matrices and processed foods are still limited. This study assessed the performance of a duplex TaqMan real-time PCR assay targeting an ultra-short *Jug r* 2 fragment for detecting walnut-derived DNA in processed and commercial food products.

Material and Methods: The assay was evaluated using representative processed food matrices, including cookie, cocoa, ketchup and soup, each spiked with walnut. The effect of thermal processing was assessed following dry heating (95 °C, 30 min) and autoclaving. In addition, seven commercially available food products were analyzed to assess the practical applicability of the assay. A plant-derived 18S rRNA internal amplification control was used to monitor amplification performance and potential PCR inhibition.

Results and Conclusion: Walnut-derived DNA was successfully detected in all processed food matrices and thermally treated samples. Matrix-associated inhibition varied within food types, with Ct shifts ranging from +1.72 cycles in cookie to +4.52 cycles in soup, while the internal amplification control was stableliable. Thermal processing decreased DNA detectability in a treatment-dependent manner, with Ct increases of +2.21 and +4.78 following dry heating and autoclaving, respectively. Analysis of 7 commercial food products showed complete agreement with declared walnut-containing products, whereas 1 hazelnut wafer without declared walnut generated a weak positive amplification signal. The developed duplex real-time PCR assay demonstrated reliable qualitative detection of walnut-derived DNA in processed and commercial food products. The assay represented a robust complementary molecular tool for food allergen surveillance, ingredient verification and quality control.

Conflict of interest: The authors declare no conflict of interest.

What is "already known":

- Real-time PCR is a sensitive and specific molecular method for detecting walnut-derived DNA in food products.
- Food processing and complex food matrices can reduce PCR performance through DNA degradation and amplification inhibition.
- Internal amplification controls improve the reliability of PCR-based food analysis by monitoring amplification efficiency and PCR inhibition.
- A few studies have evaluated walnut PCR assays under practical food-testing conditions using commercially processed foods.

What this article adds:	• Performance of a duplex *Jug r 2*-targeted real-time PCR assay in representative processed food matrices rather than its analytical characteristics. • Reliable qualitative detection of walnut-derived DNA despite matrix-dependent inhibition and severe thermal processing, including autoclaving. • Incorporation of an internal amplification control enabled reliable assessment of amplification performance in diverse processed food matrices and commercial products. • Practical evidence for suitability in food allergen surveillance, ingredient verification and quality control.

1. INTRODUCTION

The global prevalence of food allergies has escalated into a major public health concern, characterized by increasing incidence rate and a substantial socioeconomic burden on healthcare systems worldwide [1, 2]. Within the spectrum of food allergens, tree nuts and specifically walnut (*Juglans regia*), are recognized as primary triggers for severe, IgE-mediated clinical manifestations, often leading to life-threatening anaphylactic shocks even upon exposure to trace quantities [3]. Due to its desirable nutritional characteristics, walnut is widely used in numerous processed products, ranging from complex confectionery and bakery products to emulsified sauces and savory formulations, increasing the risk of unintentional exposure for sensitized individuals.

Since the only definitive management strategy for sensitized individuals is lifelong elimination of the offending allergen from their diets [4], accurate food labeling is critical. International regulatory standards now require the clear declaration of walnut components, necessitating the use of detection tools that are not only sensitive and specific but also robust enough to withstand the physicochemical alterations induced by food processing [5].

Traditional protein-based methods, particularly enzyme-linked immunosorbent assay (ELISA), are widely used for allergen detection; however, their performance may be compromised in processed foods because severe heat treatments induce Maillard reactions, leading to the formation of complex advanced glycation end-products (AGEs) [6]. These structural modifications can mask critical epitopes or reduce protein solubility, yielding false-negative results. In contrast, DNA-based methods targeting short and stable genomic sequences provide superior resilience against mechanical fragmentation and chemical modifications, serving as a reliable method for allergen monitoring [7]. As DNA is generally more stable within processing conditions, these methods are highly valuable for allergen detection in complex food matrices [8, 9].

Several molecular assays targeting walnut DNA have been reported; however, differences in target selection, extraction efficiency, matrix complexity and validation depth limit their comparability and routine applicability [10]. Importantly, several studies lack comprehensive validation data addressing matrix inhibition and robustness against food processing conditions, which are critical for implementation in routine food control laboratories.

To address these limitations, a *Jug r 2*-targeted real-time polymerase chain reaction (PCR) assay was recently developed and analytically validated in the current authors' laboratory. This diagnostic platform combines the target allergen gene detection with a plant-derived 18S rRNA as an internal amplification control (IAC) to assess complex food samples. Using this assay, the present study investigated its performance in processed foods, complex food matrices and commercial products to assess its practical applicability for food allergen surveillance. By utilizing an ultra-short 88-bp amplicon design, this system was specifically engineered to overcome the challenges of heat-induced DNA fragmentation and degradation during industrial processing; thereby, ensuring reliable detection of hidden walnut allergens.

Another important challenge in allergen analysis is the complexity of food matrices. Processed foods often contain compounds such as polyphenols, polysaccharides, lipids and other PCR inhibitors that can interfere with DNA extraction and amplification. This issue is especially relevant to products such as cocoa-based foods, baked foods and multi-ingredient formulations. Accordingly, the performance of a molecular assay depends not only on primer and probe design but also on the effectiveness of the DNA extraction protocol in removing inhibitory substances and recovering amplifiable DNA from complex matrices.

Although several PCR-based methods have been developed for walnut identification, most studies have focused primarily on assay development and analytical validation, including primer design, analytical specificity and sensitivity under controlled laboratory conditions [11, 12]. In contrast, comparatively few studies have systematically investigated the robustness and reliability of these assays in complex processed food matrices, following severe thermal processing or in commercially available food products, where DNA degradation and matrix-associated PCR inhibitors may adversely affect analytical performance. Furthermore, the incorporation of internal amplification controls to monitor assay reliability within diverse food matrices has been reported in a limited number of studies. Therefore, whether analytically validated assays preserve their reliability and diagnostic performance under routine food-assessment conditions is insufficiently investigated.

Based on the authors' previous analytical validation of the duplex TaqMan real-time PCR assay targeting an ultra-short *Jug r* 2 fragment, the present study aimed to investigate its performance under practical food-assessment conditions. Unlike the previous study, which established the analytical characteristics of the assay, this study systematically evaluates its robustness in thermally processed walnut samples, inhibitor-rich processed food matrices and commercially available food products, while using an internal amplification control to monitor assay reliability. By extending the evaluation from analytical validation to real-world food applications, the present study provides new evidence supporting the suitability of the assay for routine walnut allergen detection, food authenticity assessment and regulatory food safety surveillance.

2. MATERIALS AND METHODS

2.1. Samples and Matrix Preparation

This experimental study was carried out at the Department of Immunology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran, April to December 2025. All experimental procedures, including DNA extraction, real-time PCR assay development, analytical validation and evaluation of processed and commercial food samples, were carried out in the laboratory. Commercial food matrices including cocoa powder (Golha, Tehran, Iran), cookies (Naderi, Lahijan, Iran), instant soup (Amadeh Laziz, Karaj, Iran) and ketchup (Mahram, Tehran, Iran) were purchased as commercially packaged products from local retail supermarkets, Tehran, Iran. These matrices were selected as representative processed foods because they were reported to contain compounds such as polyphenols, lipids and complex carbohydrates, that might interfere with DNA extraction and PCR amplification and were therefore used to evaluate the assay performance under challenging matrix conditions [13, 14]. All matrix samples were screened prior to use to confirm the absence of walnut DNA. Since the analytical sensitivity of the assay (LOD = 100 pg) was established during the initial analytical validation, a representative contamination level (1%, w/w) was selected to specifically evaluate matrix-associated inhibitory effects under standardized experimental conditions rather than re-establish analytical sensitivity. Each mixture was thoroughly homogenized to ensure uniform distribution of walnut particles. Spiked samples were then subjected to the same DNA extraction procedure used for pure walnut samples. The extracted DNA was normalized to a uniform working concentration of 200 ng/µL, allowing for the addition of 300 ng of the template DNA into each real-time PCR reaction.

2.2. Thermal Processing

To assess the effect of heat processing on walnut DNA detectability, controlled thermal treatments were applied prior to DNA extraction on pure walnut samples. Dry heat treatment was carried out by incubating samples at increased temperatures up to 95 °C for 30 minutes. Samples were evenly distributed to ensure uniform heat exposure and allowed to cool to room temperature (RT) before analysis. For severe processing conditions, moist heat treatment was carried out by autoclaving walnut samples at 121 °C for 20 minutes to simulate industrial sterilization [15]. After cooling, DNA extraction and Real-time PCR analysis were performed under identical conditions for both processed and unprocessed samples. Targeting an ultra-short amplicon of the *Jug r 2* gene was hypothesized to preserve detectability despite heat-induced DNA fragmentation.

2.3. DNA Extraction and Quantification

Genomic DNA was extracted from 100 mg of each sample, which was ground in liquid nitrogen and transferred into sterile 2-mL microcentrifuge tubes. A total of 400 µL extraction buffer (50 mM ethylene-ediaminetetraacetic acid (EDTA) (Merck, Germany), 120 mM Tris-HCl (Merck, Germany), 1.5 M NaCl (Merck, Germany), 0.5 M sucrose (Merck, Germany), 1.5% Triton X-100 (Merck, Germany), 0.1% β-mercaptoethanol (Sigma-Aldrich, USA) and 2% cetyltrimethylammonium bromide (CTAB) (Merck, Germany) were added to the microtubes, followed by 100 µL of 1% (w/v) polyvinylpyrrolidone (PVP) (Merck, Germany), 100 µL of 1% (w/v) sodium dodecyl sulfate (SDS) (Merck, Germany) and 50 µL of 2% (w/v) sodium N-lauroyl sarcosinate (Merck, Germany). Samples were incubated at 65 °C for 45 min with intermittent inversion followed by centrifugation at 12,000× g for 15 min. The aqueous phase mixed with chloroform:isoamyl alcohol (24:1, v/v) (Merck, Germany) in a new tube and centrifuged at 13,000× g for 10 min. Then, DNA was precipitated from the aqueous phase using cold isopropanol, 5 M NaCl and 3 M ammonium acetate while incubating at -20 °C for 1 h. After centrifugation (13,000× g, 10 min), the pellet was washed twice with 70% ethanol, air-dried and the DNA pellets were resuspended in 100 µL of 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0).

To evaluate the quantity and purity of the extracted genetic material, spectrophotometric measurements were carried out using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at absorbance ratios of A_{260}/A_{280} and A_{260}/A_{230}. Then, all DNA stock solutions were meticulously normalized and diluted with sterile molecular-grade water to a uniform working concentration of 200 ng/µL to ensure consistency in later amplification steps. The quantified DNA extracts were systematically aliquoted and stored at -20 °C until further analysis.

2.4. Real-time PCR Assay and Conditions

The performance evaluation of the *Jug r 2* DNA detection was carried out using a duplex real-time PCR platform recently optimized by the current research group. To achieve maximum efficiency in screening thermal-processed samples, the primer and probe sequences were adapted from [11] (GenBank accession no. AF066055.1) to target an ultra-short 88-bp fragment between nucleotide positions 497 and 584 of the target gene. The oligonucleotide sequences used for the dual-target screening were as follows. *Jug r 2* Forward Primer: 5′-GCGCAGAGAAAGCAGAG -3′; *Jug r 2* Reverse Primer: 5′-CTCATGTCTCGACCTAATGCT -3′ and *Jug r 2* Hydrolysis Probe: 5′- FAM-ATTGTGCCTCTGTTGCTCCTCTTCCCG -BHQ -3′. The IAC system included IAC Forward Primer: 5′-TGGTGCCAGCAGCCGC -3′; IAC Reverse Primer: 5′-TCCAACTACGAGCTTTTTAACTGCA-3′ and IAC Hydrolysis Probe: 5′- HEX-CGCTATTGGAGCTGGAATTACC-BHQ -3′. While the core *Jug r 2* target sequences used this established short-amplicon architecture, the comprehensive duplex assay configuration-incorporating a plant-derived 18S rRNA IAC was implemented by the laboratory to overcome false-negative challenges in food matrix assessment. This study specifically focused on the operational robustness, matrix tolerance and commercial feasibility of this integrated diagnostic setup.

Real-time PCR assays were performed using Rotor-Gene 6000 real-time PCR system (Corbett Research, Sydney, Australia) in a total reaction volume of 20 µL, using 10 µL of 2× RealQ Plus Master Mix for Probe (Ampliqon, Odense, Denmark). Each reaction mixture was composed of 0.5 µL of each forward and reverse primers (10 pmol/µL), 0.1 µL of each TaqMan probe (10 µM) and 1.5 µL of the template DNA (standardized to 200 ng/µL, corresponding to 300 ng of DNA input per reaction), with the final volume adjusted using nuclease-free water. The optimized thermal profile was initiated with a primary denaturation and polymerase activation step at 95 °C for 5 minutes, followed by 40 amplification cycles. Each cycle consisted of a denaturation step at 95 °C for 15 seconds and a combined annealing/extension step at 60 °C for 30 seconds. To ensure diagnostic precision, all samples were analyzed in duplicate and no-template controls (NTC) were used in each run to verify the absence of reagent contamination or carry-over. The entire assay was independently replicated in three independent runs to verify method reproducibility.

Prior to amplification, the Rotor-Gene 6000 instrument was calibrated according to the manufacturer's instructions. Fluorescence signals were acquired using FAM channel for the *Jug r* 2 target and HEX channel for the IAC. Baseline and threshold settings were constant throughout all

experiments using Rotor-Gene software to ensure consistency between the assays. The HEX-labelled IAC was included in each reaction to monitor amplification performance and identify potential PCR inhibition. Since the present study was designed as a qualitative real-time PCR assay for walnut DNA detection, no Ct normalization or relative quantification was performed.

2.5. Applicability to Commercial Food Products

To evaluate the practical reliability and diagnostic performance of the optimized *Jug r 2* assay, a variety of commercial food products were purchased from local markets and analyzed. These samples were products explicitly declared to contain walnut on the ingredient label as well as walnut-free products. All commercial samples were homogenized using sterile blender to minimize the risk of cross-contamination prior to DNA extraction. Genomic DNA was extracted from each sample based on the optimized CTAB/PVP protocol described in Section 2.3. Real-time PCR analysis was carried out to verify the presence or absence of walnut DNA, comparing the results with the manufacturer's label declarations to evaluate the sensitivity of the assay to trace quantities and its specificity against potential cross-reactants in diverse commercial formulations. One commercially available product was selected for each food category (cocoa powder, cookies, instant soup and ketchup). Spiked samples were prepared at the specified concentrations and all real-time PCR reactions were in duplicate.

2.6. Statistical Analysis

All experiments were carried out in duplicate unless otherwise stated. Quantitative data were expressed as mean ±SD (standard deviation). Descriptive statistical analyses were used to evaluate assay performance, including repeatability, reproducibility and analytical sensitivity. No inferential statistical assessments were performed, as the objective of the study was analytical performance evaluation rather than hypothesis testing.

3. RESULTS AND DISCUSSION

3.1. Performance in Non-processed/Spiked Model Samples

The analytical characteristics of the duplex real-time PCR assay, including analytical sensitivity (LOD = 100 pg), amplification efficiency and specificity, were established during the initial assay validation. Therefore, the present study focused on evaluating assay performance under practical food-processing conditions rather than re-establishing its analytical characteristics. Representative amplification plots obtained at the previously determined LOD are shown in (Figure 1). Assay specificity was established through the use of a sequence-specific TaqMan probe together with analytical specificity assessment against non-target species, positive and negative controls and validation of assay performance parameters.

3.2. Performance of The Duplex Real-time PCR Assay in Complex Food Matrices

The effect of food matrix composition on the performance of the duplex real-time PCR assay was evaluated using four representative processed food matrices of cookies, cocoa, ketchup and instant soup, each spiked with 1% (w/w) of walnut. As summarized in (Table 1) and illustrated in (Figure 2), successful amplification of both the *Jug r 2* target and IAC was achieved in all tested matrices, indicating that the assay remained operational despite the presence of matrix-derived inhibitory substances. Compared with pure walnut DNA, all processed matrices showed increased Ct values for the *Jug r 2* target, reflecting various degrees of inhibition or decreased DNA recovery. The smallest Ct shift was observed in cookie samples (+1.72 cycles), whereas the largest shift occurred in the soup matrix (+4.52 cycles). Intermediate Ct shifts were recorded for cocoa (+2.26 cycles) and ketchup (+2.74 cycles). In contrast, the IAC showed only minor Ct variations (13.7–14.6), suggesting that PCR amplification remained functional throughout the assay and that complete enzymatic inhibition did not occur. These observations indicated that the delayed amplification primarily originated from matrix-associated effects on DNA extraction efficiency and template accessibility rather than direct inhibition of the DNA polymerase. Matrix-associated inhibition is a well-addressed limitation of PCR-based food analysis because processed foods frequently contain polysaccharides, lipids, polyphenols, proteins, emulsifiers and other compounds capable of reducing DNA recovery or interfering with nucleic acid amplification [16]. Similar observations have been

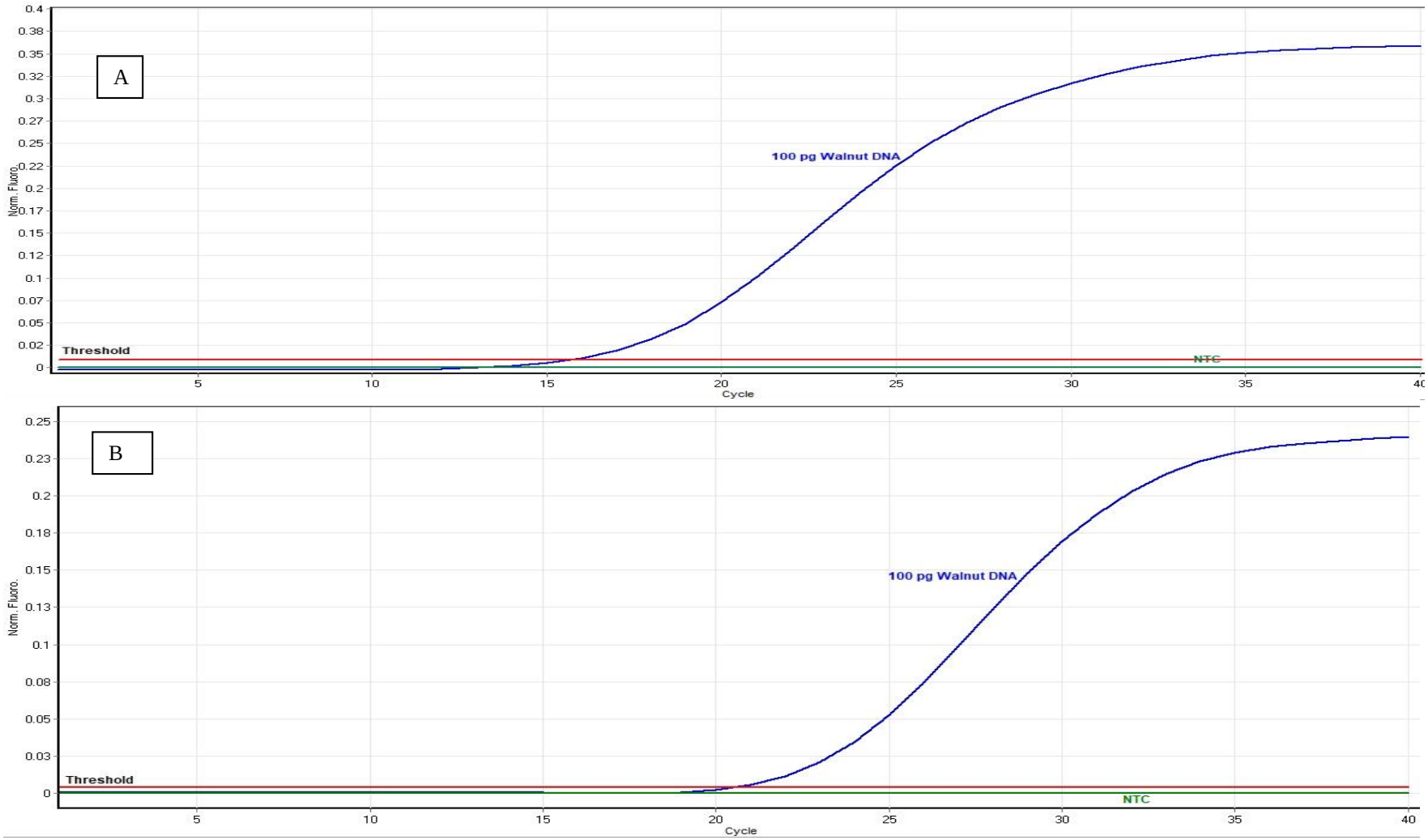

Figure 1. Representative real-time PCR amplification plots for walnut DNA detection and internal amplification control (IAC). (A) Simultaneous amplification of the internal amplification control (yellow channel/HEX) indicating the absence of PCR inhibition; and (B) detection of the *Jug r* 2 target gene (green channel/FAM) at the limit of detection (100 pg) showing a distinct amplification curve compared to the no-template control (NTC).

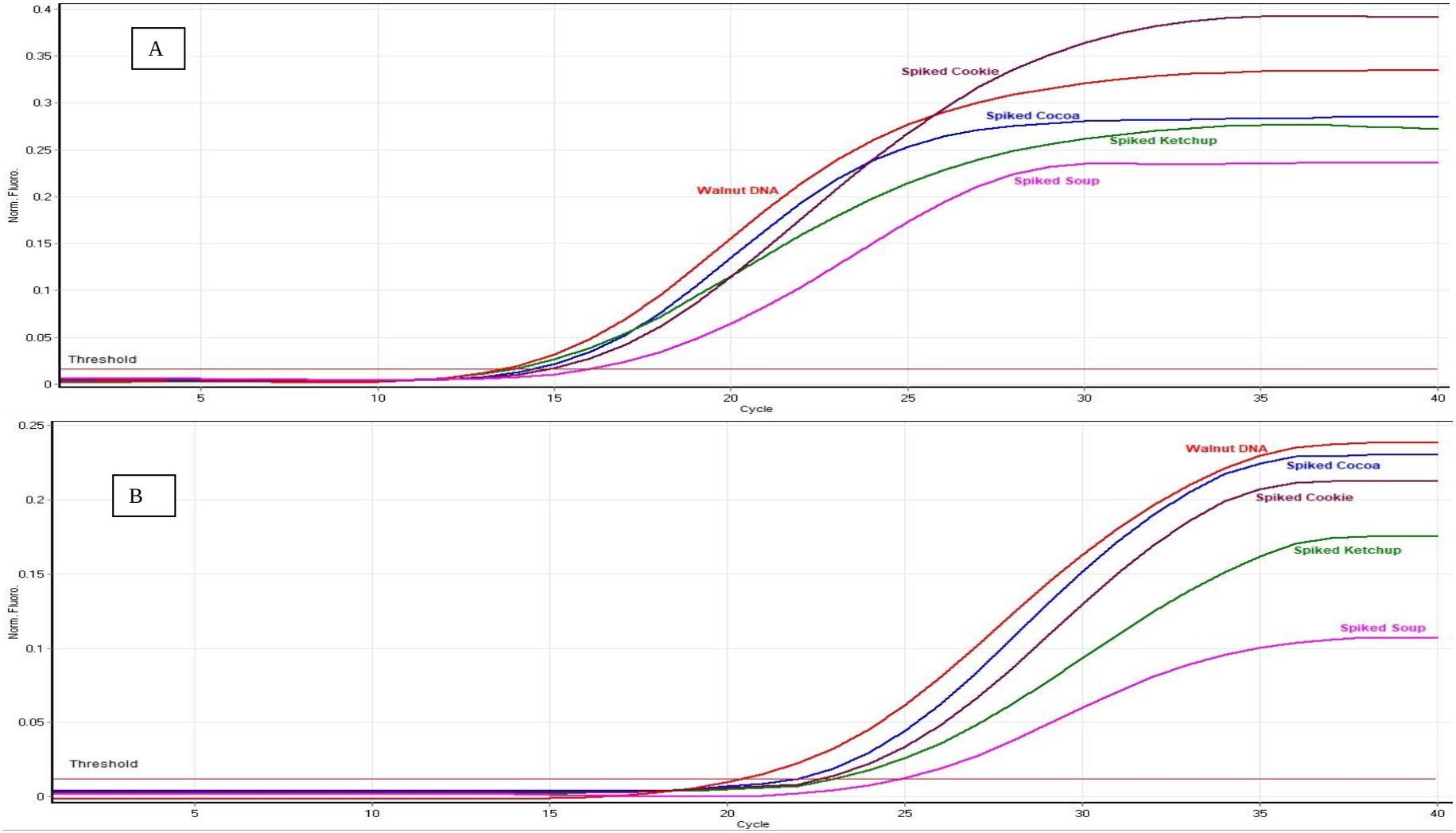

Figure 2. Effects of food matrix on the duplex qPCR assay performance. Representative amplification curves for (A) IAC and (B) Jug r 2 gene in walnut DNA (reference) and spiked complex food matrices (cookies, soup, ketchup and cocoa). The observed shifts in Ct and variations in the fluorescence intensity reflect the presence of matrix-derived inhibitors.

Table 1. Evaluation of matrix-induced inhibitory effects on the *Jug r 2* real-time PCR assay.

Matrix Type	Spiking Level	DNA input (ng/reaction)	Mean Ct ± SD (*Jug r 2*)	Mean Ct ± SD (IAC)	Ct Shift (*Jug r 2*)
Pure Walnut DNA (Control)	-	300 ng	19.31±0.15	13.2±0.10	-
Spiked Cookie	1% (w/w)	300 ng	22.03±0.32	13.7±0.18	+1.72
Spiked Cocoa	1% (w/w)	300 ng	22.57±0.28	14.0±0.25	+2.26
Spiked Ketchup	1% (w/w)	300 ng	23.05±0.17	14.2±0.22	+2.74
Spiked Soup	1% (w/w)	300 ng	24.83±0.31	14.6±0.29	+4.52

reported by Schrader et al., who demonstrated that inhibitory compounds primarily affected nucleic acid purification efficiency rather than completely suppressing PCR amplification [17]. Similarly, Janska et al. reported delayed amplification of walnut DNA in bakery products due to the presence of starch-rich ingredients and complex food components that reduced DNA accessibility [12]. Within the investigated matrices, instant soup produced the greatest Ct delay. This observation was likely explained by its heterogeneous composition, which typically included starches, hydrolyzed proteins, polysaccharides, gums and seasoning additives capable of co-precipitating with DNA during extraction [18]. Such compounds might decrease the recovery of amplifiable DNA without entirely preventing PCR amplification. The relatively small changes observed for the IAC further supported this interpretation, indicating that the extraction protocol effectively removed most inhibitory substances while preserving sufficient DNA quality for reliable amplification. The present findings highlighted the importance of integrating an internal amplification control into duplex real-time PCR assays. Unlike single-target assays, the inclusion of the plant-derived 18S rRNA IAC enabled discrimination between true negative samples and potential amplification failure caused by extraction inefficiency or matrix-derived inhibition. This feature is particularly valuable for routine analysis of processed foods, where the composition of individual products can vary significantly. Although several previous studies have reported successful PCR-based detection of walnut DNA, most analytical validations have been performed using purified DNA or relatively simple laboratory matrices [19]. In contrast, comparatively fewer investigations have systematically investigated assay performance within processed food matrices with distinct inhibitory characteristics [12, 20]. Therefore, the present study extended previous analytical validation by demonstrating that the developed duplex assay maintains reliable detectability within representative processed food formulations despite measurable matrix-dependent Ct shifts. Nevertheless, the observed Ct differences emphasized that food matrix composition could substantially affect amplification efficiency. Therefore, Ct values obtained from various food products should not be interpreted quantitatively without considering matrix-specific effects. Further studies involving naturally contaminated industrial samples, additional food categories and multiple contamination levels strengthen the applicability of the assay for routine food allergen monitoring.

3.3. Effects of Thermal Processing on Walnut DNA Detectability

Effects of thermal processing on walnut DNA detectability were evaluated by subjecting pure walnut samples to dry heating (95 °C, 30 min) and autoclaving (121 °C, 20 min) prior to DNA extraction and duplex real-time PCR analysis. As shown in (Figure 3) and (Table 2), the two thermal treatments resulted in increased Ct values for the *Jug r 2* target and IAC, indicating decreased recovery of amplifiable DNA following heat exposure. Dry heating produced a moderate Ct shift of +2.21 cycles, whereas autoclaving caused a significantly larger shift of +4.78 cycles relative to untreated walnut samples.

Table 2. Effects of thermal processing on *Jug r 2* DNA detectability in pure walnut samples.

Sample Type	Processing Condition	Observed Mean Ct ± SD (*Jug r 2*)	Observed Mean Ct ± SD (IAC)	Ct Shift (*Jug r 2*)
Raw Walnut	Unprocessed	19.50±0.25	13.2±0.10	-
Dry Heated Walnut	Mild Thermal Processing (95°C, 30 min)	21.71±0.18	14.2±0.17	+2.21
Autoclaved Walnut	Autoclaving (121°C, 20 min)	24.28±0.34	15.9±0.28	+4.78

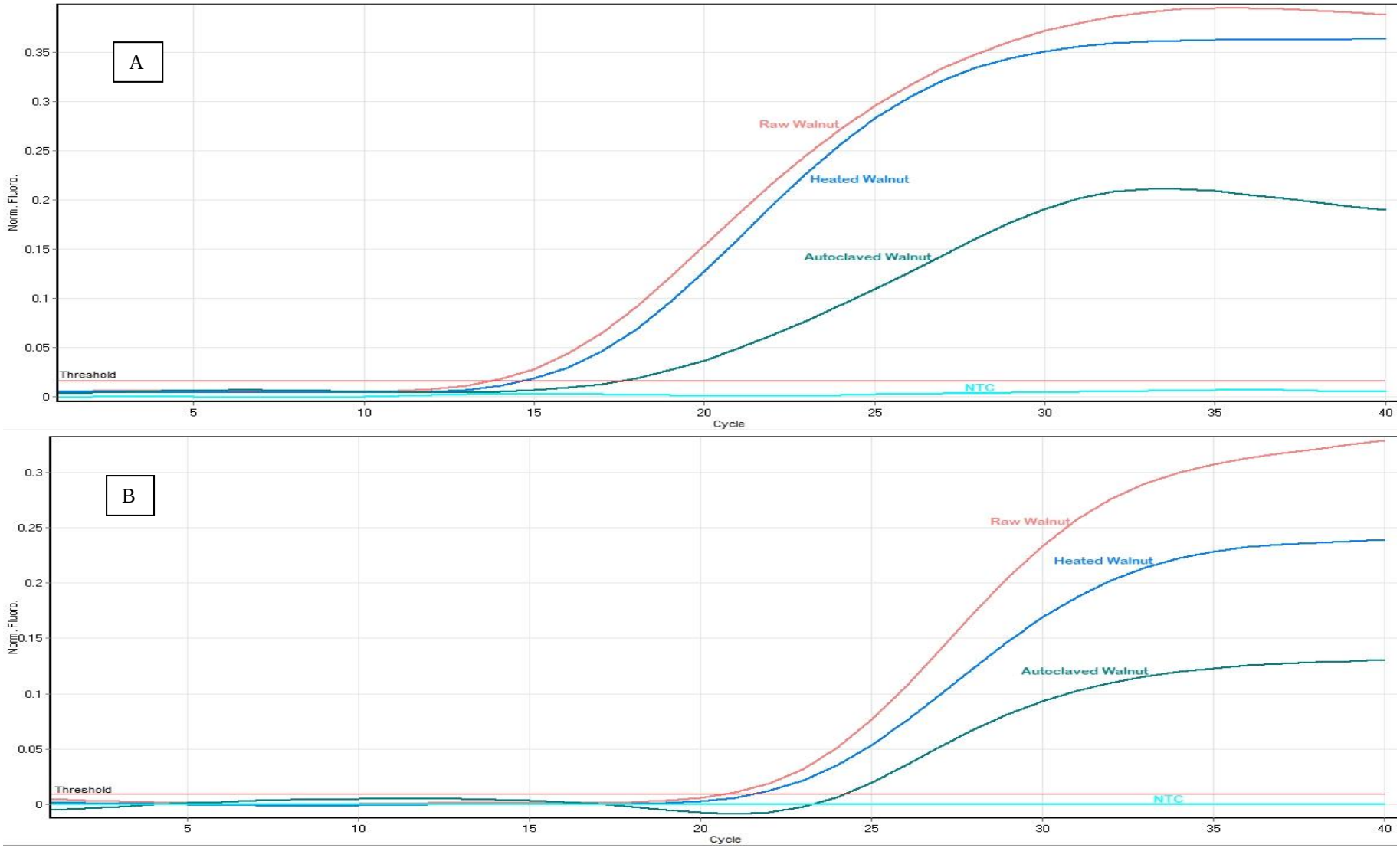

Figure 3. Effects of thermal processing on the detectability of pure walnut DNA. (A) Representative amplification curves of the IAC gene in untreated, dry heated and autoclaved walnut samples; and (B) corresponding amplification curves for the *Jug r 2* gene. The increase in Ct values and decreased fluorescence plateau in autoclaved samples reflect partial DNA fragmentation induced by high-pressure steam sterilization. NTC: No-template control.

Despite this reduction in amplifiable DNA, successful amplification was achieved in all replicates, demonstrating that the 88-bp *Jug r 2* target remained detectable even after severe thermal treatment. The preservation of amplification under autoclaving conditions highlighted appropriateness of short amplicon design for DNA-based detection in highly processed foods, where extensive fragmentation of genomic DNA is expected.

The Ct increases were consistent with the well-established effects of thermal processing on nucleic acid integrity. Increased temperature, particularly when combined with high pressure and moisture during autoclaving, accelerated hydrolytic depurination, phosphodiester bond cleavage and random fragmentation of genomic DNA; thereby reducing the number of intact template molecules available for amplification [21]. Similar processing-induced DNA degradation has been reported by Hird et al., who demonstrated substantial reductions in PCR sensitivity following heat and pressure treatments of meat products [22]. Similarly, Lopez-Andreo et al. reported that increasing processing severity progressively decreased amplifiable DNA in heat-treated foods, while short PCR targets were detectable significantly, compared to that larger amplicons were [23].

The present findings further supported the widely accepted concept that amplicon length was one of the most critical factors affecting PCR performance in processed foods. Previous studies have consistently shown that assays targeting fragments shorter than approximately 100 bp outperform assays using longer targets after intensive thermal treatment because short DNA fragments are further intact despite extensive degradation [24]. The reliable amplification of the 88-bp *Jug r 2* fragment observed in this study was therefore in agreement with these reports, demonstrating practical advantages of selecting short genomic targets for allergen monitoring in processed food products.

Nevertheless, not all published studies have reported similar levels of post-processing detectability. Several investigations have suggested that severe sterilization or repeated heat treatments may decrease DNA integrity to such an extent that reliable PCR detection becomes difficult, particularly when larger amplicons or low-copy genomic targets are used [22, 25, 26]. These conflicting observations are likely attributed to differences in food composition, extraction protocols, target sequence selection and amplicon size rather than to inherent limitations of PCR technology itself. The relatively stable performance observed in the present study probably reflected combined benefits of efficient CTAB-based DNA extraction and the use of an ultra-short amplification target that enhanced the probability of recovering amplifiable DNA from extensively processed samples.

From a practical perspective, these findings are particularly relevant because industrial food manufacturing frequently involves thermal treatments such as baking, sterilization, pasteurization, extrusion and retort processing. Under such conditions, protein-based allergen detection methods may experience decreased sensitivity owing to protein denaturation, aggregation or epitope masking during Maillard reactions [27]. In contrast, DNA-based assays can detect species-specific genomic fragments provided that sufficiently short target regions are still intact. Therefore, the duplex real-time PCR assay evaluated in this study may serve as a valuable complementary approach to immunological methods for verifying the presence of walnut-derived material in highly processed food products.

Although successful amplification was carried out after autoclaving, the Ct shift approaching five cycles indicated a significant reduction in amplifiable DNA and demonstrated that thermal processing could significantly affect analytical sensitivity. Therefore, Ct values obtained from processed and unprocessed samples should not be interpreted interchangeably, particularly in semi-quantitative applications. Further investigations involving additional processing conditions, repeated heating cycles and naturally processed industrial samples improve understanding of the relationships between food processing and walnut DNA detectability.

3.4. Applicability of The Duplex Real-time PCR Assay in Commercial Food Products

To evaluate the practical applicability of the developed duplex real-time PCR assay, seven commercially available food products representing various formulations were analyzed for the presence of walnut-derived DNA. As summarized in (Table 3), the assay successfully identified walnut DNA in all products that declared walnut as an ingredient, while no amplification was observed in products without declared walnut, with the exception of one hazelnut wafer sample that generated a weak positive

signal. The positive amplification obtained from the hazelnut wafer, despite the absence of walnut on the product label, should be interpreted cautiously. Several explanations might account for this observation, including low-level cross contacts during manufacturing, the use of shared production lines, contamination of raw materials, or the presence of undeclared walnut ingredients. Similar findings have been reported in previous market surveillance studies, where molecular assays occasionally detected allergenic species not listed on product labels, highlighting the challenges associated with allergen management in complex food production systems [20]. However, because verification analyses such as sequencing or independent extraction replicates were not carried out in the present study, the exact origin of the detected walnut DNA could not conclusively be addressed. Therefore, the result should be regarded as evidence of walnut DNA detection rather than definitive proof of undeclared walnut incorporation.

Table 3. Evaluation of the *Jug r 2* real-time PCR assay on commercial food products.

Product Category	Product Description	Declared Walnut (Label)	Real-Time PCR Result (*Jug r 2*)	IAC Result
Bakery & Confectionery	Walnut Cookie	Positive	Detected	Amplified
	Chocolate Cake	Negative	Not Detected	Amplified
	Hazelnut Wafer	Negative	Detected (Trace)*	Amplified
Sauces & Soups	Pesto Sauce	Positive	Detected	Amplified
	Instant Mushroom Soup	Negative	Not Detected	Amplified
Snacks & Bars	Mixed Nut Bar	Positive	Detected	Amplified
	Granola Bar	Precautionary (May contain)	Not Detected	Amplified

***** Trace detection corresponds to amplification at a late cycle (Ct > 35), indicating potential trace cross-contamination during commercial manufacturing.

The successful detection of walnut DNA in commercially processed foods demonstrated that the assay remains applicable within products differing substantially in composition and manufacturing processes. Commercial foods generally represent significantly more challenging analytical targets than that laboratory-prepared model samples do because they contain multiple ingredients, undergo diverse processing conditions and may include additives capable of affecting DNA extraction and amplification efficiency. The reliable amplification achieved in the present study therefore supports the robustness of the duplex assay under conditions representative of routine food analysis.

These findings were similar to those of previous studies, demonstrating the utility of real-time PCR for detecting allergenic plant species in processed commercial foods. Several studies have shown that species-specific DNA targets remain detectable even when extensive food processing has decreased protein integrity, making PCR-based approaches valuable complementary tools to immunechemical methods such as ELISA [10, 28]. In particular, DNA-based methods may provide improved analytical performance when allergenic proteins have been denatured, aggregated or chemically modified during food processing, conditions that can decrease antibody recognition and lead to false-negative immunoassay results [29]. Nevertheless, PCR detects species-specific DNA rather than allergenic proteins; therefore, positive amplification verifies presence of walnut-derived genetic material but does not directly indicate the quantity or immunological activity of walnut allergens. Hence, PCR and immunelogical methods should be addressed as complementary analytical approaches rather than interchangeable techniques for allergen monitoring.

Although the assay demonstrated excellent qualitative agreement with declared product labels for the majority of tested samples, the limited number of commercial products evaluated represented an important limitation of the present study. Broader validation, involving a larger variety of food categories, manufacturers, production batches and naturally contaminated samples, can provide stronger evidence regarding the assay performance under routine industrial conditions.

Overall, the analysis of commercial food products confirm that the developed duplex real-time PCR assay is appropriate for qualitative detection of walnut-derived DNA in processed foods and supports its potential application in food quality control, allergen surveillance and verification of ingredient labeling. The results further demonstrate that combining an ultra-short *Jug r 2* target with an internal amplification control provides a reliable strategy for molecular monitoring of walnut in com-

mercially manufactured food products, while highlighting the importance of interpreting unexpected positive findings within the broader context of manufacturing practices and analytical limitations.

4. CONCLUSION

This study evaluated the performance of a duplex TaqMan real-time PCR assay targeting an ultra-short *Jug r2* fragment for the detection of walnut-derived DNA in processed and commercial food products. Despite matrix-dependent Ct shifts and DNA degradation caused by thermal processing, the assay consistently detected walnut DNA within all tested matrices and heat-treated samples. The inclusion of an internal amplification control enhanced assay reliability by enabling the monitoring of amplification performance and decreasing the risk of false-negative results.

Analysis of commercial food products further demonstrated the applicability of the assay for the qualitative detection of walnut-derived DNA in complex food matrices, highlighting its potential utility for food allergen surveillance, ingredient verification and quality control. However, the observed effects of food matrix composition and processing conditions highlight the importance of careful interpretation of Ct values, particularly when analyzing highly processed food products.

Although the present findings support the potential applicability of the assay for routine food analysis, broader validation involving a wider range of commercial food products, naturally contaminated samples and inter-laboratory assessments is warranted before its widespread implementation in regulatory assessment. Such studies further strengthen confidence in the assay robustness and applicability under diverse analytical conditions.

In addition to walnut detection, this study has highlighted the growing importance of robust molecular analytical tools for improving food safety in increasingly complex food production and distribution systems. The performance evaluation strategy may provide a practical framework for assessing molecular assays targeting other priority food allergens; thereby, contributing to the continuously advancement of food allergen surveillance, regulatory compliance and consumer protection worldwide.

5. DECLARATION STATEMENTS

5.1. Acknowledgment

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5.3. Conflict of Interest

The authors declare no known competing financial interests or personal relationships that could have appeared to affect the study.

5.4. Data Sharing

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable requests.

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ارزیابی عملکرد یک آزمون واکنش زنجیره‌ای پلی مراز ریل تایم هدف‌گیرنده *Jug r 2* در ماتریس‌های غذایی فرآوری شده و تجاری: مدیریت ریسک آلرژن

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چکیده

سابقه و هدف: گردو یکی از مهم‌ترین آلرژن‌های گروه مغزهای درختی محسوب می‌شود و شناسایی قابل‌اعتماد آن در مواد غذایی فرآوری‌شده برای مدیریت خطر آلرژن و رعایت الزامات نظارتی ضروری است. اگرچه روش‌های متعددی مبتنی بر واکنش زنجیره‌ای پلیمراز (PCR) برای شناسایی گردو توسعه یافته‌اند، اطلاعات مربوط به عملکرد این روش‌ها در ماتریس‌های غذایی پیچیده و مواد غذایی فرآوری‌شده همچنان محدود است. هدف از این مطالعه، ارزیابی عملکرد یک آزمون duplex TaqMan real-time PCR با هدف‌گیری قطعه بسیار کوتاهی از ژن *Jug r 2* برای شناسایی DNA مشتق از گردو در محصولات غذایی فرآوری‌شده و تجاری بود.

مواد و روش ها: عملکرد آزمون با استفاده از ماتریس‌های غذایی فرآوری‌شده شامل کوکی، پودر کاکائو، کچاپ و سوپ، که هر یک با گردو اسپایک شده بودند، ارزیابی شد. اثر فرآوری حرارتی پس از حرارت‌دهی خشک (۹۵ درجه سانتی‌گراد به مدت ۳۰ دقیقه) و اتوکلاو (۱۲۱ درجه سانتی‌گراد به مدت ۲۰ دقیقه) بررسی شد. علاوه بر این، هفت محصول غذایی تجاری موجود در بازار برای ارزیابی کاربردپذیری عملی آزمون مورد بررسی قرار گرفتند. از کنترل داخلی تکثیر مبتنی بر ژن 18S rRNA گیاهی برای پایش عملکرد تکثیر و شناسایی مهار احتمالی PCR استفاده شد.

یافته ها و نتیجه‌گیری: DNA مشتق از گردو با موفقیت در تمامی ماتریس‌های غذایی فرآوری‌شده و نمونه‌های تحت فرآوری حرارتی شناسایی شد. میزان مهار مرتبط با ماتریس در انواع مختلف مواد غذایی متفاوت بود و تغییرات Ct از ۱٫۷۲+ سیکل در کوکی تا ۴٫۵۲+ سیکل در سوپ مشاهده شد، در حالی که کنترل داخلی تکثیر پایدار بود (Ct: ۱۳/۷-۱۴/۶) که نشان‌دهنده تکثیر قابل‌اعتماد بود. فرآوری حرارتی به‌صورت وابسته به نوع تیمار موجب کاهش قابلیت شناسایی DNA شد؛ به‌طوری‌که افزایش Ct به‌ترتیب ۲٫۲۱+ و ۴٫۷۸+ سیکل پس از حرارت‌دهی خشک و اتوکلاو مشاهده شد. بررسی هفت محصول غذایی تجاری، تطابق کامل نتایج را با محصولات حاوی گردوی اعلام شده در برچسب نشان داد؛ در حالی که یک نمونه ویفر فندق فاقد اعلام گردو، سیگنال تکثیر ضعیف مثبت ایجاد کرد. آزمون duplex TaqMan real-time PCR توسعه‌یافته، شناسایی کیفی قابل‌اعتماد DNA مشتق از گردو را در محصولات غذایی فرآوری شده و تجاری نشان داد. این آزمون می‌تواند به‌عنوان یک ابزار مولکولی مکمل و قابل‌اتکا برای پایش آلرژن‌های غذایی، تأیید ترکیبات و کنترل کیفیت مورد استفاده قرار گیرد. با این حال، انجام اعتبارسنجی گسترده‌تر با استفاده از ماتریس‌های غذایی بیشتر و مطالعات بین‌آزمایشگاهی توصیه می‌شود.

واژگان کلیدی: آلرژن گردو؛ *Juglans regia*؛ غذاهای فراوری شده؛ Real-time PCR؛ ایمنی غذا