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Research Article

Method development and validation for detection of *bar* gene encoding phosphinothricin N-acetyl transferase in milk products using real-time PCR technique

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Abstract

Transferring herbicide resistance genes to crop varieties is one of the most effective methods to control weed growth on agricultural land. The bar gene isolated from *Streptomyces hygroscopicus* was found to confer glufosinate resistance to crops. This gene encodes the enzyme phosphinothricin N-acetyltransferase (PAT), which is capable of inactivating phosphinothricin-containing herbicides (PPT) by promoting the acetylation of glufosinate ammonium, the main active ingredient in many herbicides. The most common genetically modified crops are soybeans, corn, rice, and cotton. Soybeans and corn are also the main crops used in processed foods. In this study, the authors applied a standardized real-time PCR method issued by the European Commission to detect the bar gene in milk products, including soy milk, corn milk, and fresh milk. The method is based on the amplification of the *bar* gene, showing high sensitivity with a detection limit of 0.05%, while achieving 100% specificity and accuracy.

Keywords: *expansion ratio, hardness, oil absorption, taro snack, weight loss.*

1. INTRODUCTION

Weeds are among the primary biotic factors adversely affecting agricultural productivity [1, 2]. Chemical herbicide application is the most cost-effective and efficient weed management strategy, capable of reducing both crop yield losses and labor costs [3, 4]. However, most chemical herbicides are selective and only control the growth of certain weed species. Moreover, selective herbicides can have phytotoxic effects on crops, potentially reducing crop yields. With the advent of modern biotechnology, genetically modified (GM) crops have become a promising solution to these challenges. Herbicide-tolerant GM crops not only enhance weed control efficiency but also contribute to soil and water conservation and mitigate greenhouse gas emissions by reducing the need for tillage [5]. Therefore, the cultivation of genetically modified (GM) crops with herbicide resistance offers a promising solution to weed management in agricultural lands. Among herbicide resistance traits, tolerance to glufosinate is one of the most widely adopted. To date, eight GM crop varieties with glufosinate resistance have been approved for commercial use [6]. All of these are engineered to express the enzyme phosphinothricin N-acetyltransferase (PAT), which acetylates to detoxify L-phosphinothricin (L-PPT), the active ingredient in glufosinate-based herbicides [7]. Two glufosinate-resistance genes used commercially include bar and pat, which were isolated from *Streptomyces hygroscopicus* and *Streptomyces viridochromogenes* in 1987 and 1988, respectively [8 - 10]. The number and diversity of GM crops, as well as their cultivated areas, have steadily increased worldwide [11]. As of 2018, the global cultivation area of GM crops reached 191.7 million hectares. Soybean, maize, cotton, and canola accounted for 99% of this total, with GM variants comprising 78% of soybean, 30% of maize, 76% of cotton, and 29% of canola acreage. In Vietnam, by 2020, six GM crop species with 39 approved transformation events had been commercialized,

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including 17 maize events, 13 soybeans, 1 sugar beet, 4 canola, 2 cotton, and 2 alfalfa events. By 2017, more than 45,000 hectares of GM maize had been cultivated across various provinces [12]. Among these, soybean and maize are not only widely cultivated but also serve as primary ingredients in processed food, particularly in Asia [13]. Soybeans are consumed in diverse forms such as tofu, soy sauce, soybean oil, soy protein, flour, noodles, and soy milk [14]. The rising prevalence of GM crops-derived foods has raised increasing public concerns regarding biosafety and potential health risks. Consequently, many countries have implemented regulatory frameworks mandating GMO labelling for transparency and consumer protection [15]. Detection and labelling of GM content in processed foods are thus among the most pressing consumer issues related to food safety and quality [16], and they play an essential role in raising public awareness [17]. To support regulatory compliance and consumer protection, reliable screening methods for GMO detection are required. In the past decade, real-time PCR (quantitative PCR) using specific probes has emerged as a leading approach for the identification of GMO-related genes, offering high specificity, sensitivity, and simplicity [18]. In this study, a real-time PCR assay was developed and validated to target the *bar* gene encoding phosphinothricin N-acetyltransferase, in order to detect the presence of food products potentially derived from genetically modified sources.

2. MATERIALS AND METHODS

2.1. Testing samples

The study was conducted using certified reference materials, milk matrices, and randomly collected samples of three commercial milk products: corn milk, soy milk, and fresh cow's milk, obtained from various retail stores in Hanoi, Vietnam.

2.2. Reagents and Reference Materials

2.2.1. Reagents

TaqMan Universal PCR Master Mix (2X) (Thermo Fisher Scientific, USA), DNA extraction kit: TopPURE FOOD DNA Extraction Kit (Vietnam), Primers and probe (Integrated DNA Technologies, IDT), Real-time PCR optical strips (Thermo Fisher Scientific), Powder-free latex gloves (Vietnam).

2.2.2. Reference materials

Certified reference materials (CRMs) used in this study included European reference standards ERM BCR and IRMM, provided by the Directorate-General Joint Research Centre (JRC), consisting of: Bt176 maize (code BF 411f), MON 863 maize (code BF 416dk), GA21 maize (code BF 414fk), Bt11 maize (code BF 412e), GTS 40-3-2 soybean (code BF 410gk), NK603 maize (code BF 415f).

Negative control samples (e.g., food additives, rice, soybean oil, raw food ingredients, dietary supplements, corn milk, soy milk) were pre-validated to be free from genetically modified organisms (GMOs) using real-time PCR targeting the 35S promoter. These samples are maintained at the National Institute for Food Safety and Hygiene and are labelled with the following codes: 261; 445; 043; 851; 913; 446; 755; 876; 500; 685.

2.3. Equipment

Samples were homogenized, and genomic DNA was extracted and purified using the TopPURE FOOD DNA Extraction Kit (Vietnam) following the manufacturer's guidance. DNA concentration and purity were assessed spectrophotometrically using the NanoDrop 1000 at absorbance wavelengths of 260 nm (A260) and 280 nm (A280). DNA purity was confirmed by the A260/A280 ratio, which ranged between 1.8 and 2.2. DNA concentrations varied from 6.24 ng/µL to 96.67 ng/µL, indicating suitability for PCR amplification.

2.4. Methods

2.4.1. Genomic DNA Extraction

Real-time PCR system: QuantStudio 6 Flex (Thermo Fisher Scientific), DNA/protein quantification spectrophotometer: NanoDrop 1000 (Thermo Fisher Scientific), Refrigerated microcentrifuge: Mikro 200 (Hettich, Germany), Cell disruptor: Labnet, Vortex mixer: IKA, Spindown centrifuge: GeneReach, Pipettes and tips (Eppendorf, Germany): 1-10 µL, 20-200 µL, 100-1000 µL, additional standard laboratory equipment.

2.4.2. Real-time PCR amplification

Primers were referred to the *bar* gene sequence from *S.hygroscopicus*, producing a 60 bp amplicon as described by Grohmann et al., 2009 (JRC) [19], [20]. Primer sequences are listed in **Table 1**, and the PCR reaction components and thermal cycling conditions are detailed in **Table 2**.

Table 1. *Primer and probe sequences specific to the bar gene*

Primer sequences	***Amplicon size***	***Target gene***	***References***
Forward primer (*bar*-F): 5'-ACA AGC ACG GTC AAC TTC C-3' Reverse primer (*bar*-R): 5'-GAG GTC GTC CGT CCA CTC-3' Probe (*bar*-P): 5'-FAM-TAC CGA GCC GCA GGA ACC-TAMRA-3'	60 bp	*bar*	[19], [20]

Table 2. *PCR reaction components and thermal cycling conditions*

Component	TaqMan Universal PCR master mix, 2X	1X
	Teamplate DNA (100- 200 ng)	100-200 ng
	Forward primer (10 µM)	0.14 µM
	Reverse primer (10 µM)	0.14 µM
	Probe (*bar*- P, 10 µM)	0.1 µM
	Nuclease-free water	25 µL
Thermal Cycling Protocol		
Step 1	Initial denaturation	600 seconds at 95°C
Step 2	45 subsequent cycles: Denaturation Annealing/Extension	 15 seconds at 95°C 60 seconds at 60°C

2.4.3. Method validation

The performance of the real-time PCR assay was validated based on standard analytical parameters, including limit of detection (LOD), accuracy (AC ≥ 90%), specificity (SP ≥ 90%), and sensitivity (SE ≥ 90%), according to previously established criteria [21, 22].

The following formulas were used for evaluation:

$$AC = \frac{TP + TN}{N} \times 100$$

$$SE = \frac{TP}{TP + FN} \times 100$$

$$SP = \frac{TN}{TN + FP} \times 100$$

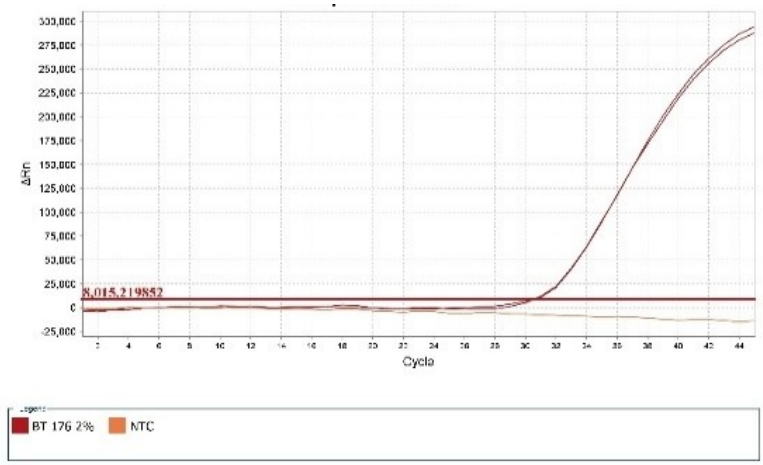
AC: Accuracy
SE: Sensitivity
SP: Specificity
TP: True positive
TN: True negative
FP: False positive
FN: False negative
N: Total Samples Analyzed

3. RESULTS AND DISCUSSION

3.1. Amplification of the *bar* gene

The certified reference material Bt176 (code BF 411e-2% GMO), extracted according to Section 2.4.1, was used along with a specific probe to examine the amplification of the *bar* gene. The results are presented in **Figure 1**.

a)



b)

Sample ...	Target ...	Task	Dyes	Ct	Ct Mean
NTC	Target 1	UNKNOWN	FAM-None	Undetermi...	
BT 176 2%	Target 1	UNKNOWN	FAM-None	31.916	31.939
BT 176 2%	Target 1	UNKNOWN	FAM-None	31.962	31.939

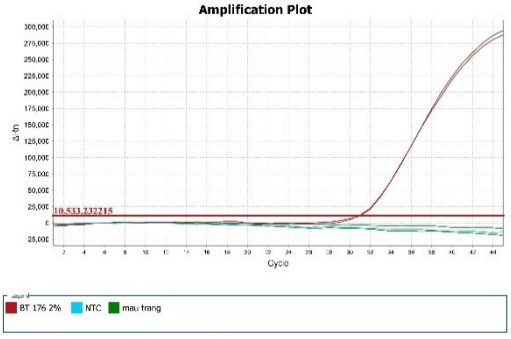
***Figure 1**. Amplification curve (a) and corresponding Ct value (b) for certified reference material Bt176*

Based on the real-time PCR results, the fluorescence amplification plot for the reference material Bt176 exhibited a typical sigmoidal curve (**Figure 1a**), indicating successful amplification of the *bar* gene. The corresponding threshold cycle (Ct) value was approximately 31.9 (**Figure 1b**).

3.2. Testing of negative control samples

A maize kernel sample collected from the laboratory was extracted according to Section 2.4.1. The extracted DNA was subjected to amplification using a specific probe and primer pair targeting the *bar* gene. The results are presented in **Figure 2**.

a)



b)

Sample ...	Target ...	Task	Dyes	Ct	Ct Mean
NTC	Target 1	UNKNOWN	FAM-None	Undetermi...	
BT 176 2%	Target 1	UNKNOWN	FAM-None	31.916	31.939
BT 176 2%	Target 1	UNKNOWN	FAM-None	31.962	31.939
mau trang	Target 1	UNKNOWN	FAM-None	Undetermi...	
mau trang	Target 1	UNKNOWN	FAM-None	Undetermi...	

***Figure 2.** Amplification curves (a) and Ct values (b) of the reference material Bt176 and the non-transgenic maize sample (negative control).*

Based on the results shown in **Figure 2a** and **2b**, the non-transgenic maize sample (negative control) did not exhibit any typical amplification curve, and the Ct value remained below the baseline threshold.

3.3. Limit of Detection (LOD) detection

The limit of detection (LOD) is defined as the lowest GMO concentration at which at least 90% of the tested samples yield a positive result. To determine the LOD, different GMO concentrations were tested: 2%, 0.2%, 0.05%, 0.02%, and 0.01%. These concentrations were used as templates in real-time PCR reactions employing the specific primer-probe set targeting the *bar* gene. The results are presented in **Figure 3** and **Table 3**.

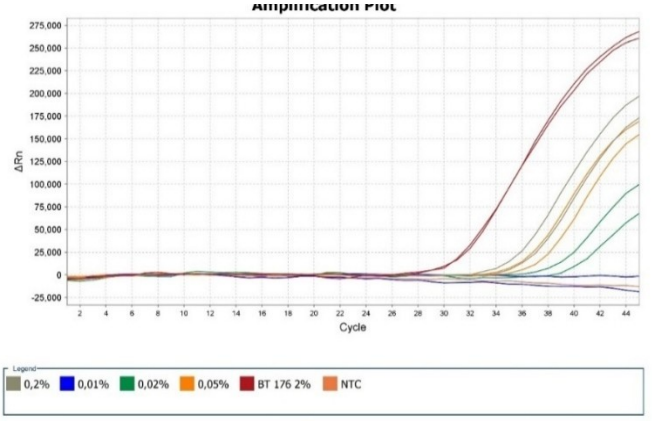
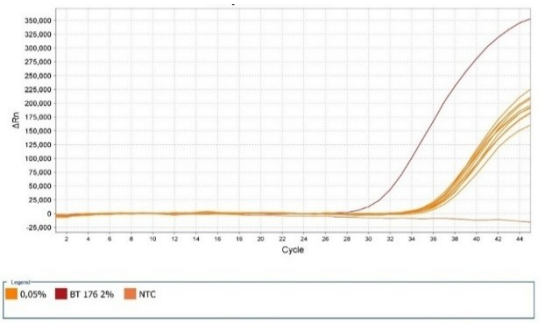


***Figure 3**. Amplification curves of samples containing different concentrations of Bt176 (2%-0.01%)*

***Table 3.** Cycle threshold (Ct) values of samples with varying Bt176 concentrations (2%-0.01%)*

No.	Sample ID	GMO content (%)	Ct Value (Ct)		Mean Ct value
			Trial 1	Trial 2	
1	B1	2%	29,04	29,25	29,14
2	B2	0,2%	33,55	34,88	34,21
3	B3	0,05%	34,60	35,96	35,28
4	B4	0,02%	37,56	39,47	38,52
5	B5	0,01%	NA	NA	
6	B6	0% (NTC)	NA		

The results from **Figure 3** and **Table 3** show that at the concentration of 0.02%, the Ct value was approximately 38.52. Therefore, 0.02% was initially predicted to be the lowest concentration at which the target gene could be detected. To confirm the limit of detection, the real-time PCR assay was repeated 10 times using a 0.02% GMO concentration. However, the number of positive detections at this concentration did not reach the required threshold of ≥90%. Consequently, the experiment was repeated 10 times at a concentration of 0.05%. The results are presented in **Figure 4**.



a

Sample ...	Target ...	Task	Dyes	Ct	Ct Mean	C
0,05%	Target 1	UNKNOWN	FAM-None	35.497	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	36.286	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	35.018	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	35.698	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	35.213	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	34.895	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	35.336	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	35.107	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	35.811	35.379	
0,05%	Target 1	UNKNOWN	FAM-None	34.929	35.379	
BT 176 2%	Target 1	UNKNOWN	FAM-None	29.626	29.626	
NTC	Target 1	UNKNOWN	FAM-None	Undetermi...		

b

***Figure 4**. Amplification curves (a) and Ct values (b) from 10 replicate reactions using the sample containing 0.05% of the bar gene*

The results in **Figure 4** show that the detection rate at the 0.05% target gene concentration was 100%. Therefore, the limit of detection (LOD) of the method is determined to be 0.05%.

3.4. Accuracy (AC), specificity (SP), and sensitivity (SE) detection

Real-time PCR reactions using the primer-probe set of the *bar* gene were performed on a total of 20 DNA samples, consisting of 10 negative samples (Group 1), which were confirmed to be free of the target gene: NK603, MON863, GA21, Bt11, GTS 40-3-2, 755, 500, 445, 043, and 261. Ten positive samples (Group 2), including 1 certified reference material Bt176 and 9 spiked samples prepared by adding Bt176 DNA (at concentrations of 2%, 1%, and 0.2%) into the 10 negative matrices from Group 1. These spiked samples were labeled: SP 603, SP 863, SP 21, SP 11, SP 40-2-3, SP 755, SP 500, SP 445, SP 043, and SP 176.

The amplification curves of both groups are shown in **Figure 5**, and the corresponding results are summarized in **Table 4**.

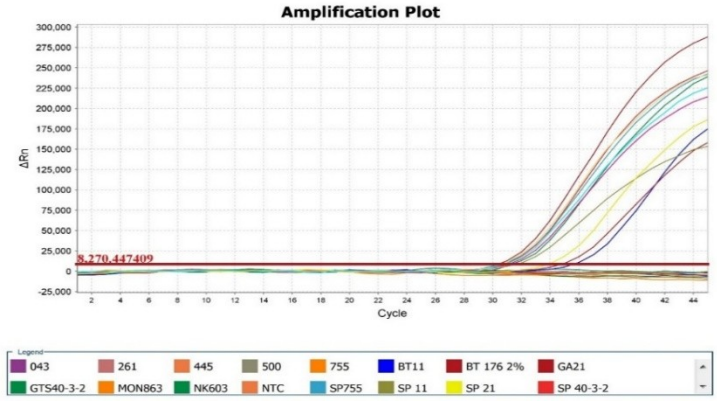


Figure 5. *Typical amplification curves of Group 1 and Group 2*

Table 4. *Cycle threshold (Ct) values of Group 1 and Group 2*

No.	Sample ID	Ct value (Ct)
Group 1: DNA samples negative for the bar gene		
1	NK603	NA
2	MON863	NA
3	500	NA
4	GA21	NA
5	Bt11	NA
6	GTS40-3-2	NA
7	755	NA
8	445	NA
9	043	NA
10	261	NA
Group 2: DNA samples positive for the bar gene		
11	SP603	30.84
12	SP863	30.56
13	SP21	33.82
14	SP11	31.63
15	SP40-3-2	30.80
16	SP755	30.58
17	SP500	31.02
18	SP445	34.77
19	SP043	35.06
20	SP261	31.44
21	NTC	NA
22	Positive control BT176 2%	30,41

The results presented in **Figure 5** and **Table 4** show that: for the group of DNA samples positive for the target gene (Group 2), all 10 samples that were spiked with Bt176 into different non-GMO matrices exhibited typical amplification curves. In contrast, for the group of DNA samples negative for the target gene (Group 1), none of the 10 samples showed a typical amplification signal. These findings confirm that the real-time PCR method, using a specific primer–probe set targeting the *bar* gene, is effective for detecting the presence of this transgene. The performance parameters, including accuracy (AC), specificity (SP), and sensitivity (SE), as calculated according to the formulas described in Section 2.4.3, were all determined to be 100%.

3.5. Limit of Detection (LOD) in corn milk matrix spiked with *bar* gene

Genomic DNA extracted from a bar-negative corn milk sample was spiked with the *bar* gene from the reference material to obtain various GMO concentrations. The method was evaluated at the following levels: 2%, 0.2%, 0.05%, 0.02%, and 0.01%. The results are presented in **Figure 6** and **Table 5**.

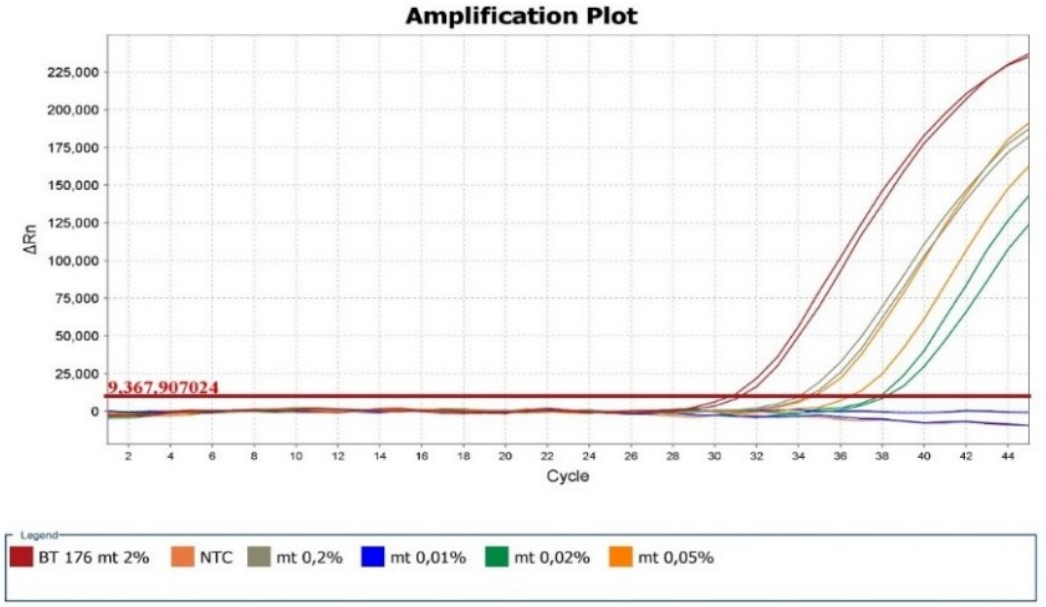
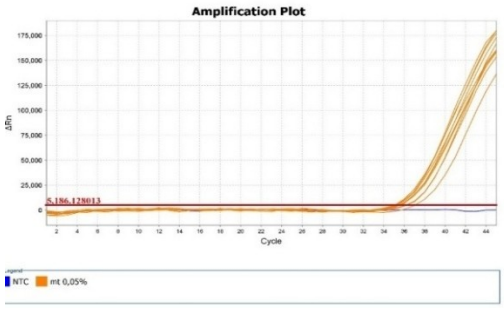


Figure 6. *Amplification curves in corn milk matrix spiked with different concentrations of reference material Bt176 (2%-0.01%)*

Table 5*. Cycle threshold (Ct) values in corn milk matrix spiked with concentrations of reference material Bt176 (2%-0.01%)*

No.	Sample ID	GMO content (%)	Ct value (Ct)		Mean Ct value
			Trial 1	Trial 2	
1	Bmt1	2%	31.19	30.65	30.92
2	Bmt2	0.2%	33.92	34.37	34.14
3	Bmt3	0.05%	36.43	34.65	35.54
4	Bmt4	0.02%	38.11	37.73	37.92
5	Bmt5	0.01%	NA	NA	
6	Bmt6	0% (NTC)	NA		

The results from **Figure 6** and **Table 5** show that at the 0.02% concentration level, the Ct value was approximately 37.92. Therefore, the limit of detection (LOD) was determined to be 0.05% of the content. To confirm the LOD, genomic DNA extracted from *bar*-negative corn milk was spiked with *bar* from reference material to a final concentration of 0.05% GMO and tested in 10 replicates. The results are shown in **Figure 7**. All 10 replicates yielded positive amplification, indicating a 100% detection rate in the corn milk matrix at this concentration.



a

Sample ...	Target ...	Task	Dyes	Ct	Ct Mean
mt 0,05%	Target 1	UNKNOWN	FAM-None	35.534	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	35.503	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	35.392	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	37.124	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	35.230	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	35.593	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	36.322	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	35.131	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	35.160	35.730
mt 0,05%	Target 1	UNKNOWN	FAM-None	36.313	35.730
NTC	Target 1	UNKNOWN	FAM-None	Undetermi...	

b

***Figure 7**. Amplification curves (a) and Ct values (b) from 10 replicate reactions in corn milk matrix spiked with reference material Bt176 at 0.05% GMO concentration*

3.5. Practical analysis

An assessment was conducted to determine the presence or absence of the target gene in commercially available milk products collected from the Hanoi market. A total of 24 samples were randomly collected from local stores, including 10 corn milk samples, 9 soy milk samples, and 5 fresh milk samples. The results are presented in **Table 6**.

***Table 6**. Summary of collected corn milk, soy milk, and fresh milk samples from the local market*

No.	Sample ID	Code	Main Matrix Composition
1	Young corn milk	11500	Water, young corn (100 g/L), sugar, milk powder (28 g/L), vegetable fat, maltodextrin, soluble fiber, stabilizers, artificial flavoring, colorant, salt, vitamins.
2	Corn milk	26021	Not disclosed
3	Corn milk	26022	Not disclosed
4	Corn milk	26023	Not disclosed
5	Soy milk	26024	Not disclosed
6	Soy milk	26025	Not disclosed
7	Corn milk	04031	Not disclosed
8	Corn milk	04032	Not disclosed
9	Mixed nut milk (9 types of nuts)	04033	Nut milk (94.4%) [extracted from crushed almonds, whole soybeans, water, vegetable oil, ground peanuts, red bean extract, oat flour, walnut extract, black/red/green/white beans], sugar (4.7%), emulsifier, minerals, artificial flavoring, salt, vitamins, acidity regulators.
10	Corn milk	04034	Not disclosed
11	Soy–red bean milk	04035	Extracted from whole soybeans (63.8%), water, sugar (6%), red bean extract (16.6 g/L), vegetable oil, emulsifier, coconut milk powder, synthetic ingredients, salt, vitamins.
12	Fresh soy milk	04036	Extracted from soybeans (50.3%), water, fresh milk (10%), sugar, vegetable oil, stabilizers, calcium carbonate, artificial green bean and vanilla flavors, acidity regulator, salt, vitamins.
13	Soy–walnut milk	04037	Extracted from whole soybeans (48.5%), water, sugar (5%), vegetable oil, ground peanuts, walnut extract (5.1 g/L), salt, synthetic ingredients, other bean extracts, vitamins, acidity regulator.
14	Pasteurized strawberry-flavored milk	04038	Fresh cow's milk (93.5%), sugar, natural strawberry flavoring, natural carmine color.
15	Pure soy milk	04039	Extracted from soybeans (71%), water, sugar, emulsifier, artificial flavoring, acidity regulator, salt.

No.	Sample ID	Code	Main Matrix Composition
16	Pasteurized fresh milk	040310	Fresh milk (97.4%), sugar (2.5%), synthetic milk flavoring.
17	Pasteurized pure fresh milk	06031	100% fresh milk
18	UHT fresh milk	06032	97% pure fresh cow's milk, sugar (2.8%), stabilizer.
19	UHT fresh milk	06033	97.1% fresh milk, sugar (2.7%), stabilizers (471, 460(i), 407, 466), vitamins (A, D3), minerals.
20	Corn milk	17031	Not disclosed
21	Corn milk	17032	Not disclosed
22	Corn milk	17033	Not disclosed
23	Soy milk	17034	Water, extracted soybean solution (40%), sugar (8.5%), calcium (0.18%), stabilizer, soybean oil, nature-identical flavoring, vitamins, minerals, salt, acidity regulator.
24	Soy milk	17035	Extracted soybean solution (96.2%: water, soybean), sugar (3.5%), stabilizer, nature-identical flavoring, salt.

Six samples coded as: 11500, 26021, 26022, 26023, 26024, and 26025 were analyzed. Genomic DNA was extracted according to Section 2.4.1, and target gene amplification was performed using real-time PCR as described in Section 2.4.2, targeting the *bar* gene. The results are presented in **Figure 8** and **Table 7**.

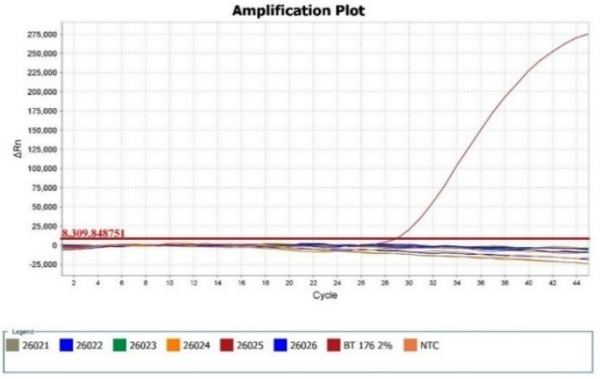
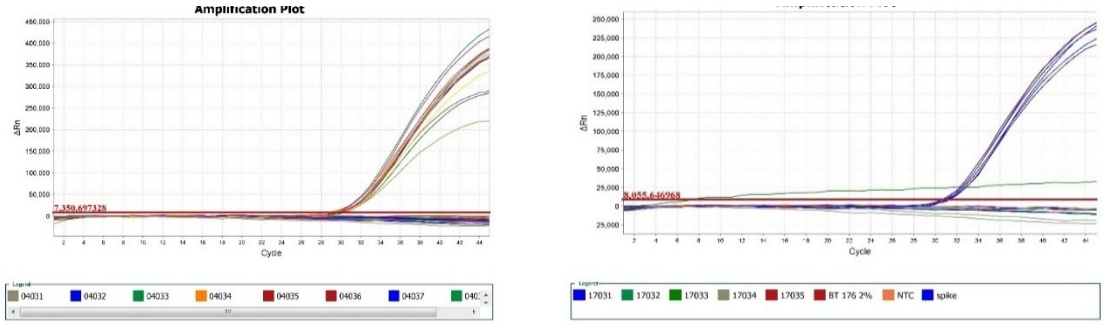


***Figure 8**. Amplification curves of six samples for the bar gene.*

***Table 7**.Cycle threshold (Ct) values of six samples for the bar gene.*

No.	Sample code	Ct value	
		Trial 1	Trial 2
1	NTC (No Template Control)	NA	
2	Positive Control Bt176 2%	29.82	
3	26021	NA	NA
4	26022	NA	NA
5	26023	NA	NA
6	26024	NA	NA
7	26025	NA	NA
8	26026	NA	NA

The results shown in **Figure 8** and **Table 7** indicate that the first six collected samples did not contain the *bar* gene. Therefore, an additional set of 18 samples, coded as: 04031, 04032, 04033, 04034, 04035, 04036, 04037, 04038, 04039, 040310, 06031, 06032, 06033, 17031, 17032, 17033, 17034, and 17035,was examined. For each of these samples, both native (non-spiked) and spiked variants (with added reference material Bt176 *bar* gene) were tested to assess the potential matrix effect on target gene detection. The results are presented in **Figure 9** and **Table 8**.



a b

Figure 9. *Amplification curves of 18 samples for the bar gene:(a) 15 samples; (b) 5 samples.*

Table 8. *Cycle threshold (Ct) values of 18 samples and corresponding spiked samples for the bar gene*

No.	Sample code	Ct value	
		Trial 1	Trial 2
1	NTC (No Template Control)		NA
2	Positive Control Bt176 2%		30.26
3	spike04031		29.77
4	04031	NA	NA
5	spike04032		29.96
6	04032	NA	NA
7	spike04033		29.80
8	04033	NA	NA
9	spike04034		29.68
10	04034	NA	NA
11	Spike04035		29.84
12	04035	NA	NA
13	Spike04036		29.47
14	04036	NA	NA
15	Spike04037		29.98
16	04036	NA	NA
17	Spike04038		29.89
18	04038	NA	NA
19	Spike04039		29.22
20	04039	NA	NA
21	Spike040310		28.46
22	040310	NA	NA
23	Spike06031		30.05
24	06031	NA	NA
25	Spike06032		30.09
26	06032	NA	NA
27	Spike06033		29.58
28	06033	NA	NA
29	Spike17031		30.96
30	17031	NA	NA
31	Spike17032		30.71
32	17032	NA	NA
33	Spike17033		30.55
34	17033	6.84	NA
35	Spike17034		31.06
36	17034	NA	NA
37	Spike17035		31.17
38	17035	NA	NA

The results in **Figure 9** and **Table 8** show that sample code 17033 displayed a Ct value of 6.842 (**Table 6**); however, the corresponding amplification curve was flat and non-sigmoidal (**Figure 9b**), indicating a non-typical amplification pattern. This may be attributed to background noise, non-specific fluorescence signals, potential cross-contamination, or matrix-related interference. When compared with the negative control (NTC) and positive control, the result was interpreted as negative for the *bar* gene. These findings confirm that all 18 collected samples did not contain the *bar* gene, and no significant matrix effect was observed.

4. CONCLUSIONS

This study successfully developed and validated a real-time PCR protocol for the detection of the *bar* gene encoding phosphinothricin N-acetyltransferase using both certified reference materials and real food matrices. The method demonstrated high sensitivity, with a limit of detection (LOD) of 0.05%, and achieved 100% specificity and accuracy. In the corn milk matrix spiked with reference material, the method maintained a consistent LOD of 0.05%, indicating a high reliability and performance in complex food matrices.

The sensitivity of the developed method is comparable to that reported in previous studies. For instance, Elodie Piednoir et al. (2014) reported an LOD of 0.1% with over 95% confidence [23], and Lutz Grohmann et al. (2009) reported an LOD of 0.02% [24]. Different from those studies, which were conducted primarily on ideal or purified DNA matrices, the current study applied the detection method directly to commercial food samples such as corn milk, which are known to contain various PCR inhibitors. Despite this, the method maintained high specificity and accuracy (both 100%), showing comparable or superior performance. Moreover, the protocol requires a relatively low amount of template DNA (100-200 ng), contributing to reduced costs and processing time, while maintaining excellent repeatability (coefficient of variation <5%).

The successful application on real-world matrices-based on the analysis of 24 commercial milk samples-demonstrated effective DNA extraction and reliable detection of the *bar* gene in both corn and soy milk. The use of corn milk, a complex food matrix, did not inhibit the PCR reaction, indicating the strong tolerance to matrix interference of the method.

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