Original paper

Improvements in Methods to Detect Wheat in Heat-processed Foods by Real-time PCR

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Identification and detection of trace amounts of food allergens in processed foods are highly important for food safety. Currently, processed food samples are frequently found to be negative for wheat allergens by a confirmatory PCR-based test even when the ELISA screening test strongly indicates possible wheat contamination (10 mg/kg or more of wheat protein). In this study, a real-time PCR (qPCR) method with a primer pair designed based on the sequence amplified in the official notification PCR method, using TaqMan-MGB probes, was developed to improve the sensitivity of the PCR-based confirmatory test for wheat allergens. The present qPCR method showed higher sensitivity to a plasmid containing the target DNA, DNA extracted from wheat, and heat-treated model samples containing wheat flour than the official notification method. Two heat-processed real specimens that tested positive by ELISA but negative by the conventional confirmatory PCR method were determined to be positive by the present qPCR method. The qPCR method designed here was found to be useful to detect wheat allergens in heat-processed foods.

Keywords: food allergen, wheat, real-time PCR, probe, processed foods

Introduction

In recent years, the number of patients with food allergies has increased in the world. In Japan, to prevent any health problems, laws associated with the Food Sanitation Act were revised in 2001. Considering the high incidence and severity of symptoms associated with wheat, buckwheat, egg, dairy, and peanut allergies, the Act required them to be labeled as major food allergens. In 2008, shrimp and crab were also added to the food allergen list. Since 2015, with the Japanese Food Labeling Act coming into place, labeling based on the Japanese Food Labeling Standards became a requirement.

In the world, the ELISA is the standard method for detecting wheat as allergens in the processed foods (Allen *et al.*, 2014). In Japan, according to the official method for food allergen “Testing Method for Foods Containing Allergic Substances” (the Japanese official notification method) (Consumer Affairs Agency, 2015), a screening test uses two

different ELISA kits. The samples positive for wheat protein at 10 mg / kg or above are considered as positive for wheat. When the samples were positive for wheat by the ELISA, the manufacturing histories of the positive samples were checked, and if wheat is not used in the samples, the conventional PCR method is applied for further confirmation of wheat. These official methods including both ELISA and PCR is unique to Japan. When tests were performed according to the Japanese official notification method, some samples of heat-processed food such as baked foods were positive by ELISA but negative by the confirmatory test with the PCR method (Hashimoto *et al.*, 2008, Hagino *et al.*, 2010; Ishimoto *et al.*, 2013). A previous study showed, following wheat removal tests for baked sweets, some samples were positive when subjected to the ELISA test (wheat protein of 10 mg/kg or higher was detected) but were negative in the confirmatory test by the PCR method (Miyazaki *et al.*, 2017). Therefore, higher sensitivity in

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Table 1. Primers and probes used in this study

Target	Primers and Probes	List of primers and probes	T _m (°C)	Amplicon length (bp)	Reference
Plant	CP03-5'	5'-CGGACGAGAATAAAGATAGACT-3'			official notification method
	CP03-3'	5'-TTTGGGGATAGAGGGACTTG-3'			official notification method
	Chloroplast-F	5'-AAGATAGAGTCCCGTTCTACATGTCAAT-3'	60	77 bp	this study
	Chloroplast-R	5'-TTTAAAGTCGACGGATTTCTCTTA-3'	60		this study
	Chloroplast-T	5'-(FAM)-CTGGCAACAATGAAATT-(NFQ)-(MGB)-3'	70		this study
Wheat	Wtr01-5'	5'-CATCACAATCAACTTATGA-3'			official notification method
	Wtr10-3'	5'-TTTGGGAGTTGAGACGGGTGA-3'			official notification method
	triticin precursor-F	5'-ACTTATGGTGGTTGGAATGGTTAG-3'	58	130 bp	this study
	triticin precursor-R (Wtr10-3')	5'-TTTGGGAGTTGAGACGGGTGA-3'	58		this study
	triticin precursor-T	5'-(FAM)-AACATCGACGATCCCAGTC-(NFG)-(MGB)-3'	70		this study

FAM : 6-carboxyfluorescein

NFQ : non-fluorescent quencher

MGB : minor groove binder

the confirmatory PCR test for wheat allergens is considered necessary.

To improve the sensitivity of the notification method, various modifications have been reported, such as a change in the amount of template DNA and primer, a nested PCR, purification of DNA samples, and use of reagents suppressing PCR inhibitors in samples (Hashimoto *et al.*, 2008; Hashimoto *et al.*, 2009; Hagino *et al.*, 2010; Ishimoto *et al.*, 2013; Hongo *et al.*, 2015; Miyazaki *et al.*, 2017). In these methods, similar to the conventional PCR method, a positive result is based on confirmation of the amplification of DNA fragments with the target size through electrophoresis; thus, a non-specific reaction cannot be eliminated. Since determination is based on visual inspection, the criterion is ambiguous.

A testing method that solves all of these problems is real-time PCR (qPCR). The qPCR method has higher specificity and sensitivity than the normal PCR method, as represented by a test of unapproved Genetically Modified Foods. Specifically, the qPCR method that uses a TaqMan probe is typically used for the tests. Meanwhile, House Foods Corp. has already acquired a patent for a high-sensitivity qPCR method that uses an amplification range that is different from the official notification method (House Foods Corp, 2016). Since the patent method targeted for the wheat specific ITS-2 region having high copy number, the detection sensitivity of the patent method is considered to be higher compared with that of the present qPCR method targeted for the precursor of wheat allergen triticin. However, the ITS-2 region targeted by the patent method is not related to the region for wheat allergen proteins. It seems that the present qPCR method targeting the wheat allergen gene is more reasonable to confirm the results of official notification ELISA method. Therefore, to improve the specificity and sensitivity of the official notification method, the qPCR method that amplifies the DNA sequence within the DNA region stipulated by the official notification

method was developed. To shorten the test time, we examined a method that uses the TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific: “the Fast mode”). As model samples of heat-processed foods, we used rice-flour cookies containing wheat flour with different compositions and prepared by different heating conditions to confirm the applicability of the present method.

Materials and Methods

Primer pairs and probes Primer pairs for qPCR and the TaqMan-MGB probes were designed within the DNA region amplified in the official notification PCR method. The primer pair for plant detection (Chloroplast-F and Chloroplast-R) was designed using Primer Express Software Version 3.0.1 (Thermo Fisher Scientific Inc.). While referring to the nucleotide sequence information for a highly conserved DNA region of the chloroplast gene, a TaqMan-MGB probe was designed based on the sequence of the chloroplast gene of higher plants (GenBank MF579001). The probe was designed within the region amplified with the official notification PCR method primer pair (CP03-5' and CP03-3') (Watanabe *et al.*, 2008) and so that the T_m value (melting temperature) of the probe (Chloroplast-T) would be about 10 °C higher than that of primer pair. The primer pair for wheat detection (triticin precursor-F) and the TaqMan-MGB probe (triticin precursor-T) were designed based on the nucleotide sequence of the DNA region within the triticin precursor gene (GenBank S62630) amplified by the primer pair (Wtr01-5' and Wtr10-3') of the official notification method. The nucleotide sequence of triticin precursor-R was the same as that of the primer Wtr10-3'. Both primers and probes were synthesized at Thermo Fisher Scientific. Nucleotide sequences and T_m values of the primer pair and the TaqMan-MGB probes are shown in Table 1.

Preparation of the model samples We used “Kome Ko” rice flour from Kyoritsu Foods (Fukuoka, Japan), which was

confirmed negative by the ELISA method for wheat. Wheat flour “Flour” from Nisshin Seifun (Tokyo, Japan), commercially available butter, caster sugar, and eggs were used to prepare model samples.

To a 60 g mixture (25 g of butter, 20 g of sugar, and 15 g of egg yolk), 40 g of rice flour and 1 mg of wheat flour were added, mixed well, and placed in a cookie cutter (11.2 cm × 13.3 cm stainless steel container) to prepare a dough with 5 mm thickness. The prepared rice-flour cookie dough included 10 mg/kg wheat flour. Similarly, rice-flour cookie doughs with 0, 20, 30, and 100 mg/kg of wheat flour were prepared.

The cookie dough was divided into 9 rectangles (3.7 × 4.4 cm) and baked for 20 min at 180, 200, and 220 °C in a muffle furnace (Advantec Toyo KM-420). After baking at three different temperatures, cookies were cooled for 30 min at room temperature and powdered using a food processor (Panasonic MK-K48) to prepare samples for inspection. These samples were stored at −30 °C.

ELISA for wheat The FASPEK food allergen measurement kit “Wheat Gliadin” (M kit, Morinaga Institute of Biological Science, Inc.) and FASPTKIT ELISA ver. III “Wheat” (N kit, Nipponham Foods Ltd.) was used according to the official notification method to quantify wheat protein in the actual samples and model samples. According to the kits, the detection limit was 0.3 mg/kg.

Preparation of DNA Two different DNA solutions were prepared from the same sample (two parallel points) for detection of wheat by PCR and qPCR. According to the official notification method, DNA was extracted from 2 g of homogenized samples using an ion-exchange resin kit, Genomic Tip 20/G (G-tip) (QIAGEN), which is suitable for processed foods (Hizen *et al.*, 2007; Fujita *et al.*, 2012), and dissolved in 100 µL of sterilized water. The absorbance of the DNA solution was measured using a spectrophotometer at 230 nm, 260 nm, and 280 nm (Thermo Scientific NanoDrop ND-1000) to determine purity. The DNA concentration was calculated to be 50 ng/µL when the absorbance at A₂₆₀ was 1. The DNA concentration was adjusted to 20 ng/µL with 10-fold or 2-fold serial dilutions with Tris-EDTA buffer (pH 8.0; Nippongene, Japan) containing carrier DNA at 50 µg/mL herring sperm (Promega). Plasmids carrying DNA fragments including the target DNAs of the official notification methods for detecting wheat and plants supplied as positive controls in allergen checker “Wheat” (Oriental Yeast Co., Ltd.) were used as templates for positive controls of wheat and plants. The plasmid solutions were 10-fold serially diluted (from 0.01 pg/µL to 0.01 fg/µL) with Tris-EDTA buffer with carrier DNA, then serially diluted 2-fold (0.01 fg/µL ~ 0.000625 fg/µL). For each 25 µL of PCR reaction mixture, 2.5 µL of the template DNA was added.

For the actual food sample, 2 specimens of baked sweets made in Fukuoka City during 2015–2016, which were positive by the ELISA screening test but negative in the PCR

confirmatory test were used.

Detection and quantification of wheat by the official notification method and the qPCR method

Official notification method The PCR mixture was prepared in three parallel wells per reaction according to the official notification method. The reaction volume for the detection of plant material was 25 µL per well. The reaction mixture contained 1 × PCR buffer II, 0.2 mmol/L dNTP, 1.5 mmol/L MgCl₂, 0.2 µmol/L each (CP03-5' and CP03-3') primers, 0.025 Unit/µL AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific), 2.5 µL of the serially diluted DNA solution, and sterilized water. The composition of the PCR mixture for wheat detection was similar to that of the PCR mixture for plant detection, except for the primer pairs (Wtr01-5' and Wtr10-3'). The PCR was performed by heating the mixture at 95 °C for 10 min, followed by 40 cycles of amplification reactions consisting of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C in a thermal cycler (iCycler, BioRad). After the amplification reactions, mixtures were kept for 10 min at 72 °C, then stored at 4 °C. After running 10 µL of the PCR mixture in a 4 % E-gel (Thermo Fisher Scientific) agarose gel electrophoresis, DNA bands were visualized by using a gel imaging system (UVP BioDoc-It System). Amplified bands were confirmed in all 3 wells for both plant detection and wheat detection; it was determined that the target genes were detected. If either one or both of the DNA preparations from the same sample (parallel tests) produced the correct amplicon, the sample was determined to be positive. While all the other results were negative.

qPCR method For each experiment, 3 wells in parallel per reaction were used. In the Standard mode, 25 µL of the reaction mixture contained 1 × Universal Master Mix (TaqMan Universal Master Mix, Thermo Fisher Scientific), 0.9 µmol/L each of the primer pair for the plant detection (Chloroplast-F and Chloroplast-R), 0.25 µmol/L of the TaqMan-MGB probe (Chloroplast-T), 50 ng template DNA, and sterilized water. The mixture was kept at 50 °C for 2 min and then 95 °C for 10 min, followed by 50 cycles of amplification reactions consisting of 15 s at 95 °C and 2 min at 60 °C using the real-time PCR device (QuantStudio5, Thermo Fisher Scientific). The composition of the PCR mixture for wheat detection was similar to that of the PCR mixture for plant detection, except for the wheat detection primer pair (triticin precursor-F and triticin precursor-R) and the TaqMan-MGB probe (triticin precursor-T). The real-time PCR reactions for plant detection and wheat detection were performed on the same 96-well plate.

The TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) was used for Fast mode, instead of the Universal Master Mix, but the composition of the reaction mixture was the same as that of the Standard mode. After keeping the reaction mixture at 95 °C for 20 s, 50 cycles of the reaction consisting of 1 s at 95 °C and 20 s at 60 °C were conducted.

Determination of the results was made based on the

Fig. 1. Calibration curves of the real-time PCR for detection of Plant and Wheat in the Standard mode

standard of Testing Methods for Genetically Modified Foods, of which the mandatory safety assessment had not been finished (Ministry of Health, Labor and Welfare, 2012). First, amplification was confirmed when the Ct values were less than 43 in all three parallel wells with the threshold line and baseline of 0.2 and 3–15, respectively, for plant detection. After confirming the amplification of the target for plant detection, amplification of the target for wheat detection was confirmed when the Ct values in all three parallel wells were less than 43 with the threshold line and baseline were 0.2 and 3–15, respectively. All other results were considered non-detection. Amplification of the target genes was confirmed in all 3 wells for both plant detection and wheat detection; it was confirmed that the target genes were detected. If the target genes were detected in either one or both of the DNA preparations from the same sample (parallel tests), the sample was determined to be positive. All other results were negative.

Results and Discussion

Comparison of the Standard and Fast modes of the qPCR method for wheat detection Using the primer pair and the TaqMan-MGB probe designed in this study, qPCR with the Standard and the Fast modes were done with serially diluted wheat DNA (20–0.02 ng/μL) as the template. The linearity of the calibration curve and amplification rate of the results were compared. The determination coefficient of the calibration curve was 1.000 for plant detection and 0.991 for wheat detection under the Standard mode (Fig.1), while it was ascertained to be 0.998 for plant detection and 0.996 for wheat detection under the Fast mode (Fig.2). Linearity was good for the detection of plant and wheat. Amplification efficacy was 97 % for the plant detection and 90 % for the wheat detection under the Standard mode; and 100 % for the plant detection and 97 % for the wheat detection under the Fast mode. The efficacy of amplification of qPCR for wheat detection in Fast mode was

Fig. 2. Calibration curves of the real-time PCR for detectin of Plant and Wheat in the Fast mode

nearly 100 % compared to that of the Standard mode. Moreover, in the Fast mode, the reaction time was reduced from 160 min to about 50 min. Thus, the Fast mode was employed for qPCR in this study.

Comparison of detection sensitivity between the official notification method and the present qPCR The lower limits of detection for plant and wheat were compared between the official notification method and the present qPCR method. Table 2 shows the lower limits of detection of plasmids used as positive controls for plants and wheat by the official notification method and the qPCR method. In plant detection, the lower limit of detection was 0.25 fg for the official notification method and 0.025 fg for the qPCR method. In

wheat detection, it was 2.5 and 0.006 fg for the official and the qPCR methods, respectively. In both plant and wheat detections, sensitivity was higher in the qPCR method than that of the official notification method.

Table 3 shows the lower limits of detection of DNA extracted from wheat flour by the official notification method and the present qPCR method. In plant detection, the lower limits of detection were 5 and 0.003 ng for the official and the qPCR methods, respectively. In wheat detection, it was 0.05 and 0.0125 ng for the official and the qPCR methods, respectively. For both plant and wheat detection, the qPCR method showed higher sensitivity compared to the official notification method.

Table 2. Comparison of lower limit of detection of plasmid DNA between the official notification method and present qPCR

Positive control plasmid (fg)	qPCR (Ct value)		Detection by official method	
	Plant	Wheat	Plant	Wheat
25	35.53 ± 0.08	25.52 ± 0.20	+	+
2.5	38.94 ± 0.06	29.00 ± 0.01	+	+
0.25	42.24 ± 0.35	32.54 ± 0.20	-	+
0.025	46.37 ± 1.17	35.40 ± 0.31	-	+
0.0125	no Ct	36.99 ± 0.09	-	-
0.006	no Ct	36.82 ± 0.19	-	-
0.003	no Ct	no Ct	-	-
0.0016	no Ct	no Ct	-	-

(n=3)

Table 3. Comparison of lower limit of detection of DNA Extracted from wheat flour between the official notification method and present qPCR

Template DNA (ng)	qPCR (Ct Value)		Detection by official method	
	Plant	Wheat	Plant	Wheat
50	23.35 ± 0.07	25.91 ± 0.05	+	+
5	26.92 ± 0.04	29.30 ± 0.04	+	+
0.5	30.29 ± 0.10	32.34 ± 0.33	-	+
0.05	33.61 ± 0.06	36.21 ± 1.13	-	+
0.025	34.65 ± 0.12	36.63 ± 0.30	-	-
0.013	35.80 ± 0.17	40.03 ± 1.40	-	-
0.006	36.83 ± 0.15	no Ct	-	-
0.003	37.92 ± 0.17	no Ct	-	-

(n=3)

Table 4. Protein content of model samples

No.	Wheat flour content (mg/kg)	Baking temp. (°C)	Wheat protein content (mg/kg)	
			M kit	N kit
1	0	180	0	0
2	10	180	0.6	0.6
3	10	200	0.6	0.7
4	10	220	0.4	0.4
5	20	180	1.2	1.2
6	20	200	0.8	0.9
7	20	220	0.8	0.9
8	30	180	1.5	1.4
9	30	200	2.3	2.4
10	30	220	2.5	2.4
11	100	180	7.8	10.1
12	100	200	8.2	10.2
13	100	220	8.2	10.8

(LOQ:0.3 mg/kg)

Table 5. Amount and purity of DNA extracted from model samples

No.	Wheat flour content (mg/kg)	Baking temp. (°C)	DNA extracted (mg/2 g sample)	A_{260}/A_{280}	A_{260}/A_{230}
1	0	180	4.8	1.9	1.3
			10.6	1.7	1.1
2	10	180	8.2	1.8	1.0
			7.9	1.7	1.1
3	10	200	6.9	1.7	0.9
			6.3	1.8	1.2
4	10	220	3.0	1.7	0.9
			3.0	1.8	0.9
5	20	180	6.2	1.8	1.3
			10.1	1.8	1.3
6	20	200	9.6	1.6	0.7
			8.0	1.7	0.8
7	20	220	4.1	1.7	1.1
			4.6	1.6	1.0
8	30	180	9.6	1.8	1.0
			9.7	1.8	1.0
9	30	200	4.3	1.8	1.2
			9.9	1.7	1.0
10	30	220	5.3	1.8	1.0
			8.1	1.6	0.8
11	100	180	9.6	1.8	1.1
			10.0	1.8	1.0
12	100	200	5.1	1.9	1.5
			7.2	1.8	1.2
13	100	220	5.4	1.8	1.1
			4.8	1.7	1.1

The amount of wheat protein in the model samples The amount of wheat protein in the prepared model samples (No. 1 to 13 in Table 4) were analyzed using the two types of ELISA kits (N kit and M kit) based on the official notification method. The amount of wheat protein in samples with 10, 20, 30, and 100 mg/kg wheat flour was determined to be 0.5, 1, 2, and 9 mg/kg, respectively. The ratio of wheat protein in wheat flour was estimated to be approximately 4 to 11 %, and the results were consistent with the ratio (8 %) of wheat protein in wheat flour estimated from Standard Tables of Food Composition in Japan (Ministry of Education, Culture, Sports, Science and Technology, 2015).

Effects of baking temperature on DNA extraction from the model samples Table 5 shows the amount and purity of DNA extracted from model samples baked at different temperatures. The DNA extracted from the samples ranged from 3.0 to 10.6 µg/2 g sample. The amounts of DNA extracted from samples baked at different temperatures was 8.7, 7.1, 4.8 µg/2 g sample for 180, 200, and 220 °C, respectively. As the baking temperature increased, the amount of DNA decreased. The

A_{260}/A_{280} , an indicator of DNA purity (protein contamination), of the obtained DNA solution ranged from 1.6 to 1.9, suggesting the DNA samples were suitable to PCR ($A_{260}/A_{280} = 1.7$ to 2.0). Meanwhile, A_{260}/A_{230} , an index of contamination by polysaccharides, was 0.7 to 1.5, which did not satisfy the values required for PCR ($A_{260}/A_{230} = 1.8$ to 2.0). The reason for this is model samples contain 20 % sucrose in addition to 40 % rice flour containing more than 80 % carbohydrates¹²⁾, making the α -amylase treatment incomplete.

Comparison of the detection sensitivity between the official notification method and the qPCR method in the model samples Using model samples, we compared detection sensitivity of the qPCR method and the official notification method. As shown in Table 6, the sample without wheat was negative (No. 1). The reference value was 10 mg/kg for wheat protein and all samples with 100 mg/kg of wheat flour were considered positive (No. 11 to No. 13), suggesting accurate detection of added wheat based on the reference value.

Samples with wheat flour of 10, 20, and 30 mg/kg (No. 2 to No. 10), which were determined to contain wheat protein

Table 6. Comparison of wheat detection between present qPCR and PCR of official notification method on model samples containing trace amounts of wheat flour

No.	Wheat flour content (mg/kg)	Baking temp. (°C)	Lot	qPCR									Official method (PCR)					
				Ct Value									Decision Result					
				Plant			Wheat						Plant		Wheat		Decision Result	
1	0	180	1	19.91	19.83	20.04	no Ct	no Ct	no Ct	-	-	+	+	+	-	-	-	-
			2	20.22	20.03	20.05	no Ct	no Ct	no Ct	-	-	+	+	+	-	-	-	-
2	10	180	1	20.19	20.00	20.18	no Ct	38.34	37.96	-	-	+	+	+	-	-	+	-
			2	20.04	20.09	20.13	38.91	no Ct	38.89	-	-	+	+	+	-	+	-	-
3	10	200	1	20.26	20.26	20.37	38.36	no Ct	no Ct	-	-	+	+	+	-	-	+	-
			2	19.92	19.70	19.82	37.31	no Ct	37.52	-	-	+	+	+	-	-	+	-
4	10	220	1	20.88	20.60	20.70	38.98	37.69	no Ct	-	-	+	+	+	-	-	-	-
			2	20.92	20.99	21.14	no Ct	no Ct	no Ct	-	-	+	+	+	-	-	-	-
5	20	180	1	20.60	20.75	20.58	38.20	37.72	38.04	+	+	+	+	+	-	-	-	-
			2	20.09	20.11	20.05	38.15	no Ct	37.16	-	-	+	+	+	-	-	-	-
6	20	200	1	21.34	21.14	21.20	38.02	37.80	no Ct	-	-	+	+	+	-	+	+	-
			2	20.84	20.63	20.68	no Ct	39.04	40.52	-	-	+	+	+	-	-	-	-
7	20	220	1	21.50	21.61	21.59	42.05	39.09	no Ct	-	-	+	+	+	-	+	+	-
			2	21.56	21.50	21.57	no Ct	39.42	40.61	-	-	+	+	+	-	-	-	-
8	30	180	1	20.13	19.86	20.00	36.70	38.37	37.86	+	+	+	+	+	-	-	+	-
			2	19.97	19.67	19.85	37.27	37.16	38.30	+	+	+	+	+	+	-	-	-
9	30	200	1	20.72	20.95	20.94	36.19	37.00	37.05	+	+	+	+	+	-	+	+	-
			2	19.91	20.31	20.22	35.93	37.43	36.51	+	+	+	+	+	-	+	+	-
10	30	220	1	21.29	21.09	21.19	38.04	37.42	37.34	+	+	+	+	+	+	+	-	-
			2	20.72	20.57	20.93	38.19	no Ct	38.93	-	-	+	+	+	-	-	+	-
11	100	180	1	20.60	20.61	20.71	36.08	37.17	35.11	+	+	+	+	+	+	+	+	+
			2	20.75	20.58	21.09	37.59	37.06	35.39	+	+	+	+	+	+	+	+	+
12	100	200	1	19.84	19.66	19.79	35.30	34.52	35.07	+	+	+	+	+	+	+	+	+
			2	19.80	19.84	19.89	35.22	35.37	35.95	+	+	+	+	+	+	+	+	+
13	100	220	1	20.52	20.59	20.58	35.15	35.84	35.76	+	+	+	+	+	+	+	+	+
			2	20.64	20.28	20.46	35.87	35.97	34.86	+	+	+	+	+	-	-	-	-

Table 7. Comparison of wheat detection between present qPCR and PCR of official notification method on real samples containing over 10 mg/kg of wheat protein

No.	Sample	Wheat protein content (mg/kg)		DNA extracted (µg/2 g sample)	qPCR										Official method (PCR)									
		M kit	N kit		A ₂₆₀ /A ₂₈₀	A ₂₆₀ /A ₂₃₀	Ct Value								Decision Result									
							Plant				Wheat								Plant		Wheat		Decision Result	
1	Cheese cookie	17	17	54.9	1.8	1.7	20.63	19.85	20.24	38.04	39.35	37.97	+	+	+	+	+	+	-	-	+	-	-	
				98.4	1.8	1.2	20.89	21.00	21.10	39.26	38.15	no Ct	-			+	+	+	+	-	-	-		
2	Biscotti	>20	>20	33.4	1.2	0.4	20.92	21.04	21.28	36.96	37.19	36.94	+	+		+	+	+	-	+	+	-	-	
				62.6	1.3	0.3	20.97	20.76	23.06	37.11	37.06	36.89	+			+	+	+	-	-	-	-		

equivalent of 0.5, 1, and 2 mg/kg of the reference value, were all negative by the official notification method. However, by the qPCR method, samples with 30 mg/kg of wheat flour (No. 8 to No. 10) were determined to be positive, indicating higher sensitivity than the official notification method. The sample with 20 mg/kg of wheat flour (No. 5) baked at 180 °C was

positive in one sample out of two parallel tests, but both two-point parallel tests were negative at 200 and 220 °C (No. 6 to No.7). Mano *et al.* reported that longer heating time or higher heating temperature of processed food increased the DNA fragmentation, leading to the increase in lower limit value of the qPCR detection of the target DNA in the processed food.

The facts support our results on decrease in the detection rate by PCR with increase in the baking temperature from 180 °C to 220 °C.

Detection of wheat from actual food samples by the qPCR method Despite positive for wheat by the ELISA method detecting wheat protein exceeding the reference value of 10 mg/kg, two actual food samples (baked sweets) were negative by the official notification PCR method as a confirmatory test. For these samples, the present qPCR method was applied. As shown in Table 7, the result showed that both samples were positive with the qPCR method, the same result as the ELISA method.

The qPCR method as a confirmatory test for food allergen (wheat) needs to accurately determine positive samples that contain wheat protein higher than the reference value of 10 mg/kg detected by the ELISA. Higher detection sensitivity is preferred, but if it is too sensitive, false-positive results could occur due to contamination with PCR products in the laboratory. The qPCR method with the Fast mode has slightly lower sensitivity compared to the reported method (Miyazaki *et al.*, 2019). However, in model samples and actual samples that went through the heating process, detection sensitivity of the qPCR method was higher than the official notification method. Thus, the qPCR method was effective for genetic testing of wheat in actual heat-processed foods and its sensitivity is sufficient as a confirmatory test.

Conclusions

Aiming to improve the sensitivity of genetic detection for wheat allergens, a qPCR method using the TaqMan-MGB probes was developed in this study. The present qPCR method seems to be highly specific through the use of the TaqMan probe and rapid compared to the official notification method. However, it is necessary to confirm the validity of the present qPCR method in more actual food samples positive by ELISA but negative by conventional PCR. Since it is very rare to encounter such actual samples, we will collect actual food samples positive by ELISA but negative by conventional PCR and test the present qPCR in the near future.

In tests using a plasmid including DNA extracted from wheat flour, model samples that went through a heating process had a higher detection sensitivity using the qPCR method than the official method. Two actual baked food specimens containing wheat protein of 10 mg/kg or higher detected by the ELISA were negative by the confirmatory PCR test of the official notification method. These two samples were positive with the present qPCR method. The qPCR method seems effective for genetic testing of wheat in actual heat-processed foods since its sensitivity is sufficient as a confirmatory test. Although the present qPCR method is an improvement of the notification method, we need to evaluate the present method transferability to introduce the present method to different research institutions. Errors depending on the difference in the

device and laboratory skills must be within set criteria. Furthermore, a full validation in a collaborative trial is required.

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