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Applicability of the Quantification of Genetically Modified Organisms to Foods Processed from Maize and Soy

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The applicability of quantifying genetically modified (GM) maize and soy to processed foods was investigated using heat treatment processing models. The detection methods were based on real-time quantitative polymerase chain reaction (PCR) analysis. Ground seeds of insect resistant GM maize (MON810) and glyphosate tolerant Roundup Ready (RR) soy were dissolved in water and were heat treated by autoclaving for various time intervals. The calculated copy numbers of the recombinant and taxon specific deoxyribonucleic acid (DNA) sequences in the extracted DNA solution were found to decrease with time. This decrease was influenced by the PCR-amplified size. The conversion factor (C_f), which is the ratio of the recombinant DNA sequence to the taxon specific DNA sequence and is used as a constant number for calculating GM% at each event, tended to be stable when the sizes of PCR products of two DNA sequences were nearly equal. The results suggested that the size of the PCR product plays a key role in the quantification of GM organisms in processed foods. It is believed that the C_f of the endosperm (3*n*) is influenced by whether the GM originated from a paternal or maternal source. The embryos and endosperms were separated from the F1 generation seeds of five GM maize events, and their C_f values were measured. Both paternal and maternal GM events were identified. In these, the endosperm C_f was lower than that of the embryo, and the embryo C_f was lower than that of the endosperm. These results demonstrate the difficulties encountered in the determination of GM% in maize grains (F2 generation) and in processed foods from maize and soy.

KEYWORDS: ***Zea mays*; *Glycine max*; genetically modified; MON810; Roundup Ready; processed food; endosperm; embryo; heat treatment; GMO detection; quantitative analysis**

INTRODUCTION

A rapid increase in the growth of genetically modified organisms (GMO) as well as dependence on foodstuffs including these materials have occurred in recent years (*1*). Some consumers are concerned about the use of advanced technology and potential health and environmental risks associated with GMOs (*2*). One particular matter of concern to consumers has been the proper labeling of food products that have been produced using GM technology. Labeling systems have been introduced for GM foods in the European Union (EU), Korea, Japan, Australia, and other countries; however, the labeling of GM foods is not compulsory in the United States and Canada. Under current legislation, the presence of GM material in conventional food products does not have to be labeled, provided that such use can be shown to be adventitious and technically unavoidable. The governments of the following countries have announced the following threshold levels for the unintentional mixing of GMOs with food products: 0.9% in the EU (*3*), 3% in Korea (*4*), and 5% in Japan (*5*).

Polymerase chain reaction (PCR) is a widely used technique to confirm the existence of GMOs and to ensure the reliability of labeling systems (*6*−*18*). For initial use in GMO screening, qualitative PCR, which determines whether more than a particular detectable limit of GMOs is present in a sample, is considered the method of first choice (*6*−*12*); real-time PCR

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or quantitative competitive PCR is a useful technique for obtaining more precise and numerical information determining the amount of GMOs in a sample (*13*−*18*). We previously reported a novel method for the detection and quantification of relative amounts of five GM maize products and a GM soy product (*19*, *20*). This analytical method was adopted as a standard (the official method) in Japan and Korea (*21*−*24*). However, application of this method is limited to use on grains and ground materials obtained from maize and soy. In Japan, 30 processed food products derived from maize, soy, and potato have been identified as products that should be labeled according to the labeling system. However, quantitative methods for determining the comingling levels of GMOs in processed foods still need to be developed in order to determine whether the current identity-preserved system of handling non-GM raw materials is appropriately implemented.

DNA degradation is the primary obstacle to GM quantification in processed foods, because the GM% is calculated from the ratio of the copy numbers of the recombinant DNA sequence to the taxon specific DNA sequence. Therefore, we are at present unable to eliminate the possibility that the degree of degradation of these two DNA sequences differs and thus to reliably determine whether the GM% in processed foods is inconsistent with the true values in the raw materials.

Several actions have been taken to detect the presence of GMOs in processed foods. In one qualitative analysis using a heat treatment processing model, the target sequence was successfully amplified by electrophoresis using a shorter region than that used for raw materials (*25*). One report mentioned the possibility of using real-time PCR for the GM quantification of processed food, where the relevant values were determined from the GM% measured before, as well as after, model processing (*26*).

In the present report, MON810 maize and Roundup Ready (RR) soy, used as representative GM crops, were processed with heat treatment in order to investigate the applicability of the quantification methods considered here. Furthermore, the reliability of these improvements was evaluated by the change in the conversion factor (C_f), which represents the ratio of the recombinant DNA sequence to the taxon specific DNA sequence.

In addition, we took into account the biological backgrounds of the seeds and the progeny grains. For this analysis, it was necessary to consider the tissue type that was used to produce the processed food from albuminous plants such as maize. This is because the nuclear phase of the embryo (germ) is diploid and that of the endosperm (albumen) is triploid; the 2*n* out of 3*n* in the endosperm originates from the maternal source. Moreover, it was also necessary to investigate whether the current GM quantifying method for seeds could be applied to processed foods, because almost all of the DNA in processed foods is thought to be derived from an endospermic fraction. Additionally, it was important to take into account the genotype differences of samples in the zygotic phase with respect to F1 hybrid seeds and the progeny used as food materials. We clarified several findings in this study that are demonstrative of such differences in biological backgrounds.

MATERIALS AND METHODS

Maize, Soy, and Other Cereal Samples. Genuine seeds of the appropriate varieties derived from five events of GM maize (*Zea mays*) and RR soy (*Glycine max*) were identical with those used in our previous paper (*19*). As a conventional non-GM maize, Dairyland 1412 was directly imported from the United States. As a conventional non-GM soy, the grain produced in Ohio in 1998 was directly imported. The rice (*Oryza sativa*) variety Kinuhikari, the wheat (*Triticum aestivum*) variety Haruyutaka, and the barley (*Hordeum vulgare*) variety Harrington were used to study the specificity of the designed primer pairs.

Heat Treatment Processing Model. MON810 maize seeds were ground with a Rotor-Speed Mill P14 (Fritsch GmbH, Idar-Oberstein, Germany) with a 0.2 mm sieve ring. RR soy seeds were ground with a Rotor-Speed Mill P14 with a 0.5 mm sieve ring. One gram of powder was suspended in 7.5 mL of distilled water and autoclaved with a high-pressure Steam Sterilizer BS-245 (TOMY Seiko Co., Ltd., Tokyo, Japan) at 110 °C for 0, 5, 15, 30, and 60 min ($n = 2$) (not including the time required for raising and decreasing temperature). DNAs were extracted from the heat-treated samples.

DNA Extraction from Samples. A silica membrane method was used for DNA extraction. Using the DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany), 1 g of the sample was incubated for 1 h at 65 °C with the addition of 5 mL of buffer AP1 and 10 μL of RNase for maize and, for the soy samples, with the addition of 10 mL of buffer AP1 and 20 μL of RNase. The following steps were carried out according to previously described methods (*18*). The eluted fraction was treated with 2-propanol precipitation, and the precipitant was rinsed with 70% ethanol, dried, and resuspended with 100 μL of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA)]. The concentration of DNA in solution was calculated from the absorbance at 260 nm, as measured by a UV spectrometer (DU7000; Beckman Coulter Inc., Fullerton, CA).

Oligonucleotide Primers and Probes. To investigate the stability of the GM% by heat treatment, PCR systems SSIIb 2-4 and Le1n03, which amplify the inside region of the PCR systems SSIIb 1 and Le1n02, were designed to detect taxon specific DNA sequences in maize and soy, respectively (**Table 1**). Schematic diagrams of PCR systems SSIIb 1-4 and Le1n02-03 are shown in **Figure 1**. Seven sets of primer pairs and Taq-Man probes used for construct specific and universal GM quantitation were identical with those described in our previous paper (*19*). Among these primer pairs and Taq-Man probes, M810 2 (M810 2-5′ and M810 2-3′ with M810-Taq), Bt11 3 (Bt11 3-5′ and Bt11 3-3′ with Bt11-2-Taq), GA21 3 (GA21 3-5′ and GA21 3-3′ with GA21-2-Taq), T25 1 (T25 1-5′ and T25 1-3′ with T25-2-Taq), E176 2 (E176 2-5′ and E176 2-3′ with E176-Taq), and RRS 01 (RRS 01-5′ and RRS 01-3′ with RRS−Taq) were used for the quantitation of construct specific sequences, while P35S 1 (P35S 1-5′ and P35S 1-3′ with P35S-Taq) was used for the quantitation of universal GM sequence. All primers and Taq-Man probes labeled with 6-carboxy-fluorescein (FAM) and 6-carboxytetramethyl-rhodamine (TAMRA) at the 5′- and 3′-ends, respectively, were synthesized using Fasmac Co., Ltd. (Kanagawa, Japan).

Qualitative PCR. A 25 μL volume of the reaction solution contained 25 ng of template DNA, 2.5 μL of PCR buffer II [Applied Biosystems (ABI), Foster City, CA], 200 μM dNTP, 1.5 mM $MgCl_2$, 0.625 U AmpliTaq Gold DNA Polymerase (ABI), and 0.5 μM of the primer pair. The reaction was carried out using a PTC-200 DNA engine (MJ Research Inc., Waltham, MA), with the following PCR step−cycle program: preincubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min, followed by a final extension at 72 °C for 7 min.

Quantitative PCR. A 25 μL volume of the reaction mixture contained 12.5 μL of Universal Master Mix (ABI), 0.5 μM primer pair (Fasmac Co.), 0.2 μM probe (except for the p35S, 0.1 μM) (Fasmac Co.), and 50 ng of template DNA. Sample DNA was diluted to 20 ng/μL in TE buffer (pH 8.0), and 2.5 μL of diluted DNA was added to the reaction mixture. The probe was labeled with fluorescent dye, and the kinetic analysis during amplification of the target sequence was monitored using an ABI PRISM 7700 (ABI) system. The reaction conditions for real-time PCR for all PCR systems was set with the following PCR step−cycle program: preincubation at 50 °C for 2 min and 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing, and extension at 59 °C for 1 min. Standard curves were calibrated using five concentrations of reference molecules, i.e., 20, 125, 1500, 20000, and 250000 copies per reaction. A

Table 1. List of Primers and TaqMan Probes for Real-Time PCR Systems

target	PCR system	name	sequence	specificity	length (bp)
z*SSIIb* (maize taxon specific)	SSIIb 1	SSIIb 1-5′	CTC CCA ATC CTT TGA CAT CTG C	z*SSIIb*/sense primer	151
		SSIIb 1-3′	TCG ATT TCT CTC TTG GTG ACA GG	z*SSIIb*/antisense primer	
		SSIIb-Taq	5′-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3′	z*SSIIb*/sense probe	
	SSIIb 2	SSIIb 2-5′	TCC CAA TCC TTT GAC ATC TGC T	z*SSIIb*/sense primer	133
		SSIIb 2-3′	GAC AGG AGC TGA TGG ATG ATC AG	z*SSIIb*/antisense primer	
		SSIIb-Taq	5′-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3′	z*SSIIb*/sense probe	
	SSIIb 3	SSIIb 3-5′	CCA ATC CTT TGA CAT CTG CTC C	z*SSIIb*/sense primer	114
		SSIIb 3-3′	GAT CAG CTT TGG GTC CGG A	z*SSIIb*/antisense primer	
		SSIIb-Taq	5′-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3′	z*SSIIb*/sense probe	
	SSIIb 4	SSIIb 3-5′	CCA ATC CTT TGA CAT CTG CTC C	z*SSIIb*/sense primer	83
		SSIIb 4-3′	GGT GCT CGC GCT GCT G	z*SSIIb*/antisense primer	
		SSIIb-Taq	5′-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3′	z*SSIIb*/sense probe	
Le1 (soy taxon specific)	Le1n02	Le1n02-5′	GCC CTC TAC TCC ACC CCC A	*Le1*/sense primer	118
		Le1n02-3′	GCC CAT CTG CAA GCC TTT TT	*Le1*/antisense primer	
		Le1-Taq	5′-FAM-AGC TTC GCC GCT TCC TTC AAC TTC AC-TAMRA-3′	*Le1*/sense probe	
	Le1n03	Le1n03-5′	GGA CAA AGA AAC CGG TAG CGT	*Le1*/sense primer	89
		Le1n03-3′	GCC CAT CTG CAA GCC TTT T	*Le1*/antisense primer	
		Le1-Taq	5′-FAM-AGC TTC GCC GCT TCC TTC AAC TTC AC-TAMRA-3′	*Le1*/sense probe	

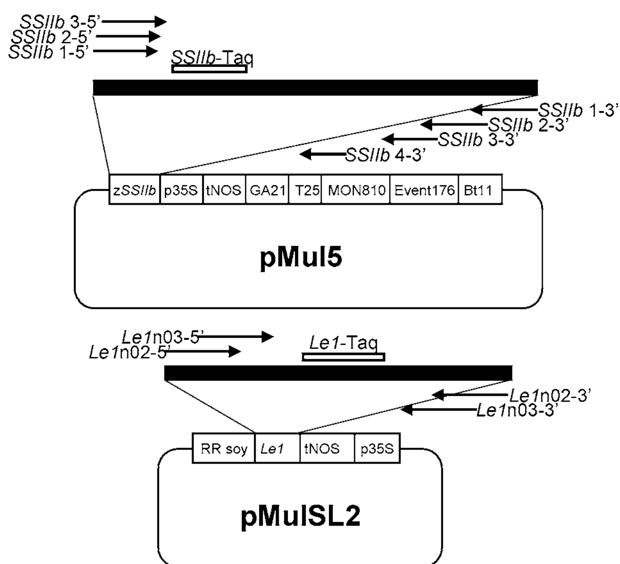

Figure 1. Schematic diagrams of the PCR systems designed to detect the taxon specific sequences of maize and soy in the construction of pMul5 and pMulSL2, respectively. SSIIb 2-5′&3′, SSIIb 3-5′&3′, and SSIIb 3-5′&4-3′ were designed within the sequence amplified by SSIIb 1-5′&3′ possessing the same TaqMan probe, i.e., SSIIb-Taq. These primer pairs were used to amplify the *zSSIIb* sequence in the pMul5 plasmid as the standard material. Le1n03-5′&3′ was designed within the sequence amplified by Le1n02-5′&3′ possessing the same TaqMan probe, i.e., Le1-Taq. This primer pair was used to amplify the *Le1* sequence in the pMulSL2 plasmid as the standard material.

no-template control (NTC) was also prepared as a negative control for the analysis. In this study, "GM Maize or Soy Detection Plasmid Set-ColE1/TE-" (Nippon Gene Co., Tokyo, Japan), which contained six concentrations (including NTC) of the plasmid pMul5 or pMulSL2 (*19*, *20*) diluted with the TE buffer (pH 8.0), including 5 ng/μL of ColE1 plasmid, was used as the reference molecule. In the reaction plate, real-time PCR was performed in triplicate using three wells for each template DNA (NTC, five concentrations of reference molecules, and extracted DNA from the samples). The copy numbers of each sequence were calculated in a fixed manner according to that described in our previous report (*19*).

Measurement of C_f. According to our previous report (*19*), the copy numbers of recombinant and taxon specific DNA sequences in DNA solution extracted from samples, seeds, embryos, and endosperms were quantitated. The ratio of the copy number of the recombinant DNA sequence to that of the taxon specific sequence was defined as the conversion factor (C_f) (in our previous report, we defined this value as the coefficient value, C_v).

Agarose Gel Electrophoresis. Extracted DNA was electrophoresed at a constant voltage (100 V) with 3% agarose gel supplemented with 0.5 μg/mL ethidium bromide (Sigma Chemical Co., St. Louis, MO) in TAE buffer [40 mM Tris-HCl (pH 8.0), 40 mM acetic acid, and 1 mM EDTA (pH 8.0)]. The gel was scanned using a Molecular Imager FX system (Bio-Rad Laboratories Inc., Hercules, CA).

Separation of Embryo and Endosperm from Single Seeds. Before separation, the seeds were washed with 1% sodium dodecyl sulfate (SDS) and were rinsed 10 times with distilled water. After they were dried on a paper, the seed coat was peeled from the seed with sharp-edged tweezers. The peeled seed was cut into two parts with a dissecting blade along the line extending from the dent to the black layer. Some of the yellow portion was collected as endosperm, and a few yellowish-white parts near the black layer were collected as the embryo using tweezers and a blade.

Grinding of Single Seed, Embryo, and Endosperm. The grinding of single seed, embryo, and endosperm was performed using a Multi-Beads Shocker (Yasui Kikai Co., Osaka, Japan) with a 12 mL tube holder (type SH-123) at 1800 rpm for 30 s (1 s for embryos). Before grinding, the seeds were washed with 1% SDS, rinsed 10 times with distilled water, and dried to remove powder and broken pieces of other seeds.

DNA Extraction from Single Seed, Embryo, and Endosperm. DNA was extracted from ground single seed, embryo, and endosperm using a DNeasy Plant Mini Kit (QIAGEN GmbH), according to the manufacturer's instruction, except that elution was carried out using distilled water.

RESULTS AND DISCUSSION

Specificities of the Newly Designed PCR Systems. The specificities of the newly designed SSIIb 2-4 and Le1n03 were confirmed by qualitative and quantitative PCR. No unexpected

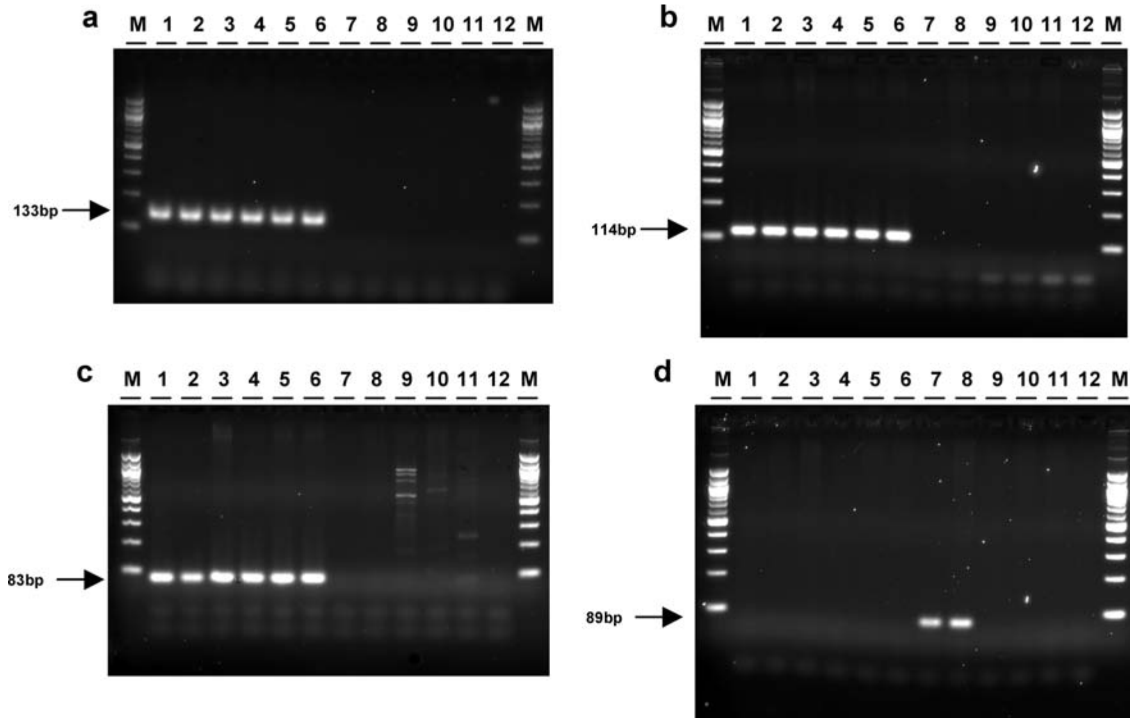

Figure 2. Agarose gel electrophoresis of PCR products amplified from maize, soy, and other cereal genomic DNAs. The arrows indicate the expected PCR amplification products. Primer pairs were used for the detection of SSIIb 2-5′&3′ (**a**), SSIIb 3-5′&3′ (**b**), SSIIb 3-5′&4-3′ (**c**), and Le1n03-5′&3′ (**d**). Lanes 1–6, amplification of maize DNAs from non-GM maize, Bt11, GA21, T25, Event176, and MON810, respectively; lanes 7 and 8, amplification of non-GM soy and RR soy, respectively; lanes 9–11, amplification of rice, wheat, and barley, respectively; lane 12, negative control (no template DNA); and M, 100 bp ladder size marker.

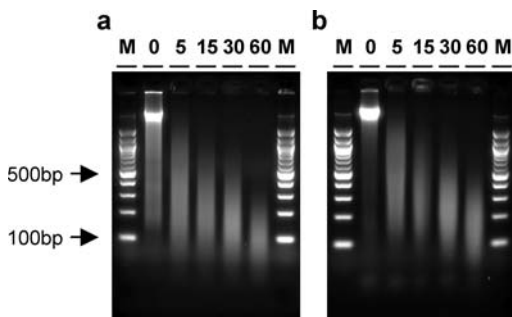

Figure 3. Agarose gel electrophoresis of DNAs extracted from heat-treated seed of maize (**a**) and soy (**b**). Lane M, 100 bp ladder size marker; lanes 0, 5, 15, 30, and 60, DNA from samples subjected to heat treatment for each of these time periods.

PCR products were observed by qualitative PCR using genomic DNA extracted from non-GM soy, RR soy, rice, wheat, and barley in the case of PCR systems SSIIb 2-4 and using genomic DNA extracted from non-GM maize, GM maize, rice, wheat, and barley in the case of PCR system Le1n03 (**Figure 2**). The specificity of the PCR primer pairs and probes was also confirmed by real-time PCR using the above genomic DNA samples. Amplification was observed only for the corresponding template genomic DNA (data not shown). Therefore, these results suggested that the PCR systems that were newly designed to detect the taxon specific DNA sequences of maize and soy exhibited sufficient specificity for use in the present qualitative and quantitative PCR analyses.

Heat Treatment Processing Model. Results of the electrophoresis showed that DNA from maize and soy was degraded to smaller sizes with increasing processing time (**Figure 3**). As compared to the molecular marker, the size of the DNA fragments in the sample DNA that was heat treated for 60 min was less than approximately 500 bp. Copy numbers that were quantitated in each 50 ng sample of the extracted DNA decreased with time (**Table 2**).

In the case of maize, the copy numbers quantitated for the taxon specific DNA sequence with four primer pairs designated as SSIIb 1, SSIIb 2, SSIIb 3, and SSIIb 4 decreased in the order of the amplified length. The C_f values consequently increased with time when SSIIb 1 was used for taxon specific sequence detection in MON810- and p35S-targeted GM quantification (**Figure 4a**,**b**). These results suggest that the current GM quantification method using SSIIb 1 could not be applied to processed foods without modification. In contrast, the C_f values obtained using SSIIb 2 or SSIIb 3 were more stable in behavior than those obtained using SSIIb 1; that is, the C_f values slowly decreased within 5−15 min, followed by a gradual increase. The C_f values of the MON810 construct specific quantification measured using SSIIb 3 were markedly improved; the changes in C_f values of heat-treated samples were within 23% of the values observed at 0 min. Moreover, the C_f values measured using SSIIb 4 showed conflicting changes; that is, the C_f values decreased with time, using SSIIb 4.

In the case of soy, the copy numbers of the taxon specific DNA sequences decreased in a manner similar to that observed in the maize experiments. The C_f values measured using Le1n02 tended to be higher than those obtained using Le1n03 in RR soy- and p35S-targeted GM quantification (**Figure 4c**,**d**). However, with regard to the observed decrease for the target recombinant DNA sequence, the C_f values decreased abruptly at 5 min and subsequently increased with time, except in the case of the Le1n02/P35S 1-targeted quantification. The changes in C_f values of heat-treated samples were within 25, 23, 58, and 14, as compared to the values observed at 0 min for Le1n02/RRS 01-, Le1n03/RRS 01-, Le1n02/P35S 1-, and Le1n03/P35S 1-targeted quantification, respectively.

Table 2. Changes in Copy Number of Each Target Sequence and C_f after Heat Treatment with an Autoclave[a]

	PCR system											
	copy number			copy number			copy number			copy number		
min	SSIIb 1	M810 2	C_f	SSIIb 2	M810 2	C_f	SSIIb 3	M810 2	C_f	SSIIb 4	M810 2	C_f
0	30691	13322	0.43	30195	13630	0.45	31802	12845	0.40	32063	13451	0.42
	100.0	*100.0*		*100.0*	*100.0*		*100.0*	*100.0*		*100.0*	*100.0*	
5	24069	10260	0.43	24963	10484	0.42	28961	9708	0.34	34698	10761	0.31
	78.4	*77.0*		*82.7*	*76.9*		*91.1*	*75.6*		*108.2*	*80.0*	
15	10510	5446	0.52	13312	5712	0.43	16350	5071	0.31	21203	5715	0.27
	34.2	*40.9*		*44.1*	*41.9*		*51.4*	*39.5*		*66.1*	*42.5*	
30	1889	1387	0.73	2832	1543	0.54	3586	1256	0.35	6112	1547	0.25
	6.2	*10.4*		*9.4*	*11.3*		*11.3*	*9.8*		*19.1*	*11.5*	
60	85	163	1.92	214	201	0.94	359	151	0.42	1081	204	0.19
	0.3	*1.2*		*0.7*	*1.5*		*1.1*	*1.2*		*3.4*	*1.5*	

	PCR system											
	copy number			copy number			copy number			copy number		
min	SSIIb 1	P35S 1	C_f	SSIIb 2	P35S 1	C_f	SSIIb 3	P35S 1	C_f	SSIIb 4	P35S 1	C_f
0	29533	12523	0.42	31630	12149	0.38	31663	11995	0.38	32068	12392	0.39
	100.0	*100.0*		*100.0*	*100.0*		*100.0*	*100.0*		*100.0*	*100.0*	
5	22427	9537	0.43	25014	9166	0.37	28867	9026	0.31	34998	9727	0.28
	75.9	*76.2*		*79.1*	*75.5*		*91.2*	*75.2*		*109.1*	*78.5*	
15	10600	5470	0.52	14123	5301	0.38	15747	4989	0.32	21061	5503	0.26
	35.9	*43.7*		*44.7*	*43.6*		*49.7*	*41.6*		*65.7*	*44.4*	
30	1779	1419	0.80	2961	1337	0.45	3363	1230	0.37	5973	1444	0.24
	6.0	*11.3*		*9.4*	*11.0*		*10.6*	*10.3*		*18.6*	*11.6*	
60	104	211	2.02	227	203	0.90	332	174	0.53	911	201	0.22
	0.4	*1.7*		*0.7*	*1.7*		*1.0*	*1.5*		*2.8*	*1.6*	

	PCR system											
	copy number			copy number			copy number			copy number		
min	Le1n02	RRS 01	C_f	Le1n03	RRS 01	C_f	Le1n02	P35S 1	C_f	Le1n03	P35S 1	C_f
0	76410	79893	1.05	81493	79429	0.97	78662	74987	0.95	84444	72516	0.86
	100.0	*100.0*		*100.0*	*100.0*		*100.0*	*100.0*		*100.0*	*100.0*	
5	44987	39878	0.89	53009	40055	0.76	46765	49250	1.05	56399	47490	0.84
	58.9	*49.9*		*65.0*	*50.4*		*59.4*	*65.7*		*66.8*	*65.5*	
15	37692	41003	1.09	49202	39949	0.81	39141	48037	1.23	50520	46272	0.92
	49.3	*51.3*		*60.4*	*50.3*		*49.8*	*64.1*		*59.8*	*63.8*	
30	26107	34136	1.31	37581	33790	0.90	26468	39785	1.50	39237	38281	0.98
	34.2	*42.7*		*46.1*	*42.5*		*33.6*	*53.1*		*46.5*	*52.8*	
60	11778	15049	1.28	19208	14793	0.77	12022	18948	1.58	19869	18705	0.94
	15.4	*18.8*		*23.6*	*18.6*		*15.3*	*25.3*		*23.5*	*25.8*	

[a] Numbers on upper row: averaged copy number of DNA sequence heat treated for specified time. Numbers on lower row in italics: percentage in terms of copy numbers of DNA sequence subjected to heat treatment for specified time as compared to that at 0 min.

It is likely that the observed differences in the rate of decrease in copy numbers resulted from the degradability of the PCR target region, which depends mainly on length, GC content, and localization on a chromosome. A longer target DNA is more easily degraded than a shorter DNA in the context of heat processing. In this study, as regards MON810 maize construct specific quantification, it was concluded that the lengths of the PCR products of the primer pairs for the taxon specific and the recombinant DNA sequences should be similar in order to determine the GM% in processed foods. However, in the early stage of heat treatment, the C_f values using SSIIb 2 and SSIIb 3 decreased. This finding indicates that target recombinant DNA sequences of M810 2 (113 bp) and P35S 1 (101 bp) were degraded to a greater extent than the target taxon specific DNA sequences that had longer PCR products than those of the target recombinant DNA sequence. DNA regions containing high GC% are generally considered to be stable when exposed to high temperatures. In fact, the GC contents of the PCR products for MON810 construct specific detection and p35S universal detection were 46 and 44%, respectively, and were lower than the GC contents of the products of SSIIb 1-4, which were 58, 60, 61, and 58%, respectively. Therefore, it is believed that the regions of MON810 and p35S were more strongly degraded than those of SSIIb in the early stages of heat treatment, and DNA degradation by random scission would be expected to subsequently occur, depending on the length of the target regions.

As shown in our previous report (*19*), the sizes of the PCR products of construct specific quantitation are between 100 and 149 bp. It was reasonable to replace primer pair SSIIb 1 with primer pair SSIIb 3 in order to quantify processed foods derived from MON810. It is possible that a PCR system for maize taxon specific genes would not be unconditionally applicable to the quantification of other GM maize events. However, because MON810 is currently the major GM maize event (*1*), it would be appropriate to investigate MON810 as a representative of maize GM events. To obtain a higher precision of GM% using SSIIb 3 for taxon specific DNA sequence detection, the PCR systems for the construct specific quantitation should be modified to one in which the amplification region is similar to

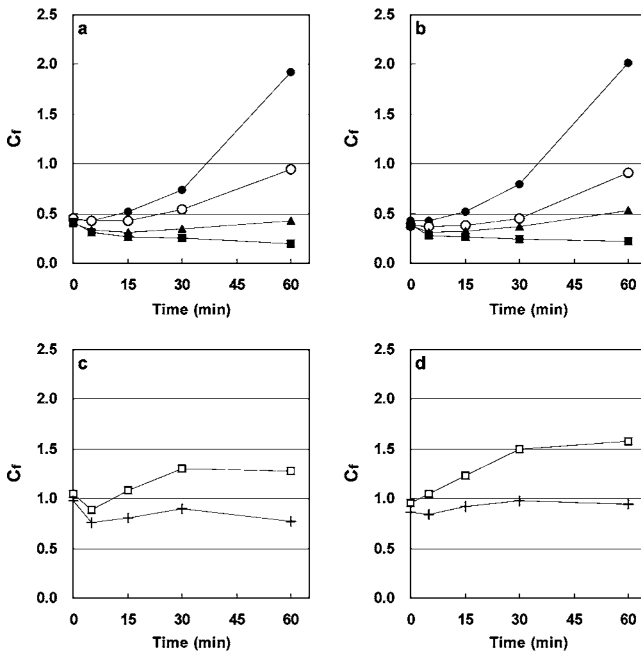

Figure 4. Changes in C_f values resulting from heat treatment in an autoclave. (**a**) Quantification using MON810 construct specific detection system; (**b**) quantification using p35S universal detection system. The target taxon specific DNA sequence was SSIIb 1 (●), SSIIb 2 (○), SSIIb 3 (▲), and SSIIb 4 (■). (**c**) Quantification using RR soy specific detection system; (**d**) quantification using p35S universal detection system. The targeted taxon specific DNA sequence was Le1n02 (□) and Le1n03 (+). The data are the means of duplicate measurements.

that of SSIIb 3; this is particularly the case with PCR systems that are to be used for T25 construct specific quantitation, in which the PCR product is 149 bp (the longest size).

In the investigation of soy, the situation becomes even more complicated. The GC contents of the PCR products of RRS 01, Le1n02, and Le1n03 were 61, 55, and 54%, respectively. Despite the GC content being higher than that of the taxon specific DNA sequence, the construct specific target region of RRS 01 (121 bp) was more degraded than that of Le1 in the early stages of heat treatment. It is difficult to account for this phenomenon by considering only the GC content. Therefore, we had to consider that factors other than the length of the PCR product and the GC content might be involved in such cases. Finally, after 30 min, a change in C_f values depending on the length of the PCR products due to random DNA degradation was observed; this was also observed in the case of maize.

C_f Values of Seed, Embryo, and Endosperm. C_f values of each fraction of the embryo and endosperm separated from eight individual seeds of five GM maize events were measured. DNA was extracted from each of these samples and eight additional individual whole seeds. The taxon specific (SSIIb 3) and construct specific DNA sequences were quantitated, and the calculated C_f values are shown in **Figure 5**. The average C_f values from whole seeds [$C_{f(seed)}$], embryos [$C_{f(emb)}$], and endosperms [$C_{f(endo)}$] were as follows. Bt11: 0.53, 0.48, and 0.54; GA21: 2.06, 2.06, and 2.28; T25: 0.41, 0.36, and 0.33; Event176: 2.43, 2.51, and 1.96; and MON810: 0.41, 0.48, and 0.33, respectively. The $C_{f(seed)}$ values for Bt11, T25, and MON810 measured using SSIIb 3 showed values similar to those reported using SSIIb 1 in our previous paper (*19*), while $C_{f(seed)}$ values for GA21 and Event176 were almost higher by 0.5 than those reported in our previous paper (*19*). Each $C_{f(seed)}$ was approximately between $C_{f(emb)}$ and $C_{f(endo)}$, except for GA21

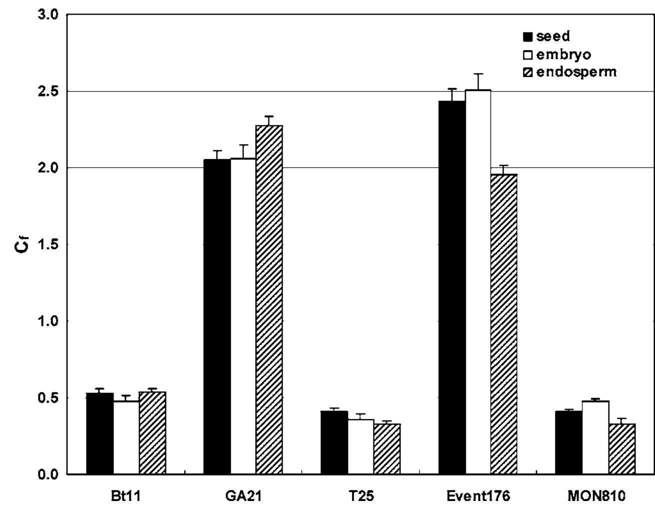

Figure 5. C_f values of the seed, embryo, and endosperm of five events of GM maize. The C_f values were calculated by the ratio of the recombinant sequence (construct specific) to the taxon specific sequence (SSIIb 3). The data are means + SD of eight preparations. The means of the C_f of the seed, embryo, and endosperm were calculated as follows. BT11: 0.53, 0.48, and 0.54; GA21: 2.06, 2.06, and 2.28; T25: 0.41, 0.36, and 0.33; Event176: 2.43, 2.51, and 1.96; and MON810: 0.41, 0.48, and 0.33, respectively.

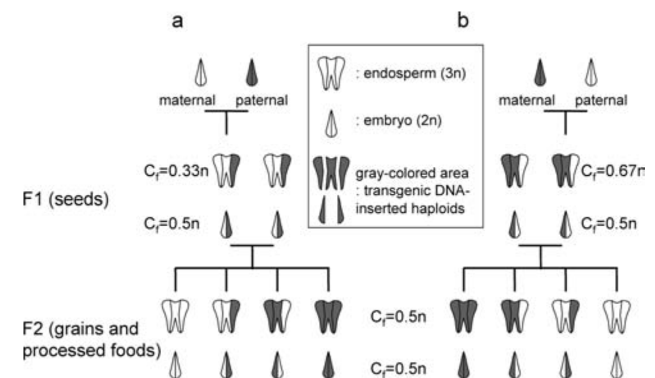

Figure 6. Illustration of the C_f determined from the endosperm and embryo of the heterosis F1 hybrid and the F2 generation in albuminous plants according to Mendelism. (**a**) F1 that was bred using paternal GM; (**b**) F1 that was bred using maternal GM; *n*, inserted copy number of the transgenic sequence. Haploids that transgenic DNA was inserted into are shown in gray.

and T25. The events showing lower $C_{f(endo)}$ than $C_{f(emb)}$ were MON810, T25, and Event176; furthermore, $C_{f(endo)}$ was higher than $C_{f(emb)}$ in Bt11 and GA21. These results suggest that the former group (MON810, T25, and Event176) is a paternal GM event, while the latter (Bt11 and GA21) is a maternal GM event.

Using the current quantification method, the GM% of an unknown sample is calculated using the C_f of DNA extracted from ground materials prepared from multiple F1 seeds. The corn grains harvested at a farm bear different seed genotypes because they are progenies of F1 hybrid seeds. Moreover, the endosperm fraction of F2 grains is milled and is primarily used as the raw materials for processed foods, such as corn snacks, after the removal of the embryos. In addition, to produce cornstarch, the seed coat, embryo, and protein-rich cornmeal fraction are removed from the corn grains. The expected C_f values from embryos and endosperms in both the F1 and the F2 generation are illustrated in **Figure 6** and are in accord with Mendelism. If a single copy of a construct of recombinant DNA was introduced to the plant genome, the $C_{f(emb)}$ of the F1 hybrid

Table 3. Applicability for the Quantification of GMOs in Foods Processed from Maize and Soy

	seed (F1)	grain (F2)	processed foods
maize	**possible**—but strictly only for GM event for which C_f was defined	**difficult**—C_f values of F2 differ from those of F1 (albuminous plant)	**very difficult**—the DNA is highly degraded; moreover, maize is an albuminous plant
soy	**possible**—but strictly only for GM event for which C_f was defined	**possible**—C_f values of F2 are the same as those of F1 (exalbuminous plant)	**difficult**—the DNA is highly degraded

seed would theoretically be expected to be 0.5 (+/−). On the other hand, the $C_{f(endo)}$ depends on differences between GM events used for F1 hybrid production; therefore, the $C_{f(endo)}$ should be 0.33 (+/−/−) or 0.67 (+/+/−) in paternally or maternally derived GM events, respectively. The C_f measured using DNA extracted from whole seeds should indicate a mean value between the $C_{f(emb)}$ and the $C_{f(endo)}$, provided the DNA amounts derived from embryo and endosperm are equivalent in each seed. On the other hand, the $C_{f(emb)}$ and the $C_{f(endo)}$ will theoretically be distributed around a value of approximately 0.5 in the F2 generation, including +/+, +/−, −/+, and −/− genotypes, in accord with Mendelism. Consequently, it may be difficult to apply the C_f of the F1 generation to F2 seeds, embryo, and endosperm.

The $C_{f(emb)}$ of T25, into which one copy of the construct of recombinant DNA was inserted, was lower than that theoretically considered at 0.5. Moreover, the $C_{f(emb)}$ of Event176 was higher than the theoretically considered values of 2.0; four copies of the construct of recombinant DNA had been inserted in Event176 strain. It is likely that these results were obtained, at least partially, due to the differences in PCR inhibition due to the DNA solution matrix or due to differences in the efficiency of DNA extraction between targeted taxon specific and recombinant DNA sequences. The present results suggest that the C_f values that were theoretically determined and the C_f values that were measured using DNA extracted from other organs would not be applicable to GM quantification, which uses the calculated ratio of taxon specific and recombinant DNA sequences. Meanwhile, the $C_{f(emb)}$ values of Bt11 and MON810 were close to the theoretically calculated value of 0.5.

In our previous collaborative study using blind samples containing appropriate amounts of GM F1 seeds with C_f values from F1 seeds, we obtained good results and thus validated the present method for practical and reliable GMO quantification in samples (*20*). On the basis of the above results, it is anticipated that the C_f of F2 grains might be distributed over a range different from that of F1 seed. Therefore, the C_f values measured using F2 grains should be applied for standard GM quantification in order to determine a more practical GM% than that obtained by considering the C_f values measured using F1 seeds because actual samples are usually planted F2 grains. However, it could be difficult to calculate the C_f using F2 grains from all of the GM events due to the contamination that occurs during cultivation in farmland. On the other hand, C_f can be theoretically determined from the introduced copy number. Some reports have adopted this approach (*14*, *17*, *27*, *28*). However, differences in the efficiency of DNA extraction or differences in PCR amplification between the two target DNA sequences may affect the quantification results. In addition, it is also possible that the C_f should be measured using DNA extracted from the leaves of plants generated from GM F1 seeds or from embryos separated from F1 seeds. However, the efficiency of DNA extraction from the leaf or embryo might differ from that of DNA extraction from seeds. It therefore remains uncertain whether a collaborative study using samples of mixed GM and non-GM DNAs extracted from leaves or from the embryo could be applied in the analysis of F2 grain samples. As a result, the current GM quantifying methods using C_f values determined from F1 seeds will continue to be the most suitable method of quantification using seeds of the albuminous plant maize.

In conclusion, on the basis of the results of the present heat processing studies, we determined that the amplification regions used to quantitate recombinant and taxon specific DNA sequences should be closely similar in terms of size in order to quantify GMOs from processed foods. To this end, primer pair SSIIb 1 was found to be less useful for the detection of maize taxon specific DNA sequence than the primer pair SSIIb 3, which was similar in size to the amplified products using a MON810 construct specific detection system. However, it is rather difficult to conclude that GM quantification for all GM events or in highly processed foods is possible.

Furthermore, in the albuminous plant maize, the C_f values determined from the F1 generation were not considered to correspond to those of F2 grains and processed foods. Theoretically, it is possible that the actual GM% might range from 0.67 [$C_{f(endo)}$ 0.33/$C_{f(emb)}$ 0.5] to 1.33 [$C_{f(endo)}$ 0.67/$C_{f(emb)}$ 0.5] times of that determined from F2 grains or processed foods, provided the ratio of DNA amounts derived from endosperm and embryo is unknown. In quantifying GM% from processed foods, we should take into account the deviation in the calculated GM% to this extent.

Considering the present results, we have summarized the expected applicability of GMO quantification to processed foods in **Table 3**. In addition, the findings of other applicability studies are available for comparison with the results of the present study (*29*).

LITERATURE CITED

(1) *Global Status of Commercialized Biotech/GM Crops: 2004, ISAAA Briefs, Vol. 32*; International Service for the Acquisition of Agri-biotech Applications: Ithaca, NY, 2004; http://www.isaaa.org/.

(2) Frewer, L.; Lassen, J.; Kettlitz, B.; Scholderer, J.; Beekman, V.; Berdal, K. G. Social aspects of genetically modified foods. *Food Chem. Toxicol.* **2004**, *42*, 1181−1193.

(3) Regulation (EC) No. 1829/2003 of the European Parliament and of the Council.

(4) *Notification No. 2000-31*; Ministry of Agriculture and Forestry of Korea: Seoul, Korea, 2000.

(5) *Notification No. 1775*; Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan: Tokyo, Japan, 2000.

(6) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Sebata, T.; Isshiki, K.; Toyoda, M.; Hino, A. A detection method for recombinant DNA from genetically modified soybeans and processed foods containing them. *J. Food Hyg. Soc. Jpn.* **1999**, *40*, 149−157.

(7) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A method of detecting recombinant DNAs from four lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2000**, *41*, 137−143.

(8) Matsuoka, T.; Kuribara, H.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2001**, *42*, 24−32.

(9) Matsuoka, T.; Kuribara, H.; Takubo, K.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J. Agric. Food Chem.* **2002**, *50*, 2100−2109.

(10) Akiyama, H.; Sugimoto, K.; Matsumoto, M.; Isuzugawa, K.; Shibuya, M.; Goda, Y.; Toyoda, M. A detection method of recombinant DNA from genetically modified potato (NewLeaf Plus− Potato) and detection of NewLeaf Plus− Potato in snack. *J. Food Hyg. Soc. Jpn.* **2002**, *43*, 24−29.

(11) Akiyama, H.; Watanabe, T.; Wakui, C.; Chiba, Y.; Shibuya, M.; Goda, Y.; Toyoda, M. A detection method of recombinant DNA from genetically modified potato (NewLeaf Y− Potato). *J. Food Hyg. Soc. Jpn.* **2002**, *43*, 301−305.

(12) Watanabe, T.; Kasama, K.; Wakui, C.; Shibuya, M.; Matsuki, A.; Akiyama, H.; Maitani, T. Laboratory-performance study of the notified methods to detect genetically modified maize (CBH351) and potato (NewLeaf Plus and NewLeaf Y). *J. Food Hyg. Soc. Jpn*. **2003**, *44*, 281−288.

(13) Terry, C. F.; Harris, N. Event-specific detection of Roundup Ready soya using two different real time PCR detection chemistries. *Eur. Food Res. Technol.* **2001**, *213*, 425−431.

(14) Taverniers, I.; Windels, P.; Van Bockstaele, E.; De Loose, M. Use of cloned DNA fragments for event-specific quantification of genetically modified organisms in pure and mixed food products. *Eur. Food Res. Technol.* **2001**, *213*, 417−424.

(15) Hübner, P.; Waiblinger, H. U.; Pietsch, K.; Brodmann, P. Validation of PCR methods for quantitation of genetically modified plants in food. *J. AOAC Int*. **2001**, *84*, 1855−1864.

(16) Brodmann, P. D.; Ilg, E. C.; Berthoud, H.; Herrmann, A. Real-time quantitative polymerase chain reaction methods for four genetically modified maize varieties and maize DNA content in food. *J. AOAC Int.* **2002**, *85*, 646−653.

(17) Permingeat, H. R.; Reggiardo, M. I.; Vallejos, R. H. Detection and quantification of transgenes in grains by multiplex and real-time PCR. *J. Agric. Food Chem.* **2002**, *50*, 4431−4436.

(18) Terry, C. F.; Shanahan, D. J.; Ballam, L. D.; Harris, N.; McDowell, D. G.; Parkes, H. C. Real-time detection of genetically modified soya using lightcycler and ABI 7700 platforms with TaqMan, Scorpion, and SYBR Green I chemistries. *J. AOAC Int.* **2002**, *85*, 938−944.

(19) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.* **2002**, *85*, 1077−1089.

(20) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. *J. AOAC Int.* **2002**, *85*, 1119−1126.

(21) Japan Agricultural Standards Testing and Analysis Handbook Series; Center for Food Quality, Labeling and Consumer Services: Saitama, Japan, 2002.

(22) *Notification No. 628001*; Department of Food Safety, Ministry of Health, Labour and Welfare of Japan: Tokyo, Japan, 2004.

(23) Guideline of Detection Methods of Genetically Modified Foods; Korean Food and Drug Administration: Seoul, Korea, 2005.

(24) Testing Manual for Genetically Modified Agricultural Products; National Agricultural Quality Management Services of Korea: Seoul, Korea, 2002.

(25) Hupfer, C.; Hotzel, H.; Sachse, K.; Engel, K. H. Detection of the genetic modification in heat-treated products of Bt maize by polymerase chain reaction. *Z. Lebensm. Unters. Forsch. A* **1998**, *206*, 203−207.

(26) Berdal, K. G.; Holst-Jensen, A. Roundup ready−Soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. *Eur. Food Res. Technol.* **2001**, *213*, 432−438.

(27) Ronning S. B.; Vaïtilingom, M.; Berdal, K. G.; Holst-Jensen, A. Event specific real-time quantitative PCR for genetically modified Bt11 maize (Zea mays). *Eur. Food Res. Technol*. **2003**, *216*, 347−354.

(28) Holst-Jensen, A.; Berdal, K. G. The modular analytical procedure and validation approach and the units of measurement for genetically modified materials in foods and feeds. *J. AOAC Int.* **2004**, *87*, 927−936.

(29) Yoshimura, T.; Kuribara, H.; Kodama, T.; Yamata, S.; Futo, S.; Watanabe, S.; Aoki, N.; Iizuka, T.; Akiyama, H.; Maitani, T.; Naito, S.; Hino, A. Comparative studies of the quantification of genetically modified organisms in foods processed from maize and soy using trial-producing. *J. Agric. Food Chem.*, **2005**, *53*, 2060−2069.

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