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 $2n$ out of $3n$ 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	

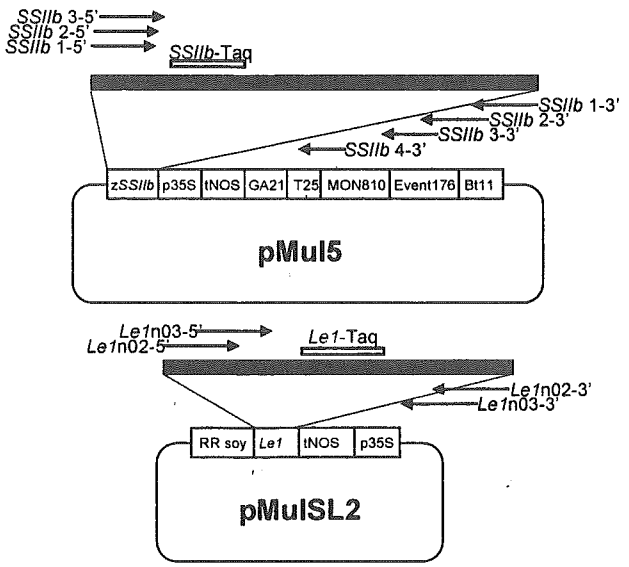

Figure 1. Schematic diagrams of the PCR systems designed to detect the taxon specific sequences of maize and soy in the construction of pMul5 and pMulSL2, respectively. SSIIb 2-5′&3′, SSIIb 3-5′&3′, and SSIIb 3-5′&4-3′ were designed within the sequence amplified by SSIIb 1-5′&3′ possessing the same TaqMan 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

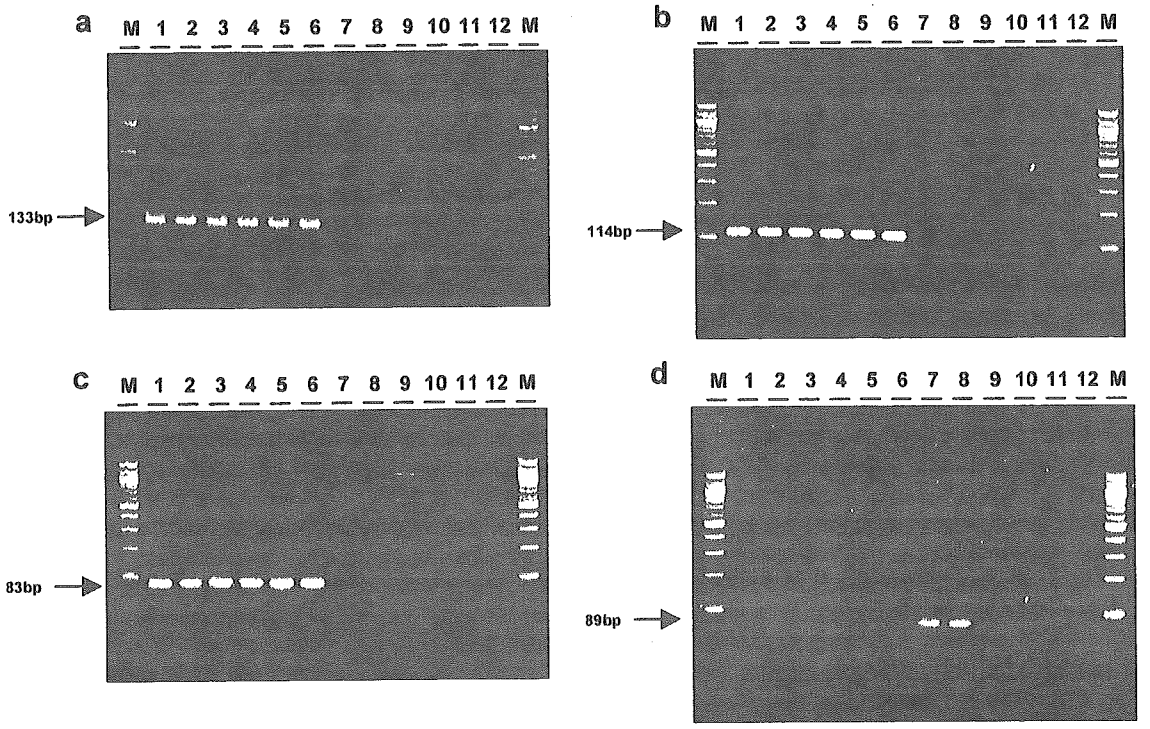

Figure 2. Agarose gel electrophoresis of PCR products amplified from maize, soy, and other cereal genomic DNAs. The arrows indicate the expected PCR amplification products. Primer pairs were used for the detection of SSIIb 2-5′&3′ (**a**), SSIIb 3-5′&3′ (**b**), SSIIb 3-5′&4-3′ (**c**), and Le1n03-5′&3′ (**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.

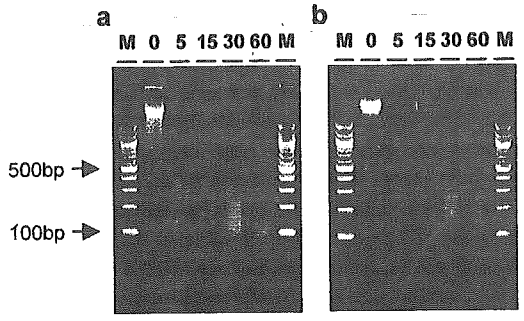

Figure 3. Agarose gel electrophoresis of DNAs extracted from heat-treated seed of maize (**a**) and soy (**b**). Lane M, 100 bp ladder size marker; lanes 0, 5, 15, 30, and 60, DNA from samples subjected to heat treatment for each of these time periods.

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_t after Heat Treatment with an Autoclave[a]

	PCR system											
	copy number			copy number			copy number			copy number		
min	SSIIb 1	M810 2	C_t	SSIIb 2	M810 2	C_t	SSIIb 3	M810 2	C_t	SSIIb 4	M810 2	C_t
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_t	SSIIb 2	P35S 1	C_t	SSIIb 3	P35S 1	C_t	SSIIb 4	P35S 1	C_t
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_t	Le1n03	RRS 01	C_t	Le1n02	P35S 1	C_t	Le1n03	P35S 1	C_t
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_t 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

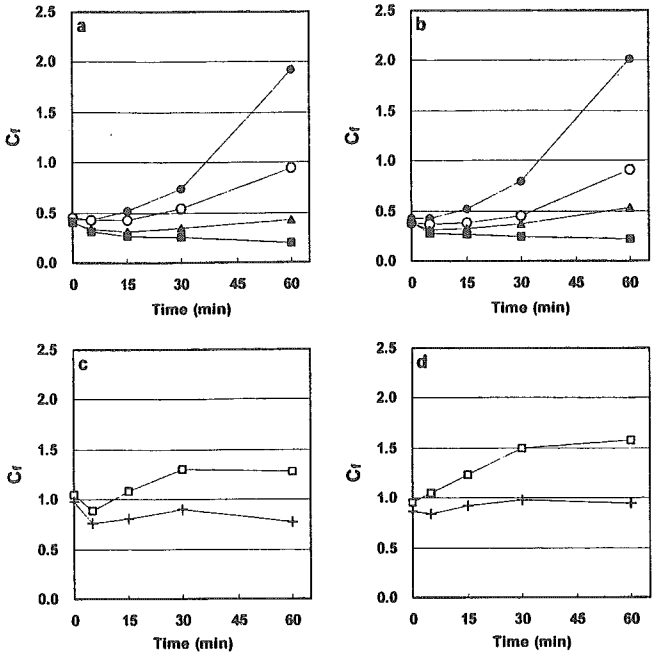

Figure 4. Changes in C_f values resulting from heat treatment in an autoclave. (**a**) Quantification using MON810 construct specific detection system; (**b**) quantification using p35S universal detection system. The target taxon specific DNA sequence was SSIIb 1 (●), SSIIb 2 (○), SSIIb 3 (▲), and SSIIb 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

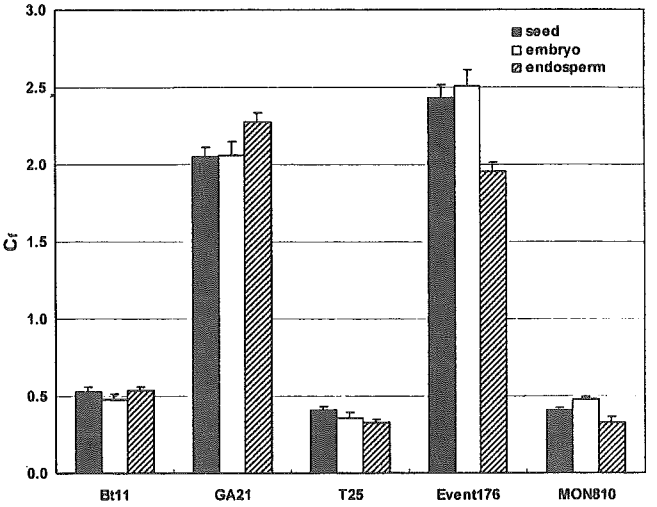

Figure 5. C_f values of the seed, embryo, and endosperm of five events of GM maize. The C_f values were calculated by the ratio of the recombinant sequence (construct specific) to the taxon specific sequence (SSIIb 3). The data are means + SD of eight preparations. The means of the C_f of the seed, embryo, 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.

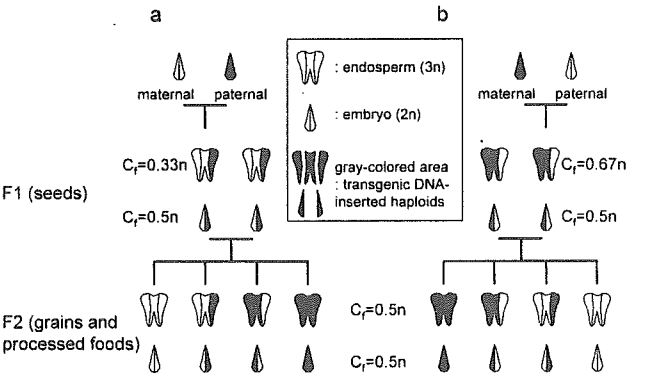

Figure 6. Illustration of the C_f determined from the endosperm and embryo of the heterosis F1 hybrid and the F2 generation in albuminous plants according to Mendelism. (**a**) F1 that was bred using paternal GM; (**b**) F1 that was bred using maternal GM; *n*, inserted copy number of the transgenic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

Received for review October 7, 2004. Revised manuscript received January 18, 2005. Accepted January 19, 2005. This study was in part supported by the Ministry of Agriculture, Forestry and Fisheries of the Japan Research Project "Assurance of Safe Use of Genetically Modified Organisms" and by a grant from the Ministry of Health, Labor and Welfare of Japan.

JF048327X

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Comparative Studies of the Quantification of Genetically Modified Organisms in Foods Processed from Maize and Soy Using Trial Producing

TOMOAKI YOSHIMURA,[†] HIDEO KURIBARA,[‡,§] TAKASHI KODAMA,[‡,§] SEIKO YAMATA,[∥] SATOSHI FUTO,[⊥] SATOSHI WATANABE,[#] NOBUTARO AOKI,[▽] TAYOSHI IIZUKA,[○] HIROSHI AKIYAMA,[¢] TAMIO MAITANI,[¢] SHIGEHIRO NAITO,[§] AND AKIHIRO HINO*[,§]

Analytical Technology Laboratory, Asahi Breweries, Ltd., 1-1-21 Midori, Moriya, Ibaraki 302-0106, Japan, Center for Food Quality, Labeling and Consumer Services, 2-1 Shintoshin, Chuo-ku, Saitama-shi, Saitama 330-9731, Japan, National Food Research Institute, 2-1-12, Kannondai, Tsukuba, Ibaraki 305-8642, Japan, Fertilizer and Feed Inspection Service, 2-1 Shintoshin, Chuo-ku, Saitama-shi, Saitama 330-9733, Japan, FASMAC Co., Ltd., 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0041, Japan, Somatech Center, House Foods Co., 1-4 Takanodai, Yotsukaido, Chiba 284-0033, Japan, Japan Food Research Laboratories, 6-11-10 Nagayama, Tama, Tokyo 206-0025, Japan, Japan Inspection Association of Food and Food Industry Environment, 3-7-4 Kyobashi, Chuo-ku, Tokyo 104-0031, Japan, and National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Seven types of processed foods, namely, cornstarch, cornmeal, corn puffs, corn chips, tofu, soy milk, and boiled beans, were trial produced from 1 and 5% (w/w) genetically modified (GM) mixed raw materials. In this report, insect resistant maize (MON810) and herbicide tolerant soy (Roundup Ready soy, 40-3-2) were used as representatives of GM maize and soy, respectively. Deoxyribonucleic acid (DNA) was extracted from the raw materials and the trial-produced processed food using two types of methods, i.e., the silica membrane method and the anion exchange method. The GM% values of these samples were quantified, and the significant differences between the raw materials and the trial-produced processed foods were statistically confirmed. There were some significant differences in the comparisons of all processed foods. However, our quantitative methods could be applied as a screening assay to tofu and soy milk because the differences in GM% between the trial-produced processed foods and their raw materials were lower than 13 and 23%, respectively. In addition, when quantitating with two primer pairs (SSIIb 3, 114 bp; SSIIb 4, 83 bp for maize and Le1n02, 118 bp; Le1n03, 89 bp for soy), which were targeted within the same taxon specific DNA sequence with different amplicon sizes, the ratios of the copy numbers of the two primer pairs (SSIIb 3/4 and Le1n02/03) decreased with time in a heat-treated processing model using an autoclave. In this report, we suggest that the degradation level of DNA in processed foods could be estimated from these ratios, and the probability of GM quantification could be experimentally predicted from the results of the trial producing.

KEYWORDS: ***Zea mays*; *Glycine max*; genetically modified organism; processed food; cornstarch; cornmeal; corn puffs; corn chips; tofu; soy milk; boiled beans; quantitative analysis**

INTRODUCTION

A new labeling system for genetically modified (GM) foods is mandatory in many countries and communities. According to these regulations, the acceptable unintended mixing of GM with non-GM materials is set at various levels ranging from 0.9 to 5% (w/w). Many qualitative and quantitative methods for GM materials have been developed to monitor the level of GM ingredients in foods, based on the presence and the amount of GM specific sequences, using polymerase chain reaction (PCR) techniques.

In our previous report (*1*), we concluded that difficulties were encountered during the quantification of GM materials in processed foods particularly in products of maize. The difficulties are not only partially due to the difference in nuclear phase between the embryo and the endosperm but also due to the

* To whom correspondence should be addressed. Tel: +81-29-838-8079. Fax: +81-29-838-7996. E-mail: akhino@nfri.affrc.go.jp.
† Asahi Breweries, Ltd..
‡ Center for Food Quality, Labeling and Consumer Services.
§ National Food Research Institute.
∥ Fertilizer and Feed Inspection Service.
⊥ FASMAC Co., Ltd..
House Foods Co..
▽ Japan Food Research Laboratories.
○ Japan Inspection Association of Food and Food Industry Environment.
¢ National Institute of Health Science.

10.1021/jf0483265 CCC: $30.25
Published on Web 03/01/2005

difference in degradation levels between the taxon specific and the recombinant deoxyribonucleic acid (DNA) sequences targeted for quantitation.

It is essential for DNA amplification by PCR that the target DNA sequence should meet several conditions involving quality, quantity, and purity. These conditions depend on the type of food, because almost all final food products in markets undergo heat, pressure, or pH treatments or are physically broken. During these treatments, genomic DNA is damaged and degraded by physical, chemical, and biological fragmentation. The degree of DNA degradation in processed foods affects the probability of detecting the target DNA by PCR. Moreover, in quantitative analysis, the difference in the ease with which endogenous and recombinant DNA sequences can be degraded affects the probability that GMO quantification can be performed in processed foods. Design that is appropriate for analysis and feasibility studies is required if correct information about the amount of GM materials in processed foods is to be obtained.

Several attempts have been taken to detect GM materials in processed foods. In the earliest investigations, it was found that the target sequence for PCR could be amplified using shorter regions than those used for raw materials in foods processed from soy (*2*−*4*), maize (*3*, *5*), and potato (*3*). It has been reported that the detection limits of Roundup Ready (RR) soy in processed meat products and trial-produced tofu were 1 (*2*) and 0.5% (*6*), respectively. A collaborative study reported that recombinant DNA sequences could be detected in various processed foods, i.e., polenta, infant formula, acidified soybeans, and biscuits, which were made from more than 2% GM raw materials (*7*). DNA degradation of processed foods was summarized in the following two reports. An investigation carried out on polenta revealed that after 65 min of processing the amplifiable DNA decreased to 40% of the amount detectable before processing (*8*). No PCR detectable DNA could be extracted from chocolate corn flakes. Various processed foods were trial produced from GM raw materials, and DNA was quantitated by PCR using primer pairs that had various amplicon sizes (*9*). In that report, it was concluded that the major degrading factor for tofu and soy milk was the mechanical treatment of soaked soybeans and that for corn masa and cooked potatoes was the thermal treatment.

In recent studies, scientists were more interested in whether the GM% obtained from processed foods reflected the original GM% of the raw materials. An attempt was made to quantify the GM% of commercial cornstarch, muesli, soy lecithin, and soybean proteins (*10*), although only the GM% of the final products was measured in the report, and it was not obvious whether the GM% of the original raw material was higher or lower than that of the processed foods. In other reports, the GM% obtained from processed foods has been compared to the original GM% using a processing model, and the possibility of quantification in processed food has been mentioned. There are two studies of trial producing using real-time PCR by LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). One study in which biscuits were produced from the soy materials including RR soy, whose GM% was known in advance, showed that the GM% values obtained for the processed foods were higher than those of the raw materials, particularly by using diluted DNA solution for real-time PCR (*11*). The other study, which produced biscuits, acidified soybeans, and infant formula from the soy materials including 10 and 100% GM soy (*12*), clarified that the GM% values obtained from the processed foods were higher than those of the raw materials, except for infant formula. Moreover, studies focused on tofu and its intermediary products (*13*), which included tofu, bean curd refuse, and soy milk made from 20 g of 1, 5, 50, and 100% GM soy seed and which compared GM% before and after processing using our previously reported GM quantification method (*14*, *15*), concluded that the GM% values obtained from the processed foods corresponded with those of the raw materials and the GM quantifying method could apply to these processed foods. However, in the above quantifying studies, it is suspicious that the original GM% values were confirmed appropriately, because it is not uncommon that the calculated GM% values were different from the theoretical GM% values because of the purities of the seeds or unexpected factors. Furthermore, even if a GM% was confirmed, the reduced sample scale (20 g) for production had a large effect on the variation of GM% in each sample preparation.

In this study, we trial produced seven processed foods (cornstarch, cornmeal, corn puffs, corn chips, tofu, soy milk, and boiled beans) with GM% values that would cause them to be considered contaminated on the commercial market (1 and 5%) from genuine source-known GM and non-GM seeds, and we statistically compared the GM% between the raw materials and the processed foods to investigate the probability of GMO quantification in the processed foods. Furthermore, to judge whether the GM% obtained from processed food is meaningful, we will discuss new indexes for processed foods throughout this study.

MATERIALS AND METHODS

Maize, Soy, and Other Cereal Samples. We obtained genuine seeds as raw materials for trial producing of the processed foods. Sixty kilograms of DK537 (GLP-0208-12885-S) and 53 kg of MON810 DKC53-32 (GLP-0208-12884-S) were used for non-GM and GM maize, respectively. Sixty kilograms of Asgrow (CON-0102-10986-S) and 20 kg of RR soy AG5602 (GRO-0006-10420-I) were used for non-GM and GM soy, respectively. All of the seeds were kindly provided by Monsanto Co. (St. Louis, MO).

DNA Extraction. A silica membrane method and an anion exchange column method were used for DNA extraction. In the silica membrane method, using the DNeasy Plant Maxi kit (DNeasy, Qiagen GmbH, Hilden, Germany), 1 g of the sample was incubated for 1 h at 65 °C after the addition of 5 mL of buffer AP1 and 10 μL of RNase A in the case of maize seeds and processed foods derived from them and 10 mL of buffer AP1 and 20 μL of RNase A for soy seeds and processed foods derived from them. The following steps were performed according to the manufacturer's instructions with the exception of a minor modification in which 1/10 volume of 3 M sodium acetate (pH 5.2) was added to the eluate for 2-propanol precipitation.

In the anion exchange column method, using Genomic-tip 20/G (G-tip)(Qiagen GmbH), 1 g of maize and soy seeds or 2 g of maize and soy processed foods were incubated for 1 h at 50 °C after the addition of 15 mL of buffer G2, 200 μL of QIAGEN Proteinase K, and 20 μL of RNase A. The following steps were performed according to the manufacturer's instructions attached to the Blood & Cell Culture DNA mini kit (Qiagen GmbH) with minor modification, namely, that the sample, after it was twice eluted with 750 μL of buffer QF, was treated with 2-propanol precipitation.

The precipitant was rinsed with 70% ethanol, dried, and then resuspended with 50 μL (20 μL for processed foods) of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA)] in both methods. The concentrations of DNA in the solutions were calculated from the absorbance at 260 nm measured by the UV spectrometer DU7000 (Beckman Coulter Inc., Fullerton, CA).

Oligonucleotide Primers and Probes. All primers and probes to quantitate taxon specific DNA sequences of maize and soy and construct specific sequences of MON810 maize and RR soy were identical with those in the previous paper (*1*, *14*, *15*). In this study, SSIIb 3 (SSIIb 3-5′ and SSIIb 3-3′ with SSIIb-Taq) and Le1n02 (Le1n02-5′ and Le1n02-3′ with Le1-Taq) were used for the quantitation of taxon

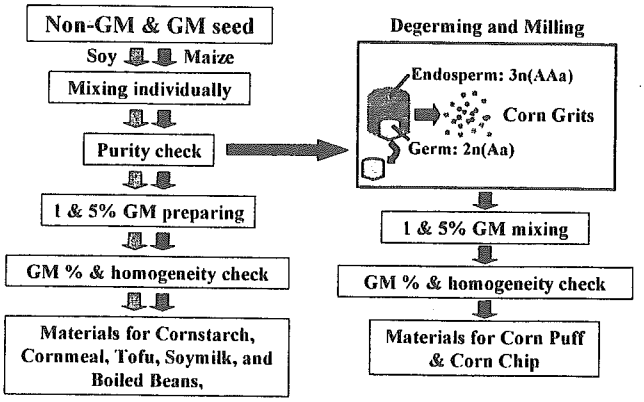

Figure 1. Flowchart of preparations for raw materials of soy and maize.

specific sequences, and SSIIb 4-(SSIIb 3-5′ and SSIIb 4-3′ with SSIIb-Taq) and Le1n03 (Le1n03-5′ and Le1n03-3′ with Le1-Taq) were also used to calculate the index of DNA degradation levels. M810-2 (M810 2-5′ and M810 2-3′ with M810-Taq) and RRS 01 (RRS 01−5′ and RRS 01-3′ with RRS-Taq) were used for the quantitation of construct specific sequences.

GM Quantification. All conditions and instruments for quantitative PCR were described in the previous paper (*1*). GM% values were calculated from the copy numbers of taxon and GM construct specific sequences in accordance with the formula described previously (*14*); however, the C_f values were newly measured in this report.

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 the Molecular Imager FX system (Bio-Rad Laboratories Inc., Hercules, CA).

Preparations for Raw Materials. A flowchart of the preparation is shown in **Figure 1**. The seeds were divided into 64 (non-GM, GM maize, and non-GM soy) or 16 (GM soy) aliquots using a chute riffler (Sample Divider, Fuji Kinzoku, Tokyo, Japan). One aliquot contained approximately 940, 840, 950, and 1250 g of seeds for non-GM maize, GM maize, non-GM soy, and GM soy, respectively. To confirm the adequacy of GM seeds, as reported previously (*14*), 32 seeds randomly sampled from one divided aliquot were individually ground with the Multi-Beads Shocker (YASUI KIKAI Co., Osaka, Japan) with a 12 mL tube holder (Type SH-123) at 1800 rpm for 30 s, and the DNA was extracted. The C_f values were measured for each seed by quantitation of the construct specific sequence and taxon specific DNA sequence. Additionally, the two randomly chosen aliquots of GM seeds were ground individually with Rotor-Speed Mill P14 (Fritsch GmbH, Idar-Oberstein, Germany) in order to determine C_f values to calculate GM% in raw materials and processed foods in this study. Moreover, the five randomly chosen aliquots of non-GM seeds were ground individually using the same mill, and the GM construct specific (MON810 and RR soy) and taxon specific DNA sequences were quantitated in order to confirm the absence of GM seeds in the aliquots.

The 1 kg of raw materials including 1 or 5% GM seeds was prepared by weighing 990 or 950 g of the non-GM seeds composed of two randomly chosen aliquots and 10 or 50 g of the GM seeds from the one randomly chosen aliquot. To produce tofu and soy milk, 10 kg of material including 1 or 5% GM seeds was also prepared as a more practical scale of production by weighing 9.9 or 9.5 kg from the remainder of the 1 kg preparation of non-GM seeds and 100 or 500 g from one randomly chosen aliquot of the GM seeds. To confirm the homogeneities in these preparations, seven 1 kg preparations of 1 and 5% GM materials were ground individually using Rotor-Speed Mill P14, DNA was extracted from the ground materials, and construct specific GM quantifications were performed with two replications for each extracted DNA. The calculated copy numbers were converted into GM% using the C_f values determined in this study. The GM% was treated with logit transformation in order to compare the between- and within-lot variances as described previously (*14*).

Corn grits, which are produced by grinding maize grains and discarding the embryos, are used as a raw material of corn puffs and corn chips. In this study, at first, the non-GM and GM corn grits were produced from non-GM and GM seeds, respectively. The 20 kg of non-GM maize seeds and the 5 kg of GM maize seeds were moisturized to a 16.7% degree of moisture and kept for 3 h at room temperature in order to make them easy to grind. The non-GM and GM seeds were independently ground with a roller type grinder (Meiji Kikai, Tokyo, Japan) and sieved with a 5 mm screen. The material that passed through the 5 mm screen was sieved with a 1800 μm screen and a 450 μm screen in turn, and the remainder of the 450 μm screen was collected. The remainder of the 1800 μm screen was reground and sieved by the same procedures. The first and second remainders of the 450 μm screen were mixed. To determine C_f to calculate GM% in processed foods derived from maize, 50 g of GM corn grits was ground, DNA was extracted, and C_f was measured by quantifying MON810 construct specifically ($n = 2$).

The 1% GM corn grits were prepared by mixing 4950 g of non-GM corn grits and 50 g of GM corn grits. Five percent GM corn grits were prepared by mixing 4750 g of non-GM corn grits and 250 g of GM corn grits. They were then divided into 64 aliquots using the divider by the same procedures described above. To confirm homogeneity in the preparation of corn grits, seven randomly chosen aliquots of the prepared 1 and 5% GM corn grits were independently ground using Rotor-Speed Mill P14. DNA was extracted from the ground corn grits, MON810 construct specific quantification was performed with two replications for each extracted DNA, and a one-way analysis of variance (*16*) was conducted as in the preparation of the 1 and 5% GM seeds.

Heat-Treated Processing Model. The 1 g of powder from 5% GM raw materials was suspended in 7.5 mL of distilled water and autoclaved with high-pressure Steam Sterilizer BS-245 (TOMY Seiko Co., Ltd., Tokyo, Japan) at 110 °C for 0, 5, 15, 30, and 60 min (each of these times does not include the time for raising and lowering the temperature). DNA was extracted from the heat-treated samples using DNeasy as described above (each for $n = 1$).

Cornstarch and Cornmeal Preparations. The 500 g of maize seeds was distributed into two polystyrene bottles, and 400 mL of sterilized water was added to each bottle. The contents were strongly shaken by hand for a few seconds, and the supernatant was disposed of. This process was repeated five times. The following steps simulated industrial cornstarch production. A volume of 650 mL of steep water including 0.2% sulfur dioxide and 0.5% lactic acid was added. The bottles were lightly shaken and heated at 53 °C for 48 h in a water bath. After decantation of the supernatant, the steeped maize seeds were transferred into a blender (TSK-953J: Tsann Kuen Japan Co., Tokyo, Japan) with 500 mL of sterilized water and were blended for 5 min.

The blended seeds were passed through the no. 6.5 (2.8 mm) mesh sieve to remove the embryos and seed coats. The remainder was spread on the mesh by a spatula and washed with 500 mL of sterilized water. The filtrate was blended for 6 min with the same blender and kept at room temperature for 45 min, and the supernatant was disposed of by decantation. The precipitate was passed through the no. 200 (75 μm) mesh sieve. The residual sample was spread on the mesh by a spatula and washed with 1 L of distilled water. The filtrate was kept at room temperature for 45 min, and the supernatant was disposed of by decantation. The precipitate was centrifuged at 3500 rpm for 15 min. The supernatant was disposed of, and cornmeal consisting of the upper part (the yellow part) of the precipitate was collected with a spatula. The collected cornmeal and remaining cornstarch (the white part) were transferred to a 0.22 μm bottle top filter, washed several times with sterilized water by vacuuming, and air-dried.

Corn Puff Preparation. The 2 kg of corn grits was puffed at 200 °C using a puff machine and cut. This process was consigned to Nippon Flour Mills Co., Ltd. (Tokyo, Japan).

Corn Chip Preparation. The 230 g of corn grits was soaked in 500 mL of 0.75% calcium hydroxide and hydrated at 49 °C for 1 h. The dough was obtained after gelatinization at 74 °C for 2 h, incubated at 60 °C for 15 h, washed by decantation, and mashed in a mortar. The dough was rolled to a sheet shape approximately 1.0 mm in thickness and was baked in an oven (RCK-10E-20 6C, Rinnai Co., Ltd., Nagoya, Japan) on both sides for 30 s each at 300 °C. The sheet

was cut, incubated at 25 °C and 70% humidity for 21 h (LH-30-02, Nagano Science Co., Ltd, Osaka, Japan), and was fried at 190 °C for 30 s with vegetable fat and oil.

Tofu and Soy Milk Preparation. The 1 kg of soy seeds was soaked in 4 L of tap water for 24 h and was then ground with Oster Blender ST-1 (Osaka Chemical, Osaka, Japan). An antifoaming agent, 0.15 g/L (final concentration) Super Emalite 300 (Riken Vitamin, Tokyo, Japan), was added, and the ground soy was heated to 95 °C, which was maintained for 5 min. The bean curd refuse "Okara" was removed with a filter cloth, and approximately 2 L of soy milk was obtained. For producing tofu, the soy milk was heated to 80 °C, and 0.28% (final concentration) glucono delta lactone (Fujiglucone, Fujisawa Pharmaceuticals Co., Osaka, Japan) was added. The mixture was kept for 1 h at room temperature in order to obtain tofu.

The 10 kg of soy seed was soaked in tap water for 20 h, ground, heated to 98 °C for 2 min with 1 kg/cm^2 steam with the antifoaming agent, and incubated at 98 °C for 3 min using NBH60S (Misuzu-co Co., Ltd., Nagano, Japan). The bean curd refuse was removed by the Screw Spin-drier MTS90-581 (Marui Industry, Osaka, Japan), and approximately 40 L of soy milk was obtained. For producing tofu, 0.75% final concentration of magnesium chloride (Akaho Kasei Co., Hyogo, Japan) was added to a portion of soy milk, kept at room temperature for 1 h, and cooled with running water.

Boiled Beans Preparation. The 1 kg of soy seeds was soaked for 15 h in a vat with a 3-fold volume of tap water after being washed in a colander and was blanched at 85 °C for 40 min. The blanched soy seeds were then washed for 10 min in a colander with running water and were distributed into 12 cans (74.0 mm inside diameter × 81.3 mm height, 318 mL volume). Following this, 120 mL of water was added, and the contents were canned under vacuum (0.4 kg/cm^2). The cans were cooled off for 15 min in tap water, following retort sterilization for 60 min at 110 °C, 1.44 kg/cm^2.

Because one can contained approximately 250 kernels (calculated as 0.33 g per kernel), it was considered that the GM% in each can varied to a large extent [95% confidence interval (*17*) of 5% GM preparation was calculated to be 2.4−7.6%]. Therefore, to extract DNA from the trial-produced boiled beans, the 12 cans were opened, and the contents were transferred into a meat chopper (MS12B-4, Matsushita Electric Industrial Co., Ltd., Osaka, Japan), followed by transfer into a 20 L large-sized mixer (460151, Aicohsha Manufacturing Co., Saitama, Japan) after being washed out with an equal volume of water.

Comparison between Processed Foods and Raw Materials. The DNA was extracted in each $n = 5$ from processed foods and their raw materials (we randomly chose five out of seven preparations for the homogeneity test) by the DNeasy and the G-tip methods. DNA degradations were confirmed by gel electrophoresis, and the GM% was quantified using primer pairs and probes of SSIIb 3 and M810 2 for maize and Le1n02 and RRS 01 for soy. In clarifying GM% differences between raw materials and processed foods, after the GM% values treated with logit transformation, the t test or the Welch test was used according to the result of the F test for homogeneity of variances. Excel 2000 (Microsoft Co., Tokyo, Japan) was used for the statistical tests.

RESULTS AND DISCUSSION

Adequacy of the Seeds. The adequacy of non-GM maize and soy seeds was investigated by determining the purity levels of the ground samples prepared from five randomly chosen aliquots using MON810 or RR soy construct specific quantification. A slight comingling of GM seeds (MON810 and RR soy) was observed in two aliquots of maize and one aliquot of soy out of five aliquots, as shown in **Table 1**. The adequacy of MON810 and RR soy seeds was studied by determining the C_f values of 32 single kernels that were randomly chosen from one aliquot of each variety. All kernels of RR soy showed the expected C_f values; however, one out of 32 seeds of MON810 did not show any amplification in construct specific quantification. The influence of the comingling mentioned above on the GM% of the raw materials prepared from the maize seeds was considered by sampling theory (*17*). The purities of GM and non-GM seeds were assumed to be 97 and 99.9%, respectively, on the assumption that the weight of a single maize seed was 0.33 g. The estimated GM% of 10 and 50 g GM seeds (consisting of 10/0.33 = 30.3 and 50/0.33 = 151.5 kernels, respectively) ranged from 91 to 100% (27.6−30.3 kernels) and 94 to 100% (142.4−151.5 kernels) at a 5% level of significance, respectively. The purity levels of both 950 and 990 g of non-GM seeds ranged from 99.8 to 100% (0−5.8 and 0−6.0 GM kernels) at a 5% level of significance. As a result, the estimated GM% in the prepared 1000 g of raw material grain samples was calculated to be 0.9−1.2% [27.6/3030 to (30.3 + 6.0)/3030] in a 1% sample and 4.7−5.2% [142.4/3030 to (151.5 + 5.8)/3030] in a 5% sample. As a result of this simulation, it was suggested that the GM mixed samples would have a rather large extent in their GM%. In conclusion, the adequacies of these seeds needed to be determined on the basis of the homogeneity test mentioned in the Homogeneity of Raw Materials section. In the case of soy preparation, the estimated range of GM% was narrower than that for maize, because the actual GM% of GM soy seed was closer to 100% than that of maize.

Table 1. Results of Purity Levels of Each Seed and C_f Values of GM Raw Materials[a]

status	material	aliquot no.	GM%	non-GM contamination (no. of kernels)	C_f
non-GM	maize	1	ND1		
		2	NA		
		3	NA		
		4	NA		
		5	ND1		
	soy	1	NA		
		2	NA		
		3	NA		
		4	ND1		
		5	NA		
GM	maize	1		1/32	0.38
		2			0.41
	soy	1		0/32	1.20
		2			1.22
	corn grits	1			0.32
		2			0.31

[a] ND1, not determined because the copy number of recombinant sequence was less than the quantitation limit (20 copies). NA, no amplification in the quantitation; blank cell, not measured.

Table 2. Evaluation of Homogeneity in Prepared 1 and 5% GM Raw Materials[a]

sample	mean GM%	95% confidence limits lower (GM%)	95% confidence limits upper (GM%)	F value	P value
maize 1%	1.2	1.1	1.4	0.96	0.51
maize 5%	5.4	4.6	6.2	1.30	0.37
soy 1%	1.1	0.69	1.8	0.13	0.99
soy 5%	6.1	5.4	6.8	0.79	0.61
corn grits 1%	0.88	0.69	1.1	0.78	0.61
corn grits 5%	4.7	4.1	5.3	2.46	0.13

[a] GM% values were quantified in each $n = 7$ using the primer pairs and probes of SSIIb 3/M810 2 for maize and Le1n02/RRS 01 for soy.

Measurement of C_f. The C_f values measured from MON810 maize, RR soy seeds, and corn grits prepared from MON810 were determined to be 0.39, 1.21, and 0.32, respectively, using the mean values of construct specific quantification as shown in **Table 1**.

Homogeneity of Raw Materials. The homogeneity of the 1 and 5% GM sample preparations was investigated by quanti-

Table 3. Concentrations and Quantitated Copy Numbers of DNA Extracted from Maize Processed Foods Using DNeasy and G-tip

	DNeasy						G-tip					
	1% GM			5% GM			1% GM			5% GM		
processed food	DNA concn (ng/μL)	copy no. taxon specific	copy no. GM specific	DNA concn (ng/μL)	copy no. taxon specific	copy no. GM specific	DNA concn (ng/μL)	copy no. taxon specific	copy no. GM specific	DNA concn (ng/μL)	copy no. taxon specific	copy no. GM specific
cornstarch	6[b]	71	<1[a]	12[b]	56	<1[a]	64	11509	61	162	15412	341
	10[b]	51	<1[a]	12[b]	55	<1[a]	78	15435	83	161	12168	313
	16[b]	30	<1[a]	14[b]	56	3[a]	82	15308	79	133	10948	312
	20	20	<1[a]	8[b]	50	<1[a]	68	15232	54	170	13026	314
	19[b]	41	<1[a]	10[b]	41	<1[a]	84	14039	55	181	13644	307
cornmeal	346	14202	67	233	10192	156	3347	17011	84	3755	15574	297
	303	13730	73	197	12353	225	3755	19850	69	3975	16225	338
	333	13841	63	298	9036	208	3755	17252	75	3676	18383	470
	395	12750	59	184	10315	208	3975	16633	71	3456	18637	446
	415	14669	67	164	8751	182	3975	19005	76	3501	15283	335
corn puff	505	<1[a]	<1[a]	639	8[a]	<1[a]	93	6[a]	<1[a]	171	446	12[a]
	875	<1[a]	<1[a]	984	7[a]	<1[a]	132	13[a]	<1[a]	111	353	10[a]
	768	<1[a]	<1[a]	597	5[a]	<1[a]	101	16[a]	<1[a]	143	485	20
	1119	<1[a]	<1[a]	746	3[a]	<1[a]	55	6[a]	<1[a]	118	625	19[a]
	906	<1[a]	<1[a]	745	6[a]	<1[a]	83	14[a]	<1[a]	170	630	19[a]
corn chip	60	9512	41	88	8367	170	2415	10692	40	2535	7448	151
	92	9230	42	69	7976	171	2610	8352	37	2230	7826	152
	44	8843	37	65	8244	175	1988	8751	34	2228	7837	162
	47	7588	29	64	9466	181	2008	9021	32	2253	7346	149
	53	6615	17[a]	199	8695	196	2160	8446	31	2238	7269	147

[a] Quantitated copy number, which was less than the quantitation limit (20 copies). [b] DNA solutions of which the DNA concentration was under 20 ng/μL.

fication of the seven aliquots. The results (F and P value and 95% confidence limit) are shown in **Table 2**. The smallest P value was 0.13, which is >0.05; therefore, the between-lot variances did not significantly differ from the within-lot variances in each preparation. Thus, we concluded that the provided seeds could be considered to be of adequate quality, and the sample preparations were sufficiently homogeneous to conduct studies for comparing GM% before and after processing.

Maize Processed Foods. The amount of DNA required for GM quantification (more than 20 ng/μL) was extracted from maize processed foods using DNeasy and G-tip with the exception of cornstarch (**Table 3**). A sufficient DNA concentration, however, was obtained from cornstarch samples with G-tip when the sample volume was changed from 2 to 5 g. The G-tip method was better suited for DNA extraction from maize processed food than the DNeasy method because excess low molecular weight saccharides are believed to inhibit the binding between DNAs and the silica membrane in the DNeasy method. The comparison of the GM% results between the raw materials and the four types of processed foods is shown in **Figure 2**. To calculate GM% for the processed foods, the C_f measured from MON810 corn grits, as shown in **Table 1**, was used because all processed foods were made from corn grits or from the remaining removed embryo.

Significant differences were observed in one out of two comparisons for cornstarch and two out of two comparisons for cornmeal using DNA extracted with the G-tip method (**Figure 2**). The GM% measured in those cases was 36−47% higher than those of the raw materials. The differences in the case of cornmeal were smaller (12 and 17%) using DNA solution with DNeasy than they were with G-tip and were not significant ($P > 0.05$).

Cornstarch and cornmeal are not directly consumed in the raw condition and are raw materials for a variety of processed foods. The most common cornstarch production method does not require heat treatment above 60 °C, except in the drying process. In this trial producing, cornstarch was dried by vacuuming and air drying, and there was no process that caused severe DNA degradation due to heating. DNA degradation could be caused by the deamination of cytosine with sulfurous acid during the steeping process (*18*).

In the comparison between cornstarch or cornmeal and raw material, the GM% values in processed foods were observed to be higher than those in raw materials, using the C_f determined from corn grits (0.32) for the calculation of GM% in the processed foods. If the C_f determined from MON810 seed (0.39) was used for cornstarch and cornmeal as well as for the raw material, the GM% was almost equivalent or slightly higher in comparison with the raw materials. Heterosis F1 GM seeds, which were heterozygous for a transgene, were used for trial producing in this study. If almost all DNAs in cornstarch and cornmeal are derived from endosperm, GM quantification using C_f determined from corn grits was reasonable. However, there is no known method for determining the exact percentage of DNA in cornstarch or cornmeal that is derived from endosperm, and there is a possibility that a small number of DNAs was eluted from the embryo during the production process. Additionally, there is no practical requirement to consider this subject in GM quantification of commercial cornstarch or cornmeal because the C_f values of F2 grains and F2 endosperm are the same in totality (*1*). In addition, as this trial producing was strictly on a laboratory scale, a study using cornstarch produced on a factory scale from F2 grain might be needed, although the procurement of pure F2 grains derived from a single F1 variety must be quite difficult.

In the case of corn puffs, the copy number of the recombinant sequence or of both recombinant and taxon specific sequences was under the quantitation limit (20 copies) regardless of the DNA extraction method used. It is assumed that considerable physical damage occurs due to the high pressure. Therefore, DNA was highly degraded (**Figure 3**) and GM% could not be quantified by this analytical method. One of the reasons for low copy number of DNA sequences might be PCR inhibition

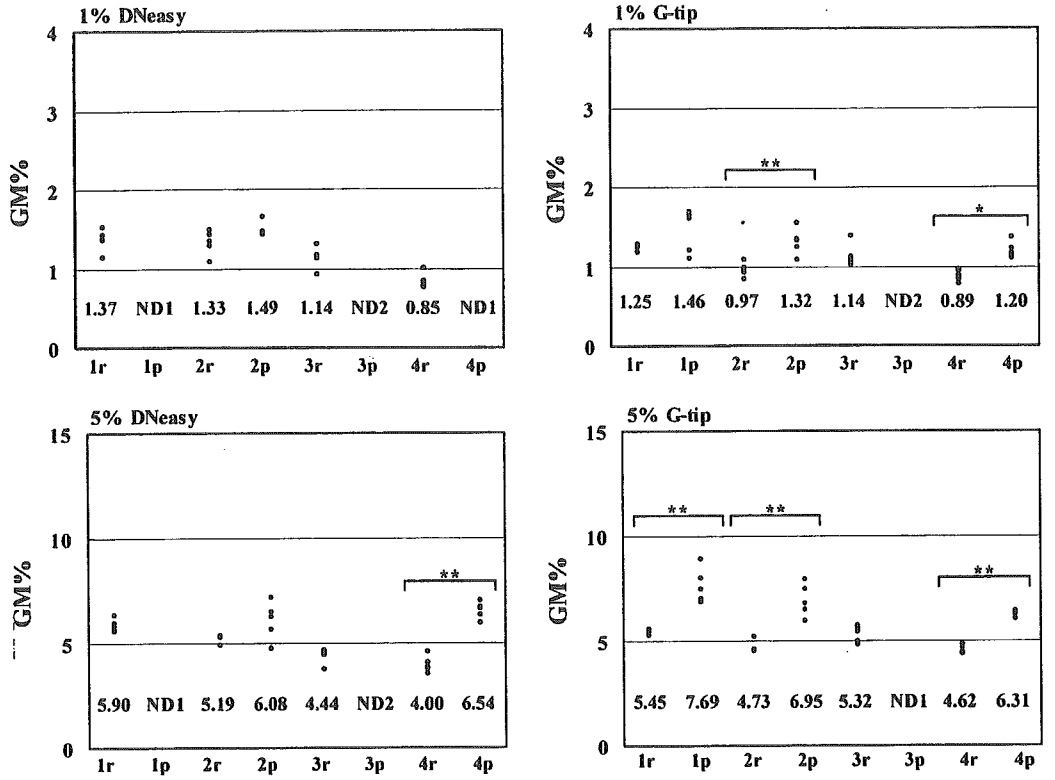

Figure 2. Comparisons of GM% between the raw materials and the processed foods in trial producing from maize. GM% values were quantified in each $n = 5$ using primer pairs and probes of SSIIb 3 and M810 2. Key: r, raw material; p, processed food; 1, cornstarch production; 2, cornmeal production; 3, corn puffs production; 4, corn chips production; *, a significant difference in the critical rate of $P < 0.05$; **, a significant difference in the critical rate of $P < 0.01$; ND1, not determined because the copy number of recombinant sequence was less than the quantitation limit (20 copies); ND2, not determined because the copy numbers of both recombinant and taxon specific sequences were less than the quantitation limit (20 copies).

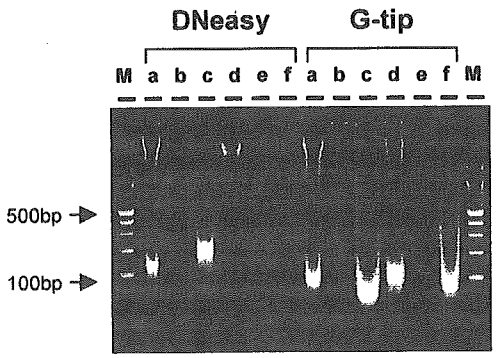

Figure 3. Agarose gel electrophoresis of genome DNAs extracted from maize and the processed foods. (a) Raw material, (b) cornstarch, (c) cornmeal, (d) corn grits, (e) corn puffs, (f) corn chips, and (M) 100 bp ladder size marker.

due to low purity. The results of the 5% sample extracted with G-tip were significantly higher than those of the raw materials, although the copy number of the recombinant sequence was under the quantitation limit. The copy number could be raised above the quantitation limit by increasing the amount of template DNA; however, it may cause PCR inhibition due to concentrated contaminants. Further investigations regarding PCR inhibition and DNA purification are needed.

In the case of corn chips, the copy number of the recombinant sequence was under the quantitation limit in the quantification of 1% GM corn chips using DNA extracted with DNeasy (only one in $n = 5$ quantification). Significant differences ($P < 0.05$) were observed in the other comparisons of 3/3 irrespective of the extraction methods. The GM% values measured in those cases were 35−64% higher than those of the raw materials. The differences were smaller using the DNA solution that was extracted with G-tip than with DNeasy; this result contradicted that of cornmeal. A sufficient amount of DNA was extracted from corn chips despite the extremely high heat condition of 300 °C for 30 s (**Table 3**), resulting in differences in the degree of degradation between taxon specific and recombinant sequences. The taxon specific sequence (SSIIb 3, 114 bp) was considerably more degraded than the recombinant sequence (M810 2, 113 bp).

In this study, corn puffs and corn chips were not seasoned with salts and seasonings, whereas a variety of salts and seasonings are used in the real product. It is believed that an inhibition of DNA extraction or PCR amplification could be caused by the contamination of the salt used; therefore, all of the commercial processed foods concerned (corn puffs and corn chips) would not show the same results.

Moreover, we quantitated the copy numbers using other primer pairs (SSIIb 4: SSIIb 3-5′ and 4-3′), in which a short amplicon size is targeted within the same taxon specific sequence. In a heat treatment study, the decreases in copy numbers with an increase in time were lower than in those using primer pairs that amplify longer regions (SSIIb 3: SSIIb 3-5′ and 3-3′), as shown in our previous study (*1*). This result showed that a longer amplicon is probably easier to degrade. Thus, there is a possibility that the ratios between the two primer sets (SSIIb 3/4) might be an index of DNA degradation.

In a heat treatment study, the GM% determined with M810 2/SSIIb 3 system was relatively stable during 60 min of treatment and did not attain a value greater than 1.3 times the GM% at 0 min (**Table 4**). At the same time, the calculated SSIIb 3/4 decreased with time and was 0.47 at 60 min in the heat treatment. Meanwhile, the GM% determined in processed foods

Table 4. Changes in GM% and the Ratio of Copy Numbers Quantitated Using Two Primer Pairs for Taxon Specific DNA Sequences by Heat Treatment Using an Autoclave[a]

	maize treatment		soy treatment	
time (min)	GM%	SSIIb 3/4	GM%	Le1n02/03
0	5.94	1.08	5.17	0.92
5	4.25	0.87	5.81	0.81
15	4.52	0.74	6.14	0.68
30	4.78	0.70	6.31	0.54
60	7.25	0.47	6.03	0.42

[a] GM% values were quantified in each $n = 1$ using primer pairs and probes of SSIIb 3/M810 2 for maize and Le1n02/RRS 01 for soy. The ratios of copy numbers of two target DNA sequences were calculated from the quantitated copy numbers in each $n = 1$ using the primers and probes of SSIIb 3/SSIIb 4 for maize and Le1n02/Le1n03 for soy.

Table 5. Ratios of Copy Numbers Quantitated for Processed Foods Produced from 5% GM Maize Using Two Primer Pairs for Taxon Specific DNA[a]

	DNeasy			G-tip		
processed	PCR system					
food	SSIIb 3	SSIIb 4	SSIIb 3/4	SSIIb 3	SSIIb 4	SSIIb 3/4
cornstarch				10779	15292	0.70
cornmeal	9110	14129	0.64	12724	18039	0.71
corn grits	22282	22043	1.01	20039	18846	1.06
corn puff	8[b]	15[b]	0.54	465	609	0.76
corn chip	6897	10035	0.69	6849	9428	0.73

[a] Copy numbers were quantitated in each $n = 1$ using the primer pairs and probe of SSIIb 3 and SSIIb 4 for the DNAs extracted using DNeasy and G-tip. [b] Quantitated copy number, which was less than the quantitation limit (20 copies).

was 1.4−1.6 times that of raw materials. Thus, it is predicted that the DNA in processed foods was more degraded than that at 60 min in the heat treatment model. However, the calculated SSIIb 3/4 of processed foods was approximately 0.6−0.7 and was not lower than that at 60 min of heat treatment, as shown in **Table 5**. Therefore, this heat treatment study was not considered to directly reflect real processing conditions; however, inclusion of an additional index as well as SSIIb 3/4 enables the prediction of the probability of GM quantification in processed foods.

Soy Processed Foods. A sufficient amount of DNA (more than 20 ng/μL) was extracted from all of the processed foods with the DNeasy and G-tip methods (**Table 6**). The comparisons of GM% values between raw materials and processed foods in trial producing from soy are shown in **Figure 4**.

Significant differences ($P < 0.01$ or $P < 0.05$) were observed in four out of eight tests in both tofu and soy milk (**Figure 4**). However, the differences in GM% values between the raw materials and the tofu or soy milk were in the range of 13 or 23%, respectively. The heating conditions were 98 °C for 3 and 5 min in 1 and 10 kg producing, respectively, and the original GM% values were retained under these conditions because a similar tendency was observed after a short duration of processing as in the heat treatment models (**Table 4**) and in our previous paper (*1*). On the other hand, no clear differences were observed in the GM% values between the 1 and 10 kg scales, except in GM5% with DNeasy, i.e., the GM% values from processed foods were lower than those from raw materials on the 10 kg scale. However, we require additional experiments to know if this difference resulted from DNA extraction methods or from trial producing method.

In the case of boiled beans, the copy numbers of the recombinant DNA sequence quantitated from the DNA extracted with the DNeasy were under the quantitation limit (20 copies), and the GM% could not be calculated. The GM% values obtained from the DNA extracted with G-tip obviously differed from those of the raw materials, e.g., in two cases, they were 63 (GM1%) and 81% (GM5%) higher than those of their raw materials. The DNA extracted from the boiled beans was highly degraded during processing at 85 °C for 40 min and at 110 °C for 60 min, as shown in **Figure 5**. These results suggest that the significant differences ($P < 0.01$) were caused by the differences in DNA degradation level between taxon specific and recombinant sequences, as shown in **Table 6**. The shift to high GM% was considered to be caused by the degree of degradation of the taxon specific DNA sequence being high as compared to that of the recombinant DNA sequence. This result suggests that a shorter region of the DNA sequence (Le1n02, 118 bp) was easier to degrade than a longer region (RRS 01, 121 bp) in this case. Therefore, we believed that factors other than the length of the DNA sequence might determine the degree of degradation.

Moreover, we quantitated the copy numbers using other primer pairs (Le1n03: Le1n03-5′ and Le1n03-3′), which have a short amplicon size targeted within the same taxon specific sequence. The decreases in the copy numbers derived using Le1n03 were lower than those derived using primer pairs that amplify longer regions (Le1n02: Le1n02-5′ and Le1n02-3′) in the previous paper (*1*). In the heat treatment studies, GM% values increased with time in comparison with the original GM% values (**Table 4**). Moreover, the calculated Le1n02/03 decreased with time and was 0.42 at 60 min. If a 20% difference in GM% values between the raw materials and the processed food was defined as the threshold for the applicability of the method, the ratio (Le1n02/03) could be permitted to attain a value of approximately 0.6 in order to quantify the GM% for processed foods. The quantitation results of Le1n02/03 in the processed foods are shown in **Table 7**. The calculated Le1n02/03 from tofu and soy milk (the determined GM% did not differ from that of raw materials) was more than 0.6, while Le1n02/Le1n03 from boiled beans (the determined GM% was approximately 1.8 times that of raw materials) was approximately 0.2. Thus, the index using Le1n02/03 might be applied to clarify the applicability of the GM quantification method, particularly for the DNA solution extracted with the DNeasy method. However, to develop a more precise index to estimate the applicability of quantification for processed foods, it is essential to design additional experiments and analyze the results.

In conclusion, variations (%) between the GM% of processed foods and those of raw materials are summarized in **Tables 8** and **9**. The maximum variations in GM% with respect to those of the raw materials were as follows: cornstarch, 41%; cornmeal, 47%; corn chips, 64%; tofu, 13%; soy milk, 23%; and boiled beans, 81%. Some significant differences were observed in all processed foods. The results suggest that it is statistically difficult to quantify GM% in processed foods in order to determine the GM% in the relevant raw materials. In addition, the quantification method could be applied as a screening assay for tofu and soy milk. However, this trial producing method was performed on a smaller scale than the methods used in factories that produce processed foods for the market, and it is only one model. Therefore, an analysis using raw materials should be performed to estimate whether a raw material was managed with appropriate identity preserved (IP) handling. Moreover, the quantification model using a compari-

Table 6. Concentrations and Quantitated Copy Numbers of DNA Extracted from Soy Processed Foods Using DNeasy and G-tip

	DNeasy						G-tip					
	1% GM			5% GM			1% GM			5% GM		
	DNA	copy no.		DNA	copy no.		DNA	copy no.		DNA	copy no.	
processed food	concn (ng/μL)	taxon specific	GM specific	concn (ng/μL)	taxon specific	GM specific	concn (ng/μL)	taxon specific	GM specific	concn (ng/μL)	taxon specific	GM specific
tofu 1 kg	101	21323	257	209	15807	948	761	37752	556	964	22226	1584
	111	20106	276	230	14241	917	759	37205	580	912	20521	1440
	119	18770	261	214	16135	1031	840	36808	581	942	21367	1523
	104	21636	275	189	17018	1123	769	39787	683	903	21711	1669
	232	23002	277	196	15047	1021	1203	32693	488	957	21016	1581
soy milk 1 kg	129	35223	407	126	19439	1348	484	57718	826	307	49683	3710
	166	26410	342	137	16361	1184	543	53726	837	318	47252	3565
	143	26788	345	142	16357	1148	525	46137	735	304	49990	4747
	181	27703	336	178	20092	1417	494	47175	708	329	51889	3014
	155	26732	317	142	15904	1118	362	55639	883	300	48813	3838
boiled beans 1 kg	271	454	12[a]	238	373	25	628	4908	97	680	4716	574
	148	877	15[a]	307	214	20	654	4539	105	690	4059	522
	209	73	3[a]	119	542	48	636	4713	79	560	4293	498
	149	819	15[a]	386	237	13[a]	790	4380	98	652	4329	487
	149	914	17[a]	261	251	18[a]	606	4996	90	876	4173	529
tofu 10 kg	205	49951	636	175	56294	2792	1005	58625	798	1015	61887	4401
	252	52624	620	156	53715	2892	1088	53667	806	1048	51998	3695
	221	50601	607	171	56213	2932	1046	62670	917	1034	54966	3940
	217	49508	627	167	51252	2780	1042	58625	874	1033	51856	3874
	215	53881	642	176	51215	2740	1151	54707	782	1041	60701	4419
soy milk 10 kg	155	50783	640	150	46767	2575	858	64486	973	793	67847	4616
	142	50421	634	146	41373	2307	807	65758	1037	937	62584	4406
	132	52721	682	187	35515	1936	916	64338	1034	844	60527	4292
	132	50052	676	133	47150	2746	862	70072	1058	892	63740	4546
	216	42992	482	180	38056	2245	887	68347	1105	880	65480	4615

[a] Quantitated copy number, which was less than the quantitation limit (20 copies).

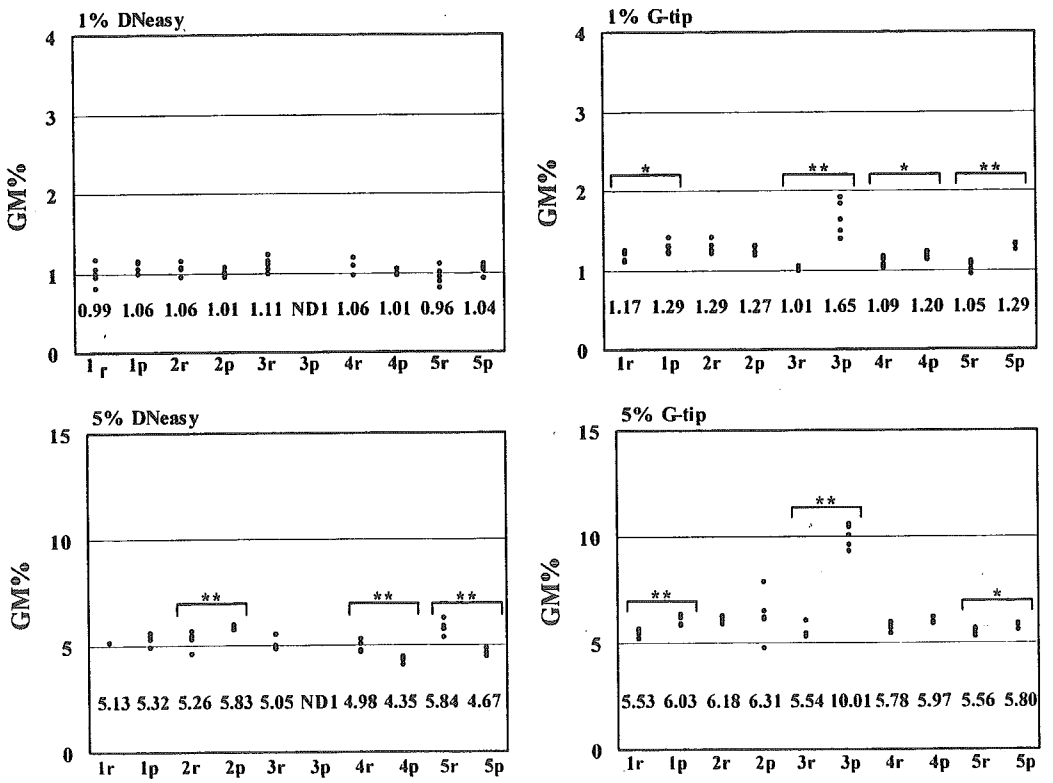

Figure 4. Comparisons of GM% between raw materials and processed foods in trial producing from soy. GM% values were quantified in each $n = 5$ using primer pairs and probes of Le1n02 and RRS 01. Key: r, raw material; p, processed food; 1, tofu 1 kg production; 2, soy milk 1 kg production; 3, boiled beans 1 kg production; 4, tofu 10 kg production; 5, soy milk 10 kg production; *, a significant difference in the critical rate of $P < 0.05$; **, a significant difference in the critical rate of $P < 0.01$; ND1, not determined because the copy number of recombinant sequence was less than the quantitation limit (20 copies).

son of Le1n02/03 could be an index for the propriety of GM quantification and a tool for managing IP handling. Further studies including development of new index(es) for processed foods will be needed.

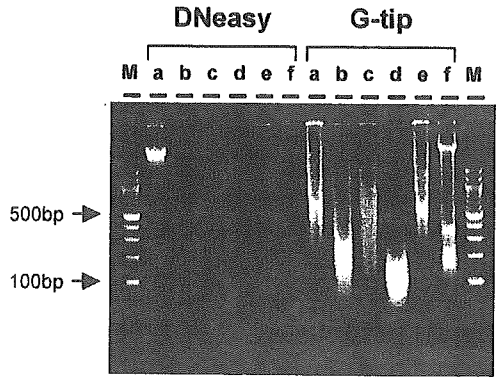

Figure 5. Agarose gel electrophoresis of genomic DNA extracted from soy and the processed foods. (a) Raw material, (b) tofu 1 kg, (c) soy milk 1 kg, (d) boiled beans 1 kg, (e) tofu 10 kg, (f) soy milk 10 kg, and (M) 100 bp ladder size marker.

Table 7. Ratios of Copy Numbers Quantitated for Processed Foods Produced from 5% GM Soy Using with Two Primer Pairs for Taxon Specific DNA[a]

	DNeasy			G-tip		
	PCR system					
processed food	Le1n02	Le1n03	Le1n02/03	Le1n02	Le1n03	Le1n02/03
tofu 1 kg	14753	20509	0.72	20680	31271	0.66
soy milk 1 kg	16686	24264	0.69	45334	75763	0.60
boiled beans 1 kg	147	754	0.20	947	4077	0.23
tofu 10 kg	53329	63166	0.84	57049	75969	0.75
soy milk 10 kg	40095	49786	0.81	39491	49675	0.79

[a] Copy numbers were quantitated in each $n = 1$ using primer pairs and probe of Le1n02 and Le1n03 for the DNAs extracted using DNeasy and G-tip.

Table 8. Variations (%) between the GM% of Maize Processed Foods and Raw Materials

	cornstarch	cornmeal	corn puff	corn chip
1% DNeasy	ND1	12	ND2	ND1
1% G-tip	17	36[b]	ND2	35[a]
5% DNeasy	ND1	17	ND2	64[b]
5% G-tip	41[b]	47[b]	ND1	37[b]

[a] Significant difference in $P < 0.05$. [b] Significant difference in $P < 0.01$. ND1, not determined because the copy number of recombinant sequence was less than the quantitation limit (20 copies); ND2, not determined because the copy numbers of both recombinant and taxon specific sequences were less than the quantitation limit (20 copies).

Table 9. Variations (%) between the GM% of Soy Processed Foods and Raw Materials

	tofu (kg)		soy milk (kg)		boiled beans (kg)
	1	10	1	10	1
1% DNeasy	7	−5	−5	8	ND1
1% G-tip	10[a]	10[a]	−2	23[b]	63[b]
5% DNeasy	4	−13[b]	11[b]	−20[b]	ND1
5% G-tip	9[b]	3	2	4[a]	81[b]

[a] Significant difference in $P < 0.05$. [b] Significant difference in $P < 0.01$. ND1, not determined because the copy number of recombinant sequence was less than the quantitation limit (20 copies).

ACKNOWLEDGMENT

We sincerely thank Monsanto Co. for providing the valuable seeds. Thanks are also due to Dr. Tetsuhisa Goto (National Food Research Institute, Ibaraki, Japan; current affiliation: Shinshu University, Nagano, Japan) for his excellent suggestions regarding the statistical aspects of this study.

LITERATURE CITED

(1) Yoshimura, T.; Kuribara, H.; Matsuoka, T.; Kodama, T.; Iida, M.; Watanabe, T.; Akiyama, H.; Maitani, T.; Furui, S.; Hino, A. Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. *J. Agric. Food Chem.* **2005**, *53*, 2052−2059.

(2) Meyer, R.; Chrdonnens, F.; Hübner, P.; Lüthy, J. Polymerase chain reaction (PCR) in the quality and safety assurance of food: Detection of soya in processed meat products. *Z. Lebensm. Unters. Forsch.* **1996**, *203*, 339−344.

(3) Greiner, R.; Konietzny, U.; Jany, K. D. Is there any possibility of detecting the use of genetic engineering in processed foods? *Z. Ernährungswiss* **1997**, *36*, 155−160.

(4) Van-Hoff, A. M. A.; Kok, E. J.; Bou, E.; Kuiper, H. A.; Keijer, J. Development and application of a selective detection method for genetically modified soy and soy-derived products. *Food Addit. Contam.* **1998**, *15* (7), 767−774.

(5) 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.* **1998**, *206*, 203−207.

(6) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, M.; 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) Lipp, M.; Bluth, A.; Eyquem, F.; Kruse, L.; Schimmel, H.; Van den Eede, G.; Anklam, E. Validation of a method based on polymerase chain reaction for the detection of genetically modified organisms in various processed foodstuffs. *Eur. Food Res. Technol.* **2001**, *212*, 497−504.

(8) Rizzi, A.; Panebianco, L.; Giaccu, D.; Sorlini, C.; Daffonchio, D. Stability and recovery of maize DNA during food processing. *Ital. J. Food Sci.* **2003**, *15*, 499−510.

(9) Kharazmi, M.; Bauner, T.; Hammes, W. P.; Hertel, C. Effect of food processing on the fate of DNA with regard to degradation and transformation capability in *Bacillus subtilis*. *Syst. Appl. Microbiol.* **2003**, *26*, 495−501.

(10) Vaïtilingom, M.; Pijnenburg, H.; Gendre, F.; Bringnon, P. Real-time quantitative PCR detection of genetically modified maximizer maize and roundup ready soybean in some representative foods. *J. Agric. Food Chem.* **1999**, *475*, 261−266.

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

(12) Berdal, G. K.; 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.

(13) Ogasawara, T.; Arakawa, F.; Akiyama, H.; Goda, Y.; Ozeki, Y. Fragmentation of DNAs of processed foods made from genetically modified soybeans. *Jpn. J. Food Chem.* **2003**, *10* (3), 155−160.

(14) 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.

(15) 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*, 1119−1125.

(16) Fisher, R. A. *Statistical Methods for Research Workers*; Oliver and Boyd Ltd.: Edinburgh, 1925.
(17) Cochran, W. G. *Sampling Techniques*, 3rd ed.; John Wiley & Sons: New York, 1977; pp 57−58.
(18) Gawienowski, M. C.; Eckhoff, S. R.; Yang, P.; Rayapati, P. J.; Binder, T.; Briskin, D. P. Fate of maize DNA during steeping, wet-milling, and processing. *Cereal Chem.* **1999**, *76* (3), 371−374.

Received for review October 7, 2004. Revised manuscript received January 17, 2005. Accepted January 19, 2005. Parts of this study were supported by the Ministry of Agriculture, Forestry and Fisheries of the Japan Research Project, "Assurance of Safe Use of Genetically Modified Organisms" and by a grant from the Ministry of Health, Labor and Welfare of Japan.

JF0483265

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Regulatory Toxicology and Pharmacology 44 (2006) 182–188
Regulatory Toxicology and Pharmacology
www.elsevier.com/locate/yrtph

Improved ELISA method for screening human antigen-specific IgE and its application for monitoring specific IgE for novel proteins in genetically modified foods

Kayoko Takagi, Reiko Teshima *, Osamu Nakajima, Haruyo Okunuki, Jun-ichi Sawada

Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received 24 August 2005
Available online 20 December 2005

Abstract

For monitoring the occurrence of IgE antibody specific for novel proteins in genetically modified (GM) foods, ELISA is the most convenient method. The levels of IgE specific for recombinant proteins, phosphinothricin-*N*-acetyltransferase (PAT), CP4-EPSPS, and Cry9C were determined by ELISA using the sera from patients allergic to known allergens. Ovalbumin (OVA) and OVA-positive patient sera were used as positive control. In the ELISA, 20-fold-diluted sera tested were mostly negative for the specific IgE. However, the PAT-specific, but not CP4-EPSPS- or Cry9C-specific IgE in some patients was apparently higher than that of the healthy volunteers. To clarify the binding specificity of the antibody, we pre-incubated the sera with soluble PAT, but the inhibition was marginal, suggesting that the binding was non-specific. Therefore, we used 1 M NaCl as a washing buffer to remove IgE non-specifically bound to the coated PAT. This washing step efficiently decreased non-specific binding. In contrast, OVA-specific IgE binding to OVA-coated plate was not affected by the washing. Finally, in this pilot study significant levels of IgE antibodies specific for the three proteins were not detected in the sera of Japanese food-allergy patients.

Keywords: Genetically modified food; Human IgE: ELISA; Phosphinothricin acetyltransferase; 5-Enolpyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4; Cry9C

1. Introduction

Prediction of potential allergenicity is one of the major issues in the safety assessments of genetically modified (GM) crops. As an important step in assessing whether a protein is potentially allergenic or not, it is recommended to compare its amino acid sequence with those of known allergenic proteins (Gendel, 2002). In a joint FAO/WHO expert consultation (2001), it was proposed that the amino acid sequence of a protein should be compared with all known allergenic proteins retrieved from protein databases to identify any stretches of 80 amino acids with more than 35% similarity or any small identical peptides of at least 6 amino acids; and that the outcome of such comparisons should then be combined with other information on allergenicity, such as its digestibility and binding to IgE in sera from allergic patients.

However, the standard method of serum test for specific IgE binding to query proteins is not fully established to date. ELISA method is convenient and sensitive method for determining antibody production in animal serum, we modified and optimized the conventional ELISA method for applying human patient serum. By using the optimized ELISA method, we performed serum test for assaying the binding capacity of serum IgE to novel proteins introduced into GM foods to investigate the cross-reactivity of specific IgE antibodies for between known allergens and novel proteins, and to assess the possibility of acquiring novel protein-specific IgE through the ingestion of GM crops.

* Corresponding author. Fax: +81 3 3707 6950.
E-mail address: rteshima@nihs.go.jp (R. Teshima).

0273-2300/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yrtph.2005.11.002

As for novel proteins, we used three recombinant proteins: phosphinothricin acetyltransferase (PAT), 5-enolpyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (CP4-EPSPS), and a *Bacillus thuringiensis* δ-toxin, Cry9C. PAT is encoded by the *pat* gene from *Streptomyces viridochromogenes*, which has been introduced into several plants and confers tolerance to a herbicide glufosinate. PAT is neither toxic nor possesses any of the characteristics associated with food allergens (Herouet et al., 2005); and it can be easily digested in the simulated gastric solution (Takagi et al., 2003). CP4-EPSPS also confers tolerance to the herbicide glyphosate on the host plants. It has been shown to be easily digested (Okunuki et al., 2002), and no immunotoxicity was found in the rats or mice fed CP4-EPSPS-containing GM soybeans (Teshima et al., 2000). Cry9C, an insecticidal protein (Bucchini and Goldman, 2002) was introduced into StarLink corn (Aventis Crop Science, USA LP). In Japan, GM foods containing PAT and CP4-EPSPS but not Cry9C were approved in 1996. Cry9C was only permitted for feed use in the United States.

2. Methods

2.1. Serum samples

Most of the human serum samples were obtained from Japanese allergic patients positive for allergen-specific IgE in the Immuno-CAP (Pharmacia Diagnostics, Uppsala, Sweden) method and clinical allergic symptoms. The allergic serum samples were collected either between 1990 and 1992 (Group A, 29 samples) or between 1999 and 2004 (Group B, 132 samples). Some sera commercially available from patients in the United States (Plasma Lab International, Everett, WA, USA) were also used (Group C, 23 samples). The sera collected between 1990 and 1992 were used as the sera of the pre-GM food period. Sera from healthy volunteers (specific IgE-negative) in Japan were used as a control group (Group D, 9 samples). Nineteen of the Group B patients (14.4%) were allergic to soybeans and two (1.5%) were allergic to corn. Informed consent was obtained from all the patients and volunteers. This study was approved by the Institutional Review Board of the National Institute of Health Sciences.

Mouse anti-phosphinothricin acetyltransferase (PAT) antiserum was used as a positive control serum for ELISA. A BALB/c mouse was immunized (ip) 3 times at a 10-day interval with 25 μg of PAT in 1 mg Alum. The IgE antibody titer was 3540 as determined by ELISA.

2.2. Recombinant proteins

Recombinant PAT and Cry9C were obtained from Bayer CropScience (Research Triangle Park, NC, USA). 5-Enolpyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (CP4-EPSPS) was produced by *Escherichia coli* clone BL21 cells transferred by CP4-EPSPS cDNA-pET23b(+) (BL21-EPPET7) and purified, as previously reported (Okunuki et al., 2002).

2.3. ELISA and inhibition assay

Assay plates (96 wells) were coated with each protein [0.2 μg/50μl of 50 mM sodium carbonate buffer (pH 9.6)/well] and incubated at 4 °C overnight. After washing with phosphate-buffered saline (PBS, pH 7.0) containing 0.05% Tween 20 (PBS-T), the plates were blocked with PBS containing 0.1% casein for 2 h at room temperature, washed with PBS-T, and incubated with serum samples diluted to 5% in 0.1% casein–PBS at 4 °C overnight. Then the wells were washed with PBS-T (or PBS-T containing 1 M NaCl in the improved method).

For fluorometric detection, the wells were incubated with rabbit anti-human IgE antibodies (Nordic Immunology, Tilburg, Netherlands; 1:1000 in PBS containing 0.1% casein). After 1 h incubation at room temperature and washing with PBS-T, a 50 μl of β-galactosidase-conjugated anti-rabbit Ig (Amersham Pharmcia Biotech; 1:1000 in 0.1% casein–PBS) was added to each well, and the plates were incubated for 1 h at room temperature and washed. The wells were incubated for 1 h at 37 °C with 100 μl of PBS containing 0.1 mM 4-methylumbelliferyl-β-galactoside (Sigma). Finally, 25 μl of 1 M sodium carbonate was added to each well. The fluorescence intensity of liberated 4-methylumbelliferone was monitored at 365 and 460 nm for excitation and emission, respectively.

For colorimetric detection, the wells were sequentially treated with peroxidase-conjugated goat anti-human IgE antibodies (Nordic Immunology, 1:1000) in 0.1% casein–PBS for 1 h at room temperature and reacted with a substrate solution (TMB reagent, Cat. No. 555214; BD Biosciences, San Diego, CA). The colorimetric intensity (OD_{450}–OD_{562}) was measured according to the manufacturer's protocol.

We judged to be ELISA-positive when the fluorescence intensity or the absorbance for a patients serum was greater than the value of the average + 5SD of control sera (Yagami et al., 1998).

For the inhibition assay, human serum (diluted at 1:20) samples were pre-incubated overnight at 4 °C with or without the novel protein to be tested before addition to the wells.

Anti-ovalbumin (OVA, A-5503, Sigma) ELISA and the inhibition assay were performed as the same method with the novel proteins.

As for murine anti-PAT ELISA, murine anti-PAT antiserum (1:2000 or 1:5000 diluted) with or without PAT were applied on the PAT-coated plates and analyzed in the same manner with human serum except using rabbit anti-mouse IgE antibodies (1:1000 in PBS containing 0.1% casein, RAM/IgE[Fc]; Nordic Immunology) as the second antibody.

2.4. Western blot analysis of serum IgE to GM or non-GM soybean extracts

Extract of GM (CP4-EPSPS-introduced) or non-GM soybeans was prepared as previously described (Okunuki et al., 2002). Each extract was loaded on a SDS–PAGE 2D-gel (167 μg protein/cm, 4–20% acrylamide) and separated by electrophoresis. Then proteins were transferred onto 0.22 μm nitrocellulose membranes (Schleicher & Schuell, Germany) by application at a constant current of 37 mA for 20 h with an electroblotter (Bio-Rad, Mini Trans-Blot Cell). To block the unoccupied sites, the membrane was incubated for 2 h at room temperature with 0.5% casein dissolved in PBS (pH 7.1). Thereafter, the transferred proteins were incubated with 4- or 40-times diluted sera for 1 h at room temperature and then overnight at 4 °C. After washing with PBS containing 0.05% Tween 20, the membrane was incubated with 500-times diluted peroxidase-conjugated goat anti-human IgE for 1.5 h at room temperature and then stained with substrate solution (Immunostain HRP-1000, Konica, Tokyo, Japan).

3. Results

3.1. ELISA conditions

IgE-ELISA conditions were investigated based on the reported method (Raybourne et al., 2003). The serum dilution and the amount of coating antigen were selected by anti-OVA IgE positive serum tests results. Fig. 1 shows the result of one of the typical OVA-specific IgE-positive sera (P4) and a control serum (C3). The resulted absorption was the most clearly distinguished at a dilution of 1:20 and the antigen-coating concentrations, 0.2 and 1.0 μg/well, resulted similar absorbance at a dilution of 1:20 in these sera. According to the OVA-ELISA method, we fixed serum

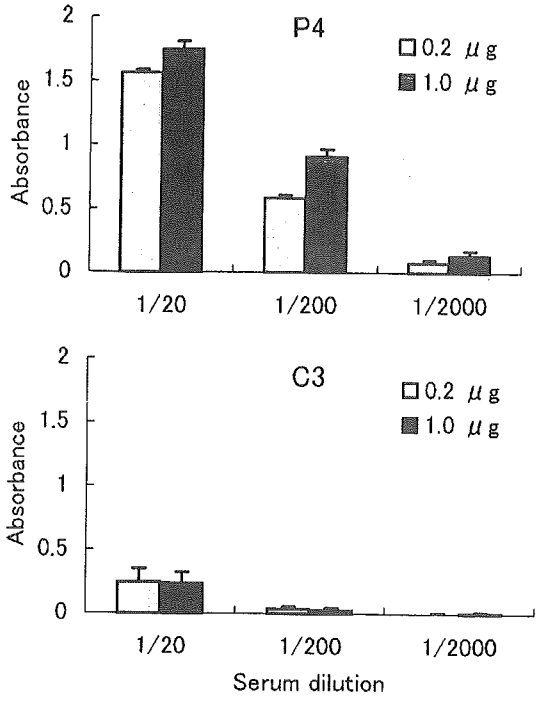

Fig. 1. Conditioning of the serum dilution and the amount of coating antigen. An OVA-positive patient serum (P4) and a control serum (C3) were diluted in PBS containing 0.1% casein at indicated dilutions and applied to OVA-coated wells [0.2 μg/50 μl/well (▨) or 1.0 μg/50 μl/well (■)]. After incubation, the wells were washed with PBS-T and the bound IgE was detected by colorimetric method. Values are the mean of triplicate analyses.

dilution of 1:20, and concentration of coating antigen at 0.2 μg/well for novel protein–ELISA.

Raybourne et al. (2003) used PBS containing 10% heat-inactivated fetal bovine serum (FBS) as a blocking reagent and PBS containing 5% FBS and 0.05% Tween 20 as a serum dilution buffer for detecting human serum IgE reactive with Cry9C in their ELISA system. In this study, we used 0.1% casein–PBS as a blocking and dilution buffer because the amino acid sequences of the three proteins tested showed no homologies with the casein sequences. The effects of 10% FBS and 0.1% casein–PBS were compared in our ELISA system, and similar results were obtained (compare A and E, or B and F in Fig. 3).

3.2. Prescreening of allergic patient sera

The sera from ca. 30 allergic patients of Group B (collected between 1999 and 2004) were preliminarily tested for specific IgE. Both ELISA methods using colorimetry and fluorometry gave almost the same results (data not shown). The data indicated that the values of absorbance or fluorescence intensity in Group B sera for Cry 9C or CP4-EPSPS was comparable to the average or less than the average + 5SD of that of Group D (healthy subjects). On the other hand, the values for PAT in 3 sera (P1, P2, and P3) of Group B were apparently higher (ca. 2-fold) than Group D average. Then, we tested the 3 sera for the occurrence of PAT-specific IgE by Western blotting, but the results were negative (data not shown).

3.3. ELISA inhibition

To clarify the reason of the non-specific binding between serum IgE from the patients and PAT, the sera were pre-incubated with PAT. The effect of pre-incubation of the sera (P1, P2, and P3) and 2 healthy volunteers (C1 and C2) with PAT on the ELISA (anti-PAT IgE-ELISA) is shown in Fig. 2A. The fluorescence intensity for serum P1 was slightly (20%) reduced only after pre-incubation with the highest concentration of PAT (1000 ng/ml), while those for sera P2 and P3 was not significantly affected by the pre-incubation. On the contrary, under similar conditions for a Der f 2 (mite allergen)-specific IgE-positive serum, a marked inhibition was observed (Fig. 2B). Namely, Der f 2 dose-dependently inhibited the binding of anti-Der f 2 IgE to the Der f 2-coated wells [IC_{50} was approximately 2.5 nM (35 ng/ml)]. The P1 patient was known to be positive for egg white-specific IgE, based on the results of Immuno-CAP (7.6 IU/ml), and the serum IgE strongly bound to the ovalbumin (OVA)-coated wells. Therefore, the inhibition by OVA was examined as a positive control. The fluorescence intensity was remarkably and dose-dependently decreased by the pre-incubation of the P1 serum with OVA [the IC_{50} was approximately 0.6 nM (25 ng/ml)].

An additional inhibition assay was performed with the system using murine anti-PAT serum and PAT-coated wells (Fig. 2D) for the positive control of specific inhibition of antibody binding to PAT. The binding of IgE in 1:2000- or 1:5000-diluted serum was clearly inhibited by pre-incubation with PAT [the IC_{50} was approximately 1.7 and 1.1 nM (35 and 22 ng/ml), respectively].

These ELISA inhibition data show that the inhibitions by the competitive antigens can be observed around nM levels for the specific IgE antibodies to known allergens in allergic patients. However, almost no significant inhibition was observed in the sera P1, P2, and P3, suggesting that binding seems to be non-specific.

3.4. Effect of plate washing on ELISA results

To further confirm the non-specific binding of antibody to the coated antigen, we added a step of washing with 1 M NaCl following incubation with patient sera.

The binding to the PAT-coated wells was clearly reduced by NaCl-washing in sera P1 and P2 (Figs. 3A and C), whereas both sera P1 and P2 (positive for OVA-specific IgE), were still reactive with OVA-coated well even after NaCl-washing (Figs. 3B and D). Reactivity of another OVA-specific IgE-positive serum (P4) was not affected by the NaCl-washing (Figs. 3A and B).

Similar results were obtained in both fluorometric and colorimetric assays. These findings indicate that the IgE binding to coated PAT for the three sera was caused by weak non-specific interactions. As for Cry9C and CP4-EPSPS, the NaCl-washing did not significantly affect the IgE binding to these proteins (data not shown). As shown in Figs. 3A–D, our colorimetric method has sufficient sensi-