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

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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

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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

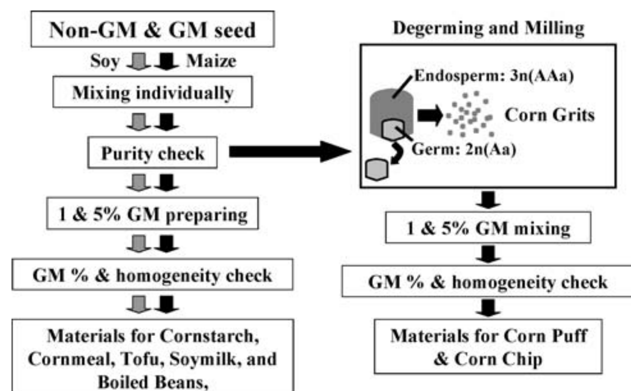

Figure 1. Flowchart of preparations for raw materials of soy and maize.

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		
	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
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

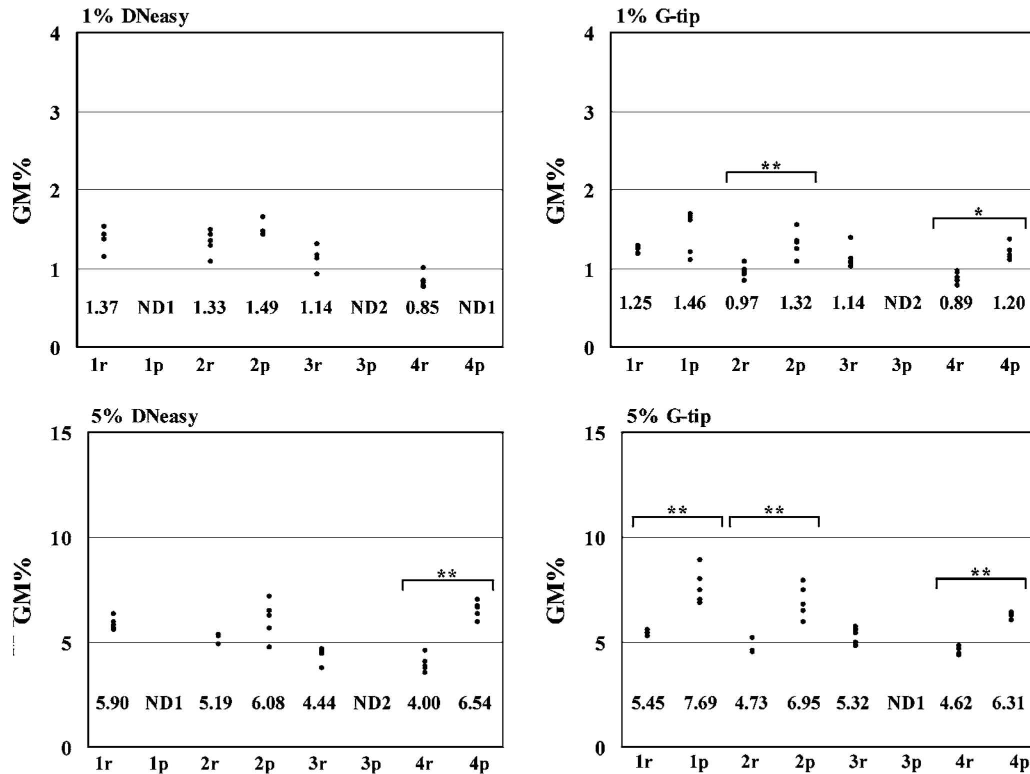

Figure 2. Comparisons of GM% between the raw materials and the processed foods in trial producing from maize. GM% values were quantified in each $n = 5$ using primer pairs and probes of SSIIb 3 and M810 2. Key: r, raw material; p, processed food; 1, cornstarch production; 2, cornmeal production; 3, corn 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).

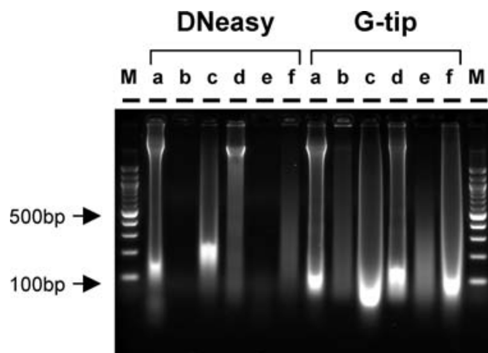

Figure 3. Agarose gel electrophoresis of genome DNAs extracted from maize and the processed foods. (a) Raw material, (b) cornstarch, (c) cornmeal, (d) corn grits, (e) corn puffs, (f) corn chips, and (M) 100 bp ladder size marker.

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		
	PCR system					
processed 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		
		copy no.			copy no.			copy no.			copy no.	
processed food	DNA concn (ng/μL)	taxon specific	GM specific	DNA concn (ng/μL)	taxon specific	GM specific	DNA concn (ng/μL)	taxon specific	GM specific	DNA 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).

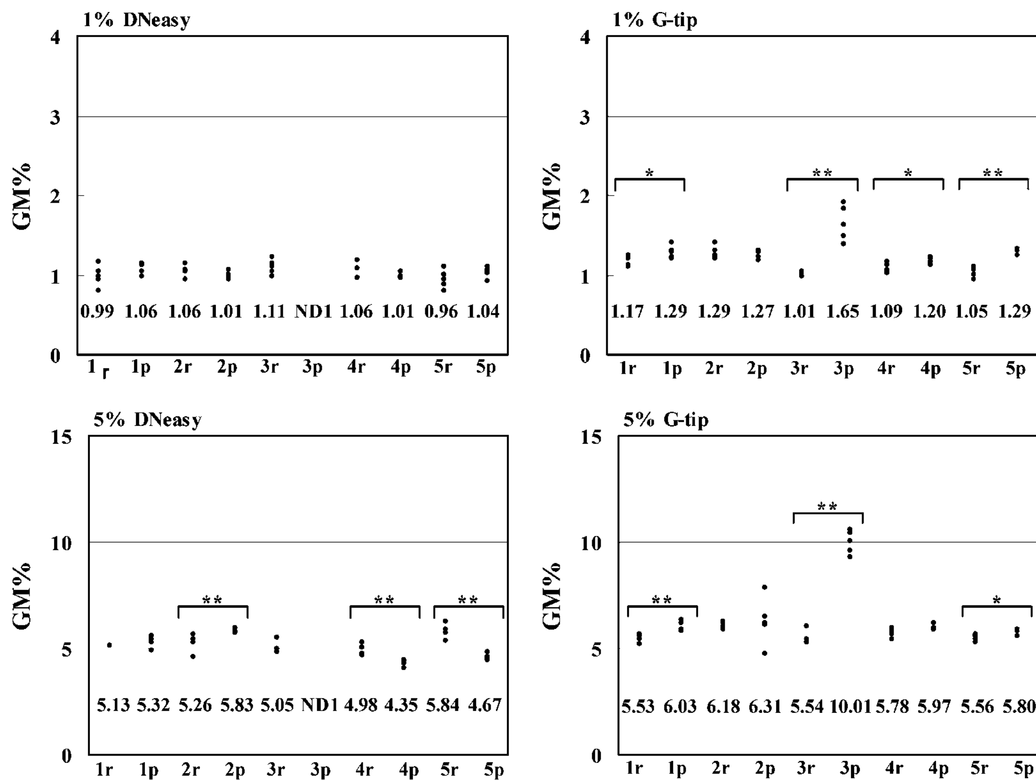

Figure 4. Comparisons of GM% between raw materials and processed foods in trial producing from soy. GM% values were quantified in each $n = 5$ using primer pairs and probes of Le1n02 and RRS 01. Key: r, raw material; p, processed food; 1, tofu 1 kg production; 2, soy milk 1 kg production; 3, boiled beans 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.

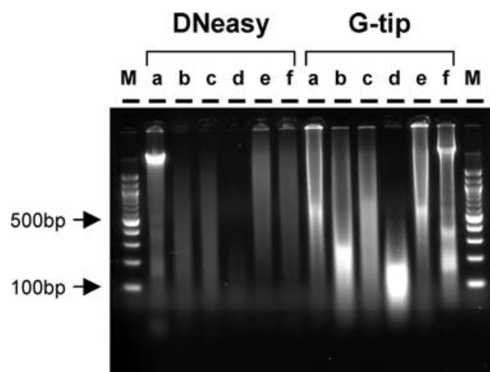

Figure 5. Agarose gel electrophoresis of genomic DNA extracted from soy and the processed foods. (a) Raw material, (b) tofu 1 kg, (c) soy milk 1 kg, (d) boiled beans 1 kg, (e) tofu 10 kg, (f) soy milk 10 kg, and (M) 100 bp ladder size marker.

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).

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