FOOD COMPOSITION AND ADDITIVES

Validation of Real-Time PCR Analyses for Line-Specific Quantitation of Genetically Modified Maize and Soybean Using New Reference Molecules

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Novel analytical methods based on real-time quantitative polymerase chain reactions by use of new reference molecules were validated in interlaboratory studies for the quantitation of genetically modified (GM) maize and soy. More than 13 laboratories from Japan, Korea, and the United States participated in the studies. The interlaboratory studies included 2 separate stages: (*1*) measurement tests of coefficient values, the ratio of recombinant DNA (r-DNA) sequence, and endogenous DNA sequence in the seeds of GM maize and GM soy; and (*2*) blind tests with 6 pairs of maize and soy samples, including different levels of GM maize or GM soy. Test results showed that the methods are applicable to the specific quantitation of the 5 lines of GM maize and one line of GM soy. After statistical treatment to remove outliers, the repeatability and reproducibility of these methods at a level of 5.0% were <13.7 and 15.9%, respectively. The quantitation limits of the methods were 0.50% for Bt11, T25, and MON810, and 0.10% for GA21, Event176, and Roundup Ready soy. The results of blind tests showed that the numerical information obtained from these methods will contribute to practical analyses for labeling systems of GM crops.

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In spite of their benefits, the rapid spread of novel foods derived from recombinant DNA (r-DNA) technologies, the so-called genetically modified organisms (GMOs), is regarded with some apprehension among certain people. Numerous opinions have been expressed, but the arguments surrounding GMO crops and their processed foods generally focus on 2 topics: safety and labeling (1–4). In light of this, many countries and international organizations have been discussing new labeling systems focused on product information intended for the general public (5–7).

Polymerase chain reaction (PCR) is a promising technique to ensure the reliability of labeling systems (8–16). As the first screening for GMO detection, qualitative analysis by PCR is considered suitable (8–10); real-time PCR or quantitative competitive PCR (QC-PCR) is a useful technique for obtaining numerical information (11–16).

The results of collaborative studies, i.e., interlaboratory evaluation of the methods, for quantitation of GMOs by QC-PCR (14) and enzyme-linked immunosorbent assay (ELISA; 17) have been published, but no information has been published regarding real-time PCR. In most previous studies, ground raw materials (Fluka Chemika, Buchs, Switzerland) were used as reference materials. The ground raw materials themselves, however, might not be suitable as refer-

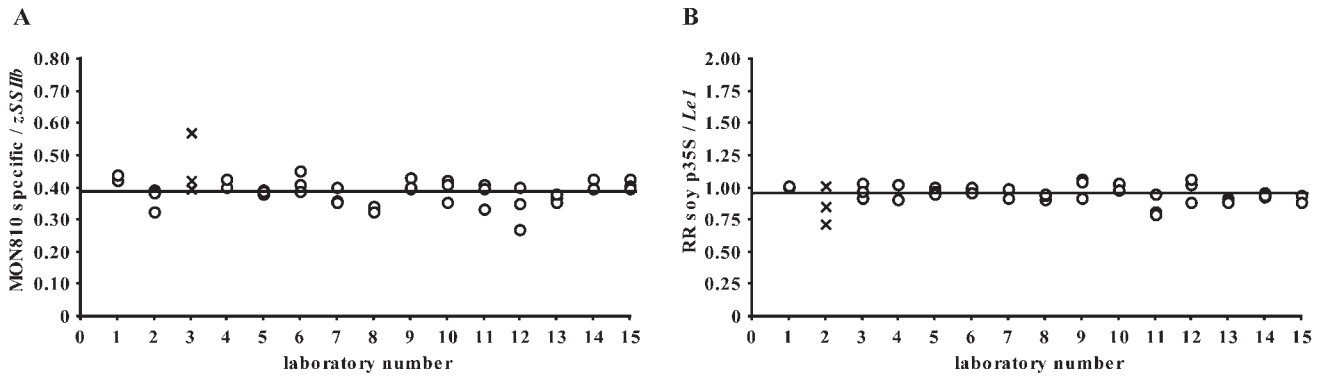

Figure 1. Summary of C_Vs submitted by participants. (A) Ratio of the line-specific sequence of MON810 and *zSSIIb*. (B) Ratio of the p35S sequence of RR soy and *Le1*. Horizontal line is the median calculated after removal of outliers. The retained laboratories are expressed as open circles. The statistical outliers are expressed as x.

ence materials for a long period from the standpoint of quality control because they are agricultural products.

Therefore, we have developed new detection methods based on a real-time quantitative PCR by using reference molecules (18). The methods are applicable to the quantitation of 5 lines of GM maize (i.e., Bt11, GA21, T25, Event176, and MON810) and one line of GM soy [Roundup Ready (RR) soy] and can provide reference materials of consistent quality to all laboratories. In this report we describe the results of interlaboratory evaluations of these methods. The studies were conducted at more than 13 laboratories in Japan, Korea, and the United States. Coefficient values (C_Vs) required for the quantitation of GM maize and GM soy, which are the ratio of r-DNA and endogenous DNA sequence in the genuine seeds of each GM line, were also measured in the interlaboratory studies.

Experimental

The materials such as *Zea mays*, *Glycine max*, oligonucleotide primers and probes, reference molecules, and test materials were the same materials as used previously (18).

DNA Extraction

DNA was extracted from the seeds according to the manual for DNA isolation from plant tissue by using the DNeasy™ Plant Maxi kit (QIAGEN GmbH; Hilden, Germany) except for minor modifications described previously (18).

Quantitative PCR

All conditions and instruments for quantitative PCR were consistent with those of a previous paper (18). A 25 μL volume of reaction solution contained 0.5 μM forward and reverse primers, 0.2 μM probe (except for CaMV 35S promoter, p35S, which contained 0.1 μM probe), and 50 ng template DNA. In the reaction plate the real-time PCR was triplicated by using 3 wells for each template DNA. The copy number of each sample was calculated as mean value of triplicated amplification results, which compared with the calibration curves established with 20, 125, 1500, 20 000, and 250 000 copies of reference molecules per reaction. The measured copy numbers were used to determine C_V or GMO amount (%) reported previously (18).

Interlaboratory Evaluation

The interlaboratory evaluation was performed in 2 separate stages. All participants were requested to follow the protocols summarized above in both stages. Primers, probes, reference molecules, extracted DNAs, and test materials were supplied to them by the National Food Research Institute (NFRI). The Universal Master Mix® (Applied Biosystems, Foster City, CA) and the DNeasy Plant Maxi Kit (QIAGEN GmbH) and other reagents required for the interlaboratory evaluations were purchased by the participants. The experimental procedures were demonstrated at NFRI for all participants before all experiments. All submitted data were treated by the Cochran's test for removal of laboratories with an extreme variation and by the Grubbs's test for removal of laboratories with an extreme average level following the guidelines for collaborative study (19).

The first stage was the test to measure the C_Vs of each GM line. All participants received the primers, probes, reference molecules, and the genomic DNAs extracted from each GM line from NFRI. The DNAs were used to measure copy numbers of each r-DNA and endogenous DNA sequence. All measurements in this stage were repeated 3 times.

Blind tests were performed as the second stage. A total of 12 maize blind samples and 12 soy blind samples simply coded were sent to the participants with 10 sets of the primers, probes, and reference molecules, which were required to detect the 5 lines of GM maize and RR soy described previously (18). The maize blind samples were designed as 6 pairs of blind duplicates, including 0, 0.10, 0.50, 1.0, 5.0, and 10.0% Bt11, GA21, T25, Event176, and MON810 maize. The blank sample, 0% GM maize, was used only to remove the invalid laboratories before statistical analysis. In the same manner, the soy blind samples were designed as 6 pairs of blind duplicates, including 0, 0.10, 0.50, 1.0, 5.0, and 10.0% RR

soy. The details of preparation and evaluation of the blind samples were described previously (18). The sample DNAs were extracted from these blind samples by the participants and were used for quantitative analysis of each GM line in their laboratories.

All participants were asked to submit their data of the real-time PCR, including the kinetics data of fluorescent signals in both stages. Neither the concentration levels of GM plants in the blind samples nor C_Vs measured at NFRI before this interlaboratory evaluation were made known to participants.

Results and Discussion

Determination of C_Vs

The interlaboratory studies were conducted to evaluate the quantitative methods for 5 lines of GM maize and one line of GM soy. Prior to the blind tests, we measured the C_Vs (the ratio of r-DNA and endogenous DNA sequence) required to calculate the GMO amount (%) in the methods. Fifteen laboratories in Japan, Korea, and the United States participated in the measurement test of C_Vs as the first stage of interlaboratory tests. All participants received the reference molecules, the 10 sets of primer pairs, probes, and the extracted DNAs from the GM maize (Bt11, GA21, T25, Event176, and MON810) and GM soy (RR soy), which were prepared and whose pertinence was tested at NFRI prior to the studies (18). Each target sequence was quantified 3 times by the above PCR systems with the extracted DNAs used as templates.

A total of 690 data was submitted from the 15 participants. In all measurements, the correlations of standard curves were acceptable ($r > 0.990$). The general guideline for collaborative study (19) was used to remove outlier laboratories with an extreme variation (Cochran's test, $p < 0.025$) and an extreme average level (Grubbs's test, $p < 0.025$) at each primer pair. The Cochran's test was applied to the variance of 3 measurements produced in one laboratory. Under guidance, the paired Grubbs's test was applied when additional outliers could not be found by the single Grubbs's test. The cycles for removal of outliers were repeated until no additional outliers were identified. After this standard procedure, one laboratory was detected as a Cochran outlier at the ratio of the line-specific sequence of MON810 and *zSSIIb* (20) and the ratio of the p35S sequence of RR soy and *Le1* (21; Figure 1). No outliers were observed at the other ratios.

The data submitted from the retained laboratories were used to calculate average and confidence interval ($\alpha = 0.05$). The average values were defined as the C_Vs for calculation of the GMO amount (%) in our blind test (Table 1). The averaged, specifically quantified C_Vs for Bt11, GA21, T25, Event176, MON810 maize, and RR soy were 0.50, 1.40, 0.34, 2.05, 0.38, and 0.95, respectively. The relative standard deviation (RSD) of the ratio was a maximum of 12.8% (C_V for T25).

The C_Vs defined in these experiments seem to reflect the construction of the foreign DNA cassettes introduced into each GM line. For example, 2 expression cassettes, one for *cryIA(b)* and the other for *pat*, are introduced into Bt11 (18). Nopaline synthase terminator (tNOS) and p35S are used in both cassettes. Their C_Vs are nearly twice that of the Bt11-specific sequence, which was designed between the *Adh 1-S* IVS 6 and *cryIA(b)* sequences. For Event176, 3 expression cassettes are introduced; 2 have *cryIA(b)* and one has p35S (18). The C_V of the Event176-specific sequence designed between *cryIA(b)* and the CaMV 35S terminator is nearly twice of that of p35S. Other GM lines have one expression cassette, and nearly equal values are observed between the promoter (or terminator) and the line-specific sequence, except for GA21 whose developer confirmed that 1 of 3 introduced cassettes may have lost its terminator in the maize cell (22). No amplification plot of tNOS for MON810 corresponds to that in the previous report (23).

Meanwhile, the C_Vs could be regarded as copies of foreign DNAs introduced into each GM line, because the C_Vs were the ratio of the copy number of foreign DNA compared with a single-copy endogenous gene. Most commercial maize varieties are sold as the first filial hybrid, and the genome of GM maize varieties is constituted from heterogeneous DNAs in the transgenes. For instance, in the case of maize, the ideal C_Vs for the single-copy foreign DNA sequence is expected to be 0.5. However, for soy, which is a typical self-pollination plant, the commercial varieties were bred to homozygosity for the transgenes; thus, the ideal C_Vs for the single-copy foreign DNAs sequence is expected to be 1.0. The results of these experiments did not conflict with those described for copies of

Table 1. Summary of the C_Vs[a]

	Number of laboratories			
	Cochran outliers	Grubbs outliers	Retained laboratories	C_Vs
p35S				
Bt11	0	0	15	0.91 ± 0.02
T25	0	0	15	0.31 ± 0.01
Event176	0	0	15	0.79 ± 0.02
MON810	0	0	15	0.39 ± 0.01
RR soy	1	0	14	0.94 ± 0.02
tNOS				
Bt11	0	0	15	0.96 ± 0.03
GA21	0	0	15	1.05 ± 0.03
RR soy	0	0	15	1.10 ± 0.04
Line-specific				
Bt11	0	0	15	0.50 ± 0.01
GA21	0	0	15	1.40 ± 0.05
T25	0	0	15	0.34 ± 0.01
Event176	0	0	15	2.05 ± 0.04
MON810	1	0	14	0.38 ± 0.01
RR soy	0	0	15	0.95 ± 0.02

[a] C_Vs are expressed as mean ± confidence interval ($\alpha = 0.05$).

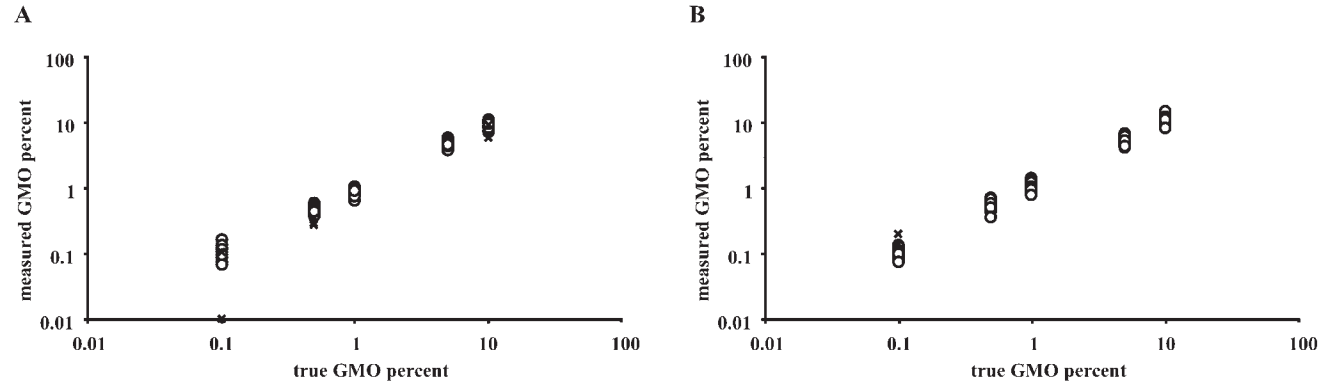

Figure 2. Summary of data for blind tests after removal of invalid laboratories. (A) Line-specific quantitation of Event176. (B) Line-specific quantitation of RR soy. The retained laboratories are expressed as open circles. The statistical outliers are expressed as x.

foreign DNAs submitted by the developers to the U.S. government for safety assessment (22, 24–28).

Removal of Outliers

For the blind tests of maize, 14 participants analyzed 168 samples by amplifying *zSSIIb* and 5 kinds of GM line-specific sequences. Further, 13 participants analyzed 156 samples for the blind test of soy by amplifying the *Le1* and RR soy-specific sequences. The GMO amount (%) of each sample was calculated by using Formula 4 described previously (18).

Typical results obtained from 0, 0.10, 0.50, 1.0, 5.0, and 10.0% of GMO levels are shown in Figure 2. Laboratories that failed to measure blank samples were judged as invalid. In these studies, laboratory Nos. 3, 8, and 14 were invalid for the measurement of GA21 maize, RR soy, and Event176 maize, respectively (Table 2). The data obtained by these laboratories were not used for any of the following statistical analyses of the GM line quantitations because contamination or other mistakes, such as replacement of the samples, may have occurred.

In all experiments, the correlations of standard curves were acceptable ($r > 0.990$). The data were first converted by the logit transformation, because GMO amount (%) is a proportion ranging from 0 to 100, and approximation to the normal distribution is appropriate for following statistical analysis (29). The formula for transformation from X (0, 100) to Y ($-\infty$, $+\infty$) is described as:

$$Y = \log\{(X/100)/(1 - X/100)\}$$

The criteria for removal conformed to the same guidelines as for C_V (19). The laboratories showing an extreme variation of the transformed data in the blind duplicate pair of each GMO level were removed as Cochran outliers for the following statistical analysis of accuracy and precision. Correspondingly, the laboratories with an extreme average of the transformed data in the blind duplicate pair of each GMO level were removed as Grubbs outliers (Table 2).

Finally, 16 Cochran outliers and 4 Grubbs outliers were detected in the 30 data of 5 mixing levels for 6 GM lines. It was suggested that 8/16 Cochran outliers and 4/4 Grubbs outliers were detected in the measurements of 0.10% samples because the measurements of high copy sequences were observed to be relatively stable (18).

Accuracy and Precision

The calculated mean, bias, repeatability standard deviation (RSD_r), and reproducibility standard deviation (RSD_R) at each mixing level and GM line are shown in Table 2. Regarding the index of precision observed in this study, the RSD_r and RSD_R at the level of 0.50% were <28.2 and 27.6%, respectively. At the level of 5.0%, the RSD_r and RSD_R were <13.7 and 15.9%, respectively. The RSD_r and RSD_R were reduced in most GM lines when the samples, including higher levels of GMO amount (%), were analyzed. The repeatability and the reproducibility limits of the methods, $2.8 \times RSD_r$ and $2.8 \times RSD_R$ (30), respectively, are also shown in Table 2. The reliability of the data obtained from 0.10% is discussed in the *Detection Limit* section, below.

A portion of these deviations could be explained by a theoretical investigation by Kay and Van den Eede (31), who mentioned that unavoidable sampling error is a result of the binomial distribution. Thus, 50 ng DNA extracted from a 0.50% blind sample would produce no better than about 20% RSD_r, even if other types of error inherent in a real analytical system were ignored. Our results indicated that the deviations derived from these methods were relatively small; therefore, we considered that the methods were sufficiently precise for practical use.

The mean values of GMO amount (%) at the level of 1.0% were 1.15, 1.20, 1.20, 0.923, 1.05, and 1.16% for Bt11, GA21, T25, Event176, MON810 maize, and RR soy, respectively. In our study, the bias (mean–true value, %) except for the 0.10% samples, ranged from –7.7 to +21.6%. On the one hand, most of the mean values calculated for Bt11, GA21, T25, and RR soy were slightly higher than their true values.

Table 2. Summary of accuracy and precision statistics for real-time quantitative PCR

	Number of laboratories				Accuracy		Precision						Detection limit
					Mean	Bias	Repeatability			Reproducibility			
%	Invalid laboratories	Cochran outliers	Grubbs outliers	Retained laboratories	GMO amount, %	True value, %	s_r[a]	r[a] (2.8 x s_r)	RSD_r, %[b]	s_R[a]	R[a] (2.8 x s_R)	RSD_R, %[b]	Below 20 copies[c]
							Bt11						
0.10	0	2	1	11	0.091	–9.0	0.020	0.057	22.3	0.016	0.046	18.0	21/22
0.50	0	0	0	14	0.510	+2.0	0.121	0.338	23.7	0.105	0.293	20.5	0/28
1.0	0	0	0	14	1.15	+14.7	0.216	0.606	18.9	0.216	0.605	18.8	0/28
5.0	0	0	0	14	6.08	+21.6	0.830	2.325	13.7	0.786	2.200	12.9	0/28
10.0	0	0	0	14	12.1	+21.1	1.258	3.524	10.4	1.389	3.889	11.5	0/28
							GA21						
0.10	1	1	0	12	0.095	–5.4	0.019	0.054	20.5	0.019	0.055	20.6	4/24
0.50	1	0	0	13	0.538	+7.7	0.068	0.189	12.6	0.117	0.329	21.8	0/26
1.0	1	0	0	13	1.20	+20.2	0.148	0.414	12.3	0.224	0.627	18.6	0/26
5.0	1	1	0	12	5.83	+16.6	0.476	1.332	8.2	0.927	2.597	15.9	0/24
10.0	1	0	0	13	11.5	+15.0	0.907	2.539	7.9	1.565	4.382	13.6	0/26
							T25						
0.10	0	2	1	11	0.139	+38.6	0.033	0.092	23.7	0.037	0.103	26.5	22/22
0.50	0	0	0	14	0.577	+15.3	0.162	0.455	28.2	0.159	0.446	27.6	1/28
1.0	0	1	0	13	1.20	+20.0	0.082	0.228	6.8	0.138	0.386	11.5	0/26
5.0	0	0	0	14	5.58	+11.6	0.690	1.932	12.4	0.827	2.317	14.8	0/28
10.0	0	0	0	14	10.8	+8.1	1.439	4.030	13.3	1.591	4.456	14.7	0/28
							Event176						
0.10	1	1	0	12	0.111	+11.3	0.018	0.051	16.3	0.024	0.066	21.3	1/24
0.50	1	2	0	11	0.492	–1.6	0.029	0.080	5.8	0.051	0.142	10.3	0/22
1.0	1	0	0	13	0.923	–7.7	0.066	0.184	7.1	0.106	0.296	11.4	0/26
5.0	1	0	0	13	5.00	0.0	0.406	1.137	8.1	0.559	1.565	11.2	0/26
10.0	1	1	0	12	9.62	–3.8	0.554	1.552	5.8	0.917	2.566	9.5	0/24
							MON810						
0.10	0	2	1	11	0.125	+25.0	0.040	0.113	32.3	0.033	0.091	26.1	19/22
0.50	0	1	0	13	0.547	+9.4	0.082	0.231	15.1	0.107	0.301	19.6	0/26
1.0	0	0	0	14	1.05	+4.6	0.124	0.347	11.8	0.158	0.443	15.1	0/28
5.0	0	1	0	13	4.78	–4.3	0.647	1.813	13.5	0.569	1.593	11.9	0/26
10.0	0	1	0	13	9.82	–1.8	1.028	2.879	10.5	1.140	3.191	11.6	0/26
							RR soy						
0.10	1	0	1	11	0.108	+8.1	0.015	0.041	13.4	0.014	0.040	13.4	4/22
0.50	1	0	0	12	0.571	+14.3	0.068	0.191	12.0	0.091	0.255	15.9	0/24
1.0	1	0	0	12	1.16	+16.1	0.129	0.362	11.2	0.161	0.451	13.9	0/24

Table 2. (*continued*)

%	Number of laboratories				Accuracy		Precision						Detection limit
					Mean	Bias	Repeatability			Reproducibility			
	Invalid laboratories	Cochran outliers	Grubbs outliers	Retained laboratories	GMO amount, %	True value, %	s_r[a]	r[a] (2.8 x s_r)	RSD_r, %[b]	s_R[a]	R[a] (2.8 x s_R)	RSD_R, %[b]	Below 20 copies[c]
5.0	1	0	0	12	5.76	+15.1	0.435	1.219	7.6	0.660	1.849	11.5	0/24
10.0	1	0	0	12	11.7	+17.2	0.993	2.779	8.5	1.246	3.489	10.6	0/24

[a] s_r, s_R, r, and R are expressed in units of % GMO.
[b] The RSD_r and RSD_R are expressed as percentage of their mean values.
[c] Below 20 copies are expressed as the ratio of the number of retained data below 20 copies/the total number of retained data.

On the other hand, the mean values of Event176 and MON810 were close to their true values. Although further investigation may be required to reduce the differences, the methods evaluated here can be applied to monitoring the mixing of GM maize and soy.

Detection Limit

In our preliminary experiments, 10 and 20 copies calibrants amplified exponentially; however, there was a large bias between amplification patterns of analyses or equipment at 10 copies (data not shown). Therefore, there was no calibrant below 20 copies, and the data below 20 copies have uncertainty in our methods (Table 2). Most measurement copies of 0.10% Bt11, T25, and MON810 were below 20 copies, whereas those of 0.10% GA21, Event176, and RR soy were sufficient for analyses. The measurement copies of 0.50% samples were over 20 copies except for 1 measurement of T25. Therefore, we concluded that the quantitation limits of the present methods were 0.50% for Bt11, T25, and MON810, and 0.10% for GA21, Event176, and RR soy. Although the quantitation results for 0.10% Bt11, T25, and MON810 were not precise, typical exponential amplifications were observed (the cycles of threshold; $C_t = 34 \sim 40$). Such significant amplifications indicate that 0.10% Bt11, T25, and MON810 were not quantifiable but were detectable by using these methods.

The C_Vs of Bt11, T25, and MON810 were relatively low and suggested low copies of r-DNA sequences in the genomes of these GM lines (Table 1). These C_Vs strongly suggested that transgenes introduced in Bt11, T25, and MON810 were predicted as a single copy per genome. According to a previous report (31), 50 ng maize DNA used for real-time quantitative PCR included 18 348 copies of the haploid *Z. mays* genome. Thus, the copy numbers experimentally measured would be smaller than 20 when a 0.10% sample of the GM line, including a single foreign expression cassette, was used for our analysis.

Therefore, we consider that the differences of detection limits between GM lines are reasonable results. In addition, the detection limits of the methods were sufficiently sensitive to judge unintended mixing of GM maize and GM soy with regard to the definition of unintended mixing in Japan, Korea, and the European Union (5, 3, and 1% GM maize or GM soy, respectively; 32–34).

Conclusions

Our methods of quantifying 5 lines of GM maize and one line of GM soy were evaluated by means of an interlaboratory study. The results showed no conflict with our in-house validation (18). Our methods are sufficiently sensitive to monitor labeling systems and have allowable levels of accuracy and precision.

One of the features of the methods evaluated here is the use of reference molecules of consistent quality, because agricultural products are not required as reference materials. The reference molecules could be easily amplified and would not be affected by common agricultural factors.

Another advantage is to be based on GM line-specific quantitation. An analytical sample may commonly include various lines of GMOs. In the case of maize, previously reported methods, such as quantitative PCRs with p35S as the target sequence, must be applied with the understanding of possible error caused by conversion into a major line. For instance, our C_Vs indicate that the same GMO amount (%) in 10% Bt11 samples will be twice that of 10% MON810 samples as a result of analysis by using previous quantitative PCR methods, because of the 100% difference of their expression cassettes, including p35S.

We conclude that the methods evaluated in this study are reliable and practical for monitoring the labeling systems for GM maize and GM soy.

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