scored as negative. Reactions with Ct values of less than 48, but without exponential amplification as judged by visual inspection of the respective ΔRn plots and multi-component plots were scored as negative.

## RESULTS AND DISCUSSION

**Detection of Unauthorized GM Papaya** To investigate the contamination with unauthorized GM papaya in commercially processed products, containing papaya as a major ingredient, in Japan, we used genomic DNA purified from the papaya-leaf-tea products as a template for the PCR test. The forward primer (p324) was designed to hybridize in the cauliflower mosaic virus (CaMV) 35S promoter sequence, which is the most common promoter used in the transformation of papaya for various GM papaya traits,[2] and the reverse primer (p323) was designed in the highly conserved sequence of the CP gene, which is cloned from various strains of PRSV (GenBank accession no.: YK, X97251; HA, S46722; Vb, AF243496.1; H1K, AF196839.1; W-CI, AY027810.2). Electrophoresis of the PCR products using p324 and p323 primers showed a single band of about 300 bp in length using DNA purified from two of the three papaya-leaf-tea products (papaya-leaf-tea B and C) (Fig. 1A). The DNA purified from non-GM papaya (Sunset) as a control and papaya-leaf-tea A generated no PCR products with the identical length. Direct sequence analysis of the PCR product and BLASTn analysis indicated that the 3′ end sequence was identical to the CP gene in a Taiwan isolate of PRSV (PRSV YK strain)[16] (Fig. 1B). Furthermore, the multiple cloning site (containing restriction sites for *Bam*HI and *Nco*I) and two amino acid mutations (methionine and alanine) between the CaMV 35S promoter and the N-terminus of CP gene were detected (Fig. 2A). According to the literature,[17] the design of this transgenic vector construct was identical to that of the GM papaya, which was generated to resist infection of the PRSV YK strain. These results suggest that the papaya-leaf-tea products were contaminated with the unauthorized GM papaya (PRSV-YK).

**Development of a Construct-Specific Detection Method for PRSV-YK** In order to qualitatively detect PRSV-YK in processed products, containing papaya as a major ingredient, with high specificity and sensitivity, we designed specific primers and a probe for a real-time PCR assay producing a short amplicon (57 bp), based on the detected transgenic construct sequence. The forward (YK-1F) and the reverse (YK-1R) primers were designed in the region between the transgenic vector backbone and the CP gene sequence. The probe

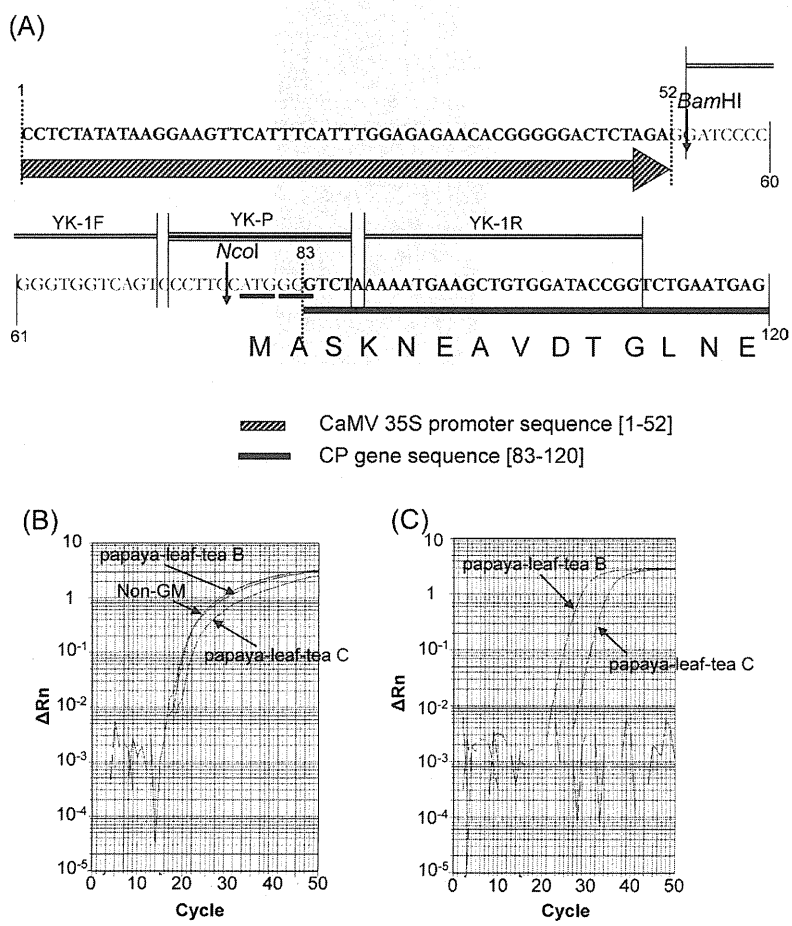


Fig. 2. Detection of PRSV-YK Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence was obtained and restriction sites were marked by vertical arrows. Design of the primers (YK-1F and YK-1R) and the probe (YK-P) for detecting construct-specific sequence of PRSV-YK is indicated by lines above the sequence. Numerals indicate the numbers of nucleotides from the 5′ terminus. (B) Endogenous *Chy* detection using a primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) (C) PRSV-YK detection using a primer set (YK-1F and YK-1R) and probe (YK-P). The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

(YK-P) was designed on the site of the initiation codon of the CP gene (Fig. 2A).

Since the forward primer sequence for detecting the papaya endogenous internal control gene, *Chy*, had an unintentional error of a single nucleotide sequence in the previous report[15] (according to personal communication), we used the right sequence for the forward primer (Q-Chy-1F2), the reverse primer (Q-Chy-2R) and the probe (Q-Chy-P). The real-time PCR assay for PRSV-YK detection confirmed that the papaya-leaf-tea products B and C were positive for PRSV-YK, producing Ct values of 25.93 and 31.88 with a threshold value of 0.2, respectively. Endogenous *Chy* detection was positive for all samples, with the papaya leaf-tea product B, C and the non-GM papaya (Sunset) producing Ct values of 21.55, 23.82 and 21.45, respectively, with a threshold value of 0.2 (Figs. 2B, C). The copy numbers of PRSV-YK construct and *Chy* sequence were calculated from Ct values using standard curves which were generated using the positive control plasmid. Papaya-leaf-tea products B and C contained 1 copy of PRSV-YK construct sequence in 27 copies and 167 copies of *Chy* sequence, respectively (data not shown). Because the genetic background of PRSV-YK used in the processed papaya products was unknown, estimation of the content of PRSV-YK in a papaya product was not possible. The non-template control and the genomic DNA derived from other crops, such as maize, rice, soybean, flax and canola, gave no amplification signals in the PRSV-YK and the endogenous *Chy* detection systems (data not shown). These results indicated that the developed method is specific for detecting PRSV-YK.

In the present study, as a result of monitoring processed products, which included papaya as a major ingredient, for contamination with unauthorized GM papaya, we found a transgenic vector construct for expression of the CP gene, which was cloned from the YK strain, in papaya-leaf-tea products. The design of a part of the transgenic vector construct was identical to the one reported in 1996.[17] We also detected PRSV-YK contamination in 1 out of 7 products of papaya jam and 2 out of 3 products of papaya pickles in real-time PCR test for PRSV-YK detection (data not shown). The origin of the GM papaya contamination in the papaya products in Japan remains to be clarified. Furthermore, we successfully developed a construct-specific real-time PCR detection method for PRSV-YK. Further studies are required to determine the detection limits, and whether the method can be used for detection in other commercially processed products containing papaya as a major ingredient.

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Validation Study

# Interlaboratory Validation of Quantitative Duplex Real-Time PCR Method for Screening Analysis of Genetically Modified Maize

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To reduce the cost and time required to routinely perform the genetically modified organism (GMO) test, we developed a duplex quantitative real-time PCR method for a screening analysis simultaneously targeting an event-specific segment for GA21 and Cauliflower Mosaic Virus 35S promoter (P35S) segment [Oguchi *et al.*, J. Food Hyg. Soc. Japan, **50**, 117-125 (2009)]. To confirm the validity of the method, an interlaboratory collaborative study was conducted. In the collaborative study, conversion factors (Cfs), which are required to calculate the GMO amount (%), were first determined for two real-time PCR instruments, the ABI PRISM 7900HT and the ABI PRISM 7500. A blind test was then conducted. The limit of quantitation for both GA21 and P35 S was estimated to be 0.5% or less. The trueness and precision were evaluated as the bias and reproducibility of the relative standard deviation ($RSD_R$). The determined bias and $RSD_R$ were each less than 25%. We believe the developed method would be useful for the practical screening analysis of GM maize.

**Key words:** screening; quantification; genetically modified (GM); duplex real-time PCR; maize (*Zea mays*)

## Introduction

The PCR technique is widely used to detect and quantify GM crops in foods and feeds. The key factor determining the specificity of a PCR-based method is the choice of a target sequence motif in the GM plant genome. The methods can be classified into at least 3 categories depending on the target; event-specific, construct-specific and screening methods. In event-specific methods, a unique sequence located at the junction between the plant genome and recombinant DNA is used as the target. Construct-specific methods target the junction between adjacent elements in an introduced gene cassette, such as a region between a promoter and a structural gene. Screening methods target commonly conserved elements among many GM events such as Cauliflower Mosaic Virus 35S promoter (P35S), nopaline synthase terminator, 5-enolpyruvylshikimate-3-phosphate synthase, phosphinothricin *N*-acetyltransferase, and so on[1)-4)]. Event-specific methods are the most specific, followed by construct-specific and screening methods. Screening methods often overestimate GM amounts, and it is generally agreed that the best estimates can be obtained by event- or construct-specific quantifications. In fact, construct-specific quantitative methods of five GM maizes (Bt11, Bt176, GA21, MON810, T25) and Roundup Ready Soy (RRS) have been adopted as Japanese standard analytical methods[*1,*2]. However, the cost of genetically modified organism (GMO) testing using specific quantifications

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*1 Notification No. 110 (Mar. 27, 2001); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2001).

will increase in parallel with the number of GM events to be examined. In this regard, screening methods are highly cost- and time-effective for routine monitoring. The Ministry of Health, Labour and Welfare (MHLW) of Japan announced a screening method combining the quantification of a P35S region and the construct-specific quantification of GA21 maize, which has been officially used as a quantitative screening method for GM maize*3. To further pursue more convenient and efficient methodology, we developed a duplex real-time PCR method which simultaneously quantifies the P35S region and an event-specific segment of GA21[5]. The developed duplex screening method will reduce both the cost and time requirement of routine GMO analysis by half compared to the current screening method. These quantitative methods are based on a real-time PCR technique for relative quantification between target and taxon-specific sequences. In many cases, extracted DNAs from processed foods are severely degraded and the degree of the degradation is not always the same among PCR-targeted sequences, so that GM quantification in processed foods by means of the PCR technique is difficult[6), 7)]. In fact, the quantification methods adopted as Japanese standard analytical methods, including the current screening method, are applicable to raw materials but not to processed foods.

In this report, we validated the duplex real-time PCR method for the screening analysis of GM maize by means of an interlaboratory study.

## Materials and Methods

*Plant materials*

The GM maize seeds, MON810 and GA21 were kindly provided by Monsanto (St. Louis, MO, USA), and Syngenta Seeds (Basel, Switzerland), respectively. MRX 3 maize was purchased from Pioneer Hi-Bred International (Johnston, IA, USA) and used as a non-GM maize sample.

*Oligonucleotide primers and probes*

All primers and probes used in this study were identical to those in the previous single laboratory evaluation[5]. The primers and probe for the event-specific detection of GA21 were as follows: GA21esp 5′-1,5′-TGGGACCTTATCGTTATGCTATTTG-3′; GA21esp3′-1,5′-CGATCCTCCTCGCGTTTCC-3′; and GA 21 es-TaqHB; 5′-CCGGACCCACCTGCTGTTGAGAAAG-3′. The primers and probe for the detection of the P35S region were as follows: P35S 1-5′,5′-ATTGATGTGATATCTCCACTGACGT-3′; P35S 1-3′,5′-CCTCTCCAAATGAAATGAACTTCCT-3′; and P35S-TaqFB, 5′-CCCACTATCCTTCGCAAGACCCTTCCT-3′. We used the maize *starch synthase IIb* (*SSIIb*)[8] gene as a maize-specific endogenous DNA for quantitative analysis, and the primers and probe for *SSIIb* were as follows: SSIIb3-5′,5′-CCAATCCTTTGACATCTGCTCC-3′; SSIIb3-3′,5′-GATCAGCTTTGGGTCCGGA-3′; and SSIIb-TaqFB, 5′-AGCAAAGTCAGAGCGCTGCAATGCA-3′. The oligonucleotide primers and TaqMan® probes were synthesized by FASMAC (Kanagawa, Japan) and Biosearch Technologies (Novato, CA, USA), respectively. All synthesized probes were labeled with Blackhole Quencher (BHQ) at the 3′ ends, and P35S-TaqFB and SSIIb-TaqFB were labeled with 6-carboxyfluorescein (FAM), while GA21es-TaqHB was labeled with hexachloro-6-carboxy-fluorescein (HEX), at the 5′ ends.

*Preparation of calibrant plasmid*

The standard plasmid pSCM which contains the specific sequence fragments from GA21, P35S and *SSIIb*, was prepared according to the previous report[5] and used as the calibrator for the quantification.

*Preparation of test samples*

Non-GM and GM maize mixing samples containing 0.50, 1.0, 5.0, and 10.0% of both GA21 and MON810 were prepared by mixing dried powders in the manner described previously[9), 10)] and used as a primary certified reference material. Briefly, washed maize seeds of non-GM, GA21 and MON810 were separately frozen, ground with a high-speed rotor mill (Fritsch, Idar-Oberstein, Germany), freeze-dried in a freeze dryer (FDU-1100; Tokyo Rikakikai, Tokyo, Japan), and then mixed on a weight-to-weight basis.

*Quantitative PCR*

All conditions and PCR instruments were identical to those in the previous report[5].

*Homogeneity of test samples*

Test samples of each GM mixing level were aliquoted (1 g each) into 200 sample tubes. Ten sample tubes were randomly selected twice from the 200 tubes prepared. DNA was extracted from each sample using the DNeasy Plant Maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's manual, and quantitative PCR was performed using GA21 and MON810 construct-specific methods[8)–10)]. The calculated copy numbers were then converted into GMO amounts (%) on a weight basis. The homogeneities of GA21 and MON810 were independently evaluated by one-way analysis of variance (ANOVA) as described previously[8)–10)].

*Interlaboratory study*

The interlaboratory study consisted of 2 separate stages, a measurement of the Cf values and a blind test. Experimental protocols were provided by the Food and Agricultural Materials Inspection Center (FAMIC). The Universal Master Mix, primers, probes, blind samples

*2 Japanese Agricultural Standard (JAS) analytical test handbook: genetically modified food quality, labeling analysis manual for individual products (2002). The Food and Agricultural Materials Inspection Center, Japan. http://www.famic.go.jp/technical_information/jashandbook/index.html

*3 Notification No. 618001; Department of Food Safety, Ministry of Health, Labour and Welfare of Japan: Tokyo, Japan, 2003.

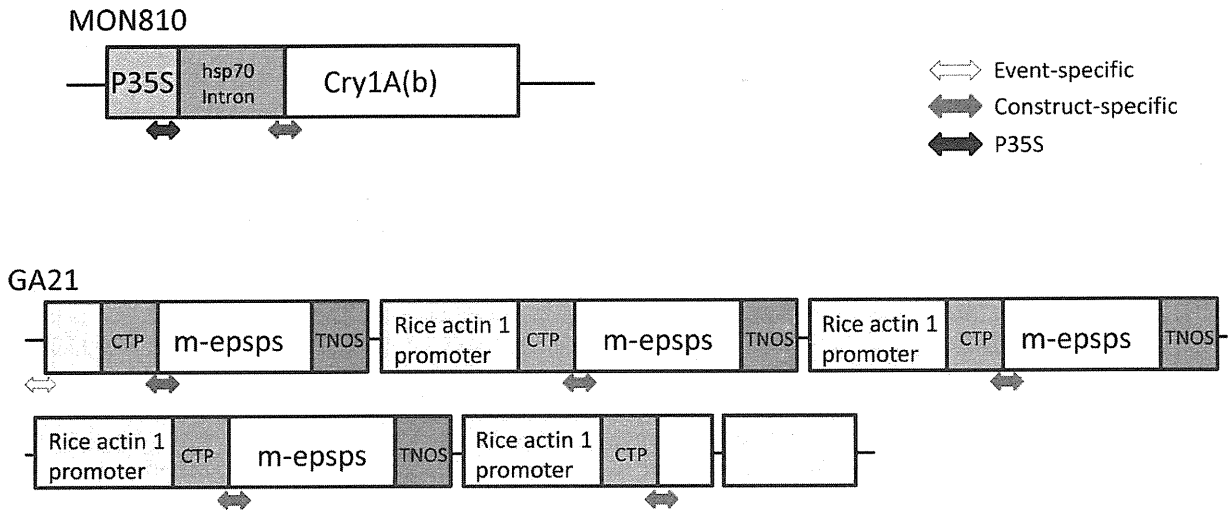


**Fig. 1.** Schematic diagrams showing the target positions in MON810 and GA21
The event-specific, construct-specific, and universal P35S target sequences are indicated with white, gray, and black double-headed arrows, respectively.

**Table 1.** Summary of the Cf values for ABI PRISM 7900 and 7500

| | 7900 | | | 7500 | | |
|---|---|---|---|---|---|---|
| | Mean | SD | RSD | Mean | SD | RSD |
| GA21 | 0.375 (0.38)[a)] | 0.046 | 12.3 | 0.332 (0.33) | 0.025 | 7.60 |
| | Mean | SD | RSD | Mean | SD | RSD |
| P35S | 0.364 (0.36) | 0.024 | 6.50 | 0.363 (0.36) | 0.013 | 3.60 |

SD: Standard deviation
RSD: Relative standard deviation
[a)] The mean values rounded to the nearest hundredth of a unit are shown in parentheses.

and DNeasy Plant Maxi kit were also supplied by FAMIC.

The first stage was measurement of the Cf values using the ABI 7900HT (AB 7900) and the ABI 7500 (AB 7500) (Life Technologies, Carlsbad, CA, USA). The Cf value is experimentally determined as the ratio of the copy number of r-DNA to the taxon-specific sequence in the GM plant genome. The Cf values for GA21 and P35 S were determined independently from the results of 12 laboratories for the AB 7900, and 5 laboratories for the AB 7500. The measurements were repeated 3 times in each laboratory, and the average values from all the submitted data were defined as the Cf values. After determination of the Cf values, one laboratory withdrew from this study.

The blind test was conducted as the second stage. All measurements were performed by 11 laboratories for the AB 7900. The maize samples were designed as blind duplicates, including 0, 0.5, 1.0, 5.0 and 10.0% of both GA21 and MON810. The blind samples sent to the participants were divided into two sets containing each concentration, and then the measurements were separately performed for each set of blind samples. DNAs were extracted from these blind samples by each participant and then quantitative analyses were carried out. All participants were requested to submit the data from the real-time PCR analyses. All submitted data were analyzed by means of Cochran's test[11)] and Grubbs' test[12), 13)]as described in the guidelines[14)].

## Results and Discussion

*Determination of Cf values for GA21 and P35S*

The Cf value for GA21 was determined by measuring the copy numbers of endogenous gene *SSIIb* and GA21 in the extracted DNA from the GA21 seed. To determine the Cf value for P35S, we used MON810 as a representative of GM maize both because it has been widely used, and because it has only one P35S segment per GM haploid, as the previous single laboratory evaluation described[5)]. The Cf values for GA21 and P 35S were measured independently with two real-time PCR instruments, the AB 7900 and AB 7500. The Cf values determined are listed in Table 1. The values for P35S with AB 7900 and AB 7500 were very close, and when rounded to the nearest hundredth of a unit both values became 0.36. GA21 contains a single insert

**Table 2.** Homogeneity of the simulated mixtures

| | % (w/w) | Measured mean, % | $F$-value[a] | $p$-value |
|---|---|---|---|---|
| GA21 | 0.50 | 0.44 | 1.01 | 0.49 |
| MON810 | 0.50 | 0.48 | 1.82 | 0.18 |
| GA21 | 1.0 | 0.74 | 0.48 | 1.00 |
| MON810 | 1.0 | 0.95 | 0.11 | 0.86 |
| GA21 | 5.0 | 3.71 | 0.47 | 0.87 |
| MON810 | 5.0 | 5.26 | 0.83 | 0.60 |
| GA21 | 10.0 | 7.67 | 1.36 | 0.32 |
| MON810 | 10.0 | 9.77 | 1.03 | 0.48 |

[a] Critical value of $F$ is 3.02 ($\alpha = 0.05$)

**Table 3.** Summary of accuracy and precision statistics for the duplex real-time PCR method

| | | Trueness | | Precision | Detection limit | | | Trueness | | Precision | Detection limit |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Means | Bias | | | | | Means | Bias | | |
| % (w/w) | Retained labs | GMO amount, % | True value, % | $RSD_R$[a], % | Below 20 copies[b] | % (w/w) | Retained labs | GMO amount, % | True value, % | $RSD_R$[a], % | Below 20 copies[b] |
| | | GA21 | | | | | | P35S | | | |
| 0.50 | 11 | 0.57 | 13.2 | 18 | 0/22 | 0.50 | 11 | 0.60 | 19.9 | 19 | 0/22 |
| 1.0 | 10 | 1.13 | 12.7 | 10 | 0/20 | 1.0 | 11 | 1.19 | 18.8 | 21 | 0/22 |
| 5.0 | 11 | 5.60 | 11.8 | 13 | 0/22 | 5.0 | 11 | 5.82 | 16.4 | 13 | 0/22 |
| 10.0 | 11 | 11.15 | 11.5 | 9.0 | 0/22 | 10.0 | 10 | 11.91 | 19.1 | 9.5 | 0/22 |

[a] $RSD_R$: Reproducibility relative standard deviation
[b] Below 20 copies refers to the ratio of the number of retained data below 20 cpies/the total number of retained data

consisting of three copies of its perfect gene cassette and three incomplete copies*4. In the whole recombinant insertion, five copies of the construct-specific segment of GA21 are supposed to be present (Fig. 1). We obtained the Cf values for the GA21 construct-specific method as between 1.40 and 2.01 in our previous studies[8)-10)]. The theoretically expected Cf value for the event-specific method would be one-fifth of these values, and thus would be between 0.28 and 0.40. Both of the Cf values determined for GA21 in this study were within this range.

*Interlaboratory validation of the duplex real-time PCR method*

After determination of the Cf values, the homogeneities of the blind samples were confirmed by one-way ANOVA. Ten tubes of each mixing sample were randomly selected twice. The DNA was then extracted from these samples, and quantitative PCR analyses of GA21 and MON810 were performed using each construct-specific method[8)-10)]. The measured copy numbers were converted into the GMO amount (%), and one-way ANOVA was then conducted on the data. The $F$- and $p$-values were calculated (Table 2). Even at the smallest value, 0.18, obtained by the MON810 specific quantification of the 0.50% sample, the $p$-values were larger than 0.05, indicating that the contents of both GA21 and MON810 of all the test samples were sufficiently homogeneous and met the requirements for the following interlaboratory study.

The developed duplex real-time PCR quantitative method was evaluated in a blind test performed by 11 laboratories using the AB 7900. The measurements of GA21 and P35S were carried out independently. A blank sample, with 0% GM content, was used to estimate invalid laboratories, and no laboratory was eliminated. All the submitted data except 0% were then handled according to the harmonized guidelines of AOAC[14)] to remove outlier laboratories with extreme variation using Cochran's test and with an extreme average level using Grubbs' test as previously described[9), 10)]. One Cochran outlier was detected in the 1.0% GA21 sample and one Grubbs outlier was detected in the 10.0% P35S sample. After removing these outliers, further statistical analyses were conducted. The trueness and precision were determined as the bias (mean-value, %) and reproducibility of relative standard deviation ($RSD_R$) for GA21 and P35S in individual samples (Table 3). The bias and $RSD_R$ of GA21 were less than 15% and 20% in all samples, respectively. Both the bias and $RSD_R$ of P35S were slightly higher than those of GA21, but were less than 20% and 25%, respectively. These obtained bias and $RSD_R$ levels were simi-

*4 Agbios database. http://www.cera-gmc.org/?action=gm_crop_database&

lar to or even less than those of previously reported GMO events[9), 10)]. In terms of the limit of quantitation (LOQ), all the measured copy numbers of the 0.5% samples were over 20 copies and there was no calibrant below 20 copies in this method. Therefore, we estimated that the LOQ for the GA21 event-specific and P 35S in the duplex PCR method was 0.50% or less.

The previously developed duplex real-time PCR method was validated in this interlaboratory study using AB 7900 instruments. The levels of obtained LOQ, trueness and precision were almost the same as those of other established methods[9), 10)] and the single laboratory evaluation[5)]. We thus consider that the duplex real-time PCR a good candidate for routine screening for GM maize commingled in agricultural crops.

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ARTICLE

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# Practicable Group Testing Method to Evaluate Weight/Weight GMO Content in Maize Grains

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Supporting Information

ABSTRACT: Because of the increasing use of maize hybrids with genetically modified (GM) stacked events, the established and commonly used bulk sample methods for PCR quantification of GM maize in non-GM maize are prone to overestimate the GM organism (GMO) content, compared to the actual weight/weight percentage of GM maize in the grain sample. As an alternative method, we designed and assessed a group testing strategy in which the GMO content is statistically evaluated based on qualitative analyses of multiple small pools, consisting of 20 maize kernels each. This approach enables the GMO content evaluation on a weight/weight basis, irrespective of the presence of stacked-event kernels. To enhance the method's user-friendliness in routine application, we devised an easy-to-use PCR-based qualitative analytical method comprising a sample preparation step in which 20 maize kernels are ground in a lysis buffer and a subsequent PCR assay in which the lysate is directly used as a DNA template. This method was validated in a multilaboratory collaborative trial.

KEYWORDS: GMO detection, detection, group testing, subsampling

## INTRODUCTION

Industrial use of genetically modified organisms (GMOs) has been advancing, and many genetically modified (GM) crops have been put on the market in the past 15 years.[1] In maize, which is one of the four major GM crops, along with soybeans, cotton, and canola, stacked-event seeds, generated by crossing two or more single GM events, have been widely used.[1] Numerous safety assessments of GM crops and their derived foods and feeds have been conducted by authorities in countries around the world, and commercially available GM crops are considered to be as safe as their conventional (non-GM) counterparts. In many countries, however, the use of GM crops is controversial among general consumers, and the demand for conventional crops is deeply rooted. To expand consumers' choices, many countries have introduced legislation requiring labels to be applied to agricultural products that happen to contain approved GMOs at more than a certain threshold level. For example, the thresholds are set as 0.9%, 3%, and 5% in the European Union (EU), Korea, and Japan, respectively.[2] For products that do not carry GM labels, compliance with these regulations is checked at various points of the supply chain, often starting with the crops.

The regulations in some countries refer to the GM material in terms of weight/weight percentages, although the most commonly used technique for GMO quantification in grain is quantitative real-time polymerase chain reaction (PCR) analysis of bulk sample homogenates, and the analysis typically measures GMO contents based on the ratio of GM DNA to plant-species DNA. Because the GM stacked events contain the GM DNA corresponding to two or more single events, the GMO content of non-GMO maize samples with a small number of stacked-event kernels measured by real-time PCR leads to an overestimation as compared to the actual weight/weight GMO content.[3] In light of the increasing use of GM stacked events, it has become virtually impossible to accurately measure low-level GMO content on a weight/weight basis with current methodology. Recently, a single-kernel-based analytical system was developed and implemented in Japan as an official method, as one possible solution for the potential overestimation due to stacked events.[3−5] In this method, individual maize kernels are analyzed to determine their GM or non-GM status, and the weight/weight GMO content is evaluated based on the assumption that the ratio of GM kernels relative to the total number of kernels is equal to the weight/weight ratio.

Since Dorfman's report on blood testing for syphilis in 1943, the group testing strategy has been exploited in epidemiology,

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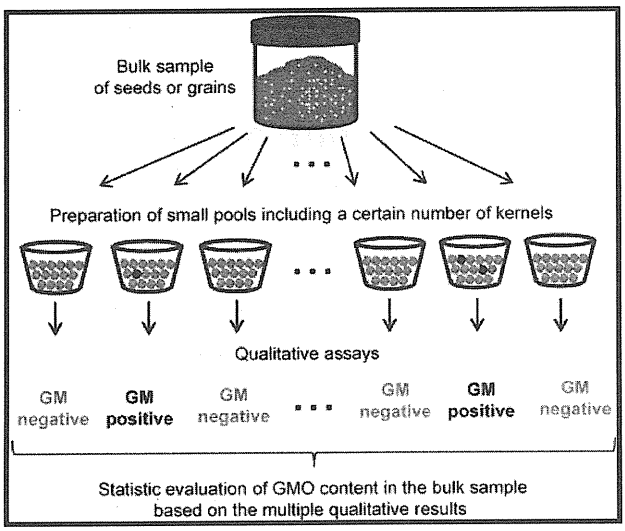


**Figure 1.** Group testing applied to GMO analysis.

genetics, blood-bank screening, drug discovery, biology, and plant pathology.[6,7] In this strategy, groups of units that make up an analytical sample are prepared. Then, qualitative analyses of multiple groups are individually performed, and the contents of the analyte are evaluated statistically.[8] When applied to GMO analysis of seeds or grains, each group contains a defined number of kernels from a larger bulk sample, and the GMO content is statistically evaluated based on qualitative results for multiple groups (Figure 1). Irrespective of the presence of stacked-event kernels, such a strategy enables the evaluation of GMO content on a weight/weight basis. Additionally, this strategy would be more efficient than a single-kernel-based strategy. In fact, the theoretical application of group testing strategy to GMO analysis has been investigated.[9−11] Meanwhile, to the best of our knowledge, a practical and accurate testing method to perform group testing for maize grains has not yet been reported. Accordingly, we present the development and validation of an efficient, easy-to-use PCR-based testing method for GMO detection in small pools of maize kernels.

## MATERIALS AND METHODS

**Cereal Materials.** The representative GM maize events used were Bt11, Event176, GA21, MON810, MON863, NK603, T25, TC1507, DAS59122, MON88017, and MIR604. F1-generation seeds of Bt11 and Event176 and ground F1-generation seeds of GA21 and MIR604 were kindly provided by Syngenta Seeds (Basel, Switzerland). F1-generation seeds of MON810, MON863, NK603, and MON88017 were kindly provided by Monsanto (St. Louis, MO, USA), and F1-generation seeds of TC1507 and DAS59122 were kindly provided by Pioneer Hi-Bred International (Johnston, IA, USA). F1-generation seeds of T25 were imported directly from the United States. Five conventional maize seeds were used as non-GM maize: DK537 and RX740 maize from Monsanto; QC9651 maize from Quality Technology International (Huntley, IL, USA); and Strike5512 and LG2265 maize, obtained in Japan. Dry conventional soybean seeds directly imported from the United States were used as non-GM soy. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) were obtained in Japan.

**Preparation of Genomic DNAs.** For the preparation of purified DNA extracts, all dry seeds were ground with a P-14 speed rotor mill (Fritsch, Idar-Oberstein, Germany). For maize, soy, wheat, barley, and rice, DNA extraction was performed using a DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) as described previously.[13] The DNA concentration of solutions was determined by measuring ultraviolet (UV) absorbance with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). DNA concentration was calculated with 1 optical density unit at 260 nm equal to 50 ng/$\mu$L. All extracted DNAs were diluted to 20 ng/$\mu$L with sterile distilled water. Genomic DNAs were analyzed using a real-time PCR array system as previously reported,[14] and the purity of the samples was confirmed.

**Preparation of Plasmid DNAs.** To establish the method for group testing, we developed two duplex real-time PCR assays: a GM maize screening assay and an experimental control assay. The GM maize screening assay was designed to detect the 35S promoter region (P35S) and NOS terminator region (TNOS) widely introduced into commercially available GM maize events. The experimental control assay was intended to detect both the starch synthase IIb gene derived from *Zea mays* (SSIIb) as the endogenous reference DNA and an artificial sequence on the pART plasmid as an internal positive control (IPC). The pUC19 plasmids harboring each of the target sequences, namely, P35S, TNOS, SSIIb, and IPC, were prepared after cloning in *Escherichia coli* DH5$\alpha$ and are denoted pP35S, pTNOS, pSSIIb, and pART, respectively. The target sequences were confirmed to be single and correct by nucleotide sequence analyses. The sequence information is included in the Supporting Information. The plasmids were purified by cesium chloride/ethidium bromide equilibrium centrifugation[15] and then diluted to the given concentration with 5 ng/$\mu$L ColE1 plasmid solution in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (Nippon Gene, Tokyo, Japan).

**PCR Assays.** The two duplex real-time PCR assays, GM maize screening and experimental control assays, were developed as described above. The reaction mixture for the GM maize screening assay consisted of 12.5 pmol of P35S 1-5′ (5′-ATTGATGTGATATCTCCACTGACGT-3′), P35S 1-3′ (5′-CCTCTCCAAATGAAATGAACTTCCT-3′), TNOS 2-5′ (5′-GTCTTGCGATGATTATCATATAATTTCTG-3′), and TNOS 2-3′ (5′-CGCTATATTTTGTTTTCTATCGCGT-3′) primers; 2.5 pmol of P35S-Taq (5′-CCCACTATCCTTCGCAAGACCCTTCCT-3′) and TNOS-Taq (5′-AGATGGGTTTTTATGATTAGAGTCCCGCAA-3′) probes; 2.5 $\mu$L of DNA template; 0.5 $\mu$L of ROX Reference Dye (Life Technologies); 0.625 units of BIOTAQ HS DNA polymerase (Shimadzu, Kyoto, Japan); and 12.5 $\mu$L of 2 × Ampdirect Plus buffer (Shimadzu) in a total volume of 25 $\mu$L. The reaction mixture for the experimental control assay consisted of 12.5 pmol of IPC 1-5′ (5′-CCGAGCTTACAAGGCAGGTT-3′), IPC 1-3′ (5′-TGGCTCGTACACCAGCATACTAG-3′), SSIIb 1-5′ (5′-CTCCCAATCCTTTGACATCTGC-3′), and SSIIb 1-3′ (5′-TCGATTTCTCTCTTGGTGACAGG-3′) primers; 2.5 pmol of IPC 1-Taq (5′-TAGCTTCAAGCATCTGGCTGTCGGC-3′) and SSIIb-Taq (5′-AGCAAAGTCAGAGCGCTGCAATGCA-3′) probes; 40 theoretical copies of the pART plasmid; 2.5 $\mu$L of DNA template; 0.5 $\mu$L of ROX Reference Dye, 0.625 units of BIOTAQ HS DNA polymerase; and 12.5 $\mu$L of 2 × Ampdirect Plus in total volume of 25 $\mu$L. The oligonucleotide DNAs for PCR primers and TaqMan probes were synthesized by Fasmac (Atsugi, Japan) and Biosearch Technologies (Novato, CA, USA), respectively. P35S-Taq, TNOS-Taq, and IPC 1-Taq were labeled with 6-carboxyfluorescein (FAM) and black hole quencher 1 (BHQ1) dyes at the 5′ and 3′ terminals, respectively. For SSIIb-Taq, hexachlorofluorescein (HEX) was used in place of FAM dye. Thermal cycling of reaction mixtures was carried out with a 7900HT real-time PCR instrument (Life Technologies, Carlsbad, CA, USA) unless otherwise specified. The thermal cycling condition was set as 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 65 °C under 9600 emulation mode. Data analysis was performed using Sequence Detection Software, version 2.3. The manual Ct mode (threshold, 0.256 for FAM and 0.064 for HEX) and manual baseline mode (start of baseline, 3; end of baseline, 15)

were set at the "Delta Rn vs. Cycle" view of the "Amplification Plot" feature. DNA amplifications with threshold-cycle (Ct) values below 40 were determined to be positive. Concerning PCR assays performed with a 7500 real-time PCR instrument (Life Technologies), all experiments were carried out as described above, except that the volume of ROX reference dye was set as 0.05 $\mu$L and Sequence Detection Software, version 1.4, was used for data analysis.

**Design of Testing Protocol.** We designed the following testing protocol:

Step 1. Groups comprising 20 maize kernels each are prepared using a grain counter plate on which only 20 holes are available (For 100 Soybeans; Fuji Kinzoku, Tokyo, Japan), and they are put into glass vessels with a capacity of 75 mL for use with a Milser 800-DG household food processor (Iwatani, Tokyo, Japan). The number of groups in one experiment is variable depending on the analyst's purpose.

Step 2. Twenty milliliters of a lysis buffer is added to each glass vessel. One liter of the lysis buffer contains of 20 mL of 1 mol/L Tris-HCl buffer solution (Nacalai Tesque, Kyoto, Japan), 10 mL of 0.5 mol/L EDTA solution (Nacalai Tesque), 80 mL of 5 mol/L sodium chloride (NaCl) solution (Nacalai Tesque), and 30 mL of 10% sodium dodecyl sulfate (SDS) solution (Nacalai Tesque) in distilled water. Each group is ground for 20 s with the household food processor. After 10 min of incubation at room temperature, the lysate in each glass vessel is vigorously shaken by hand. After 10 min of static standing to allow solid–liquid separation, 50 $\mu$L of the supernatant is moved to a plastic tube. Each portion of supernatant is diluted 2-fold with sterile distilled water. The diluted solution is centrifuged at more than 1000$g$ on a personal benchtop centrifuge for 1 min and then used for the following PCR assay.

Step 3. PCR mixtures are prepared with the supernatant for both GM maize screening and experimental control assays, and thermal cycling is performed as described above.

Step 4. The data from real-time PCR are analyzed with Sequence Detection Software as described in the PCR Assays section. If SSIIb or IPC detection is determined to be negative by the experimental control assay, the group in question is rejected. If both SSIIb and IPC are positive, the group is determined to be either GM-positive or GM-negative based on the result of the GM maize screening assay.

**Evaluation of the Testing Protocol.** To evaluate the linearity of the PCR assays, we analyzed the respective plasmid DNAs with theoretical numbers of copies of 250000, 20000, 1500, 125, and 20 ($n$ = 3). Then, we calculated the regression lines from the averages of triplicate PCR results. To evaluate the specificity of the PCR assays, we analyzed the genomic DNAs derived from various kinds of GM events and plant materials using both assays ($n$ = 6). Ct values of P35S/TNOS detection by the GM maize screening assay and those of SSIIb and IPC detection by the experimental control assay were measured. To evaluate the sensitivity of the PCR assays, we analyzed plasmid DNAs with low theoretical numbers of copies (40, 20, 10, and 0; $n$ = 21 for each dilution level) and counted the number of positive results. For the comparative analysis of PCR inhibition between P35S, TNOS, and IPC detections, we selected SDS, NaCl, EDTA, and crude maize extracts as PCR inhibitors. Under the coexistence of these possible inhibitors at various concentrations, positive and negative detections were examined both in the GM maize screening assay with 40 copies of the pP35S or pTNOS plasmid and in the experimental control assay with 40 copies of the pART plasmid. For the preparation of the crude maize extract, 1 g of the ground DK537 maize sample was incubated for 1 h with vigorous shaking at room temperature in 3 mL of the lysis buffer. After centrifugation at 15000$g$ for 1 min, the supernatant was used as the crude maize extract.

We prepared simulated groups, each of which consisted of 19 kernels of non-GM maize and one MON810 kernel. As non-GM materials, DK537, RX740, QC9651, LG2265, and Strike5512 maize were individually used. The simulated groups were analyzed in accordance with the testing protocol ($n$ = 6 for each material).

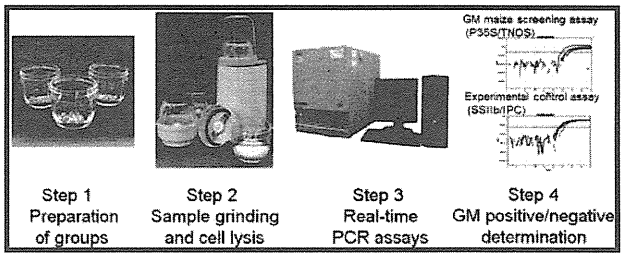


**Figure 2.** Overview of the testing protocol.

**Collaborative Trial for Method Validation.** For the collaborative trial, DK537 maize and F1-generation seeds of MON810 maize were used as non-GM and GM maize materials, respectively. All MON810 kernels were cut in half with a knife to inhibit germination, and simultaneously, approximately 2-mg fragments were scraped off individual half-cut GM kernels. To check for an adventitious presence of non-GM kernels in the GM seed lot, we suspended these fragments in 50 $\mu$L of the lysis buffer with sterile toothpicks and then analyzed them according to the testing protocol beginning with the 10-min incubation in step 2. We confirmed GM-positive detection for each MON810 kernel. The AOAC guideline specifies 10 laboratories reporting 2 analyte levels per matrix, 6 test samples per level, and 6 negative controls per matrix as the minimum criteria for the validation of qualitative methods.[16] These criteria also satisfy the requirements outlined in McClure's report.[17] Accordingly, we prepared groups consisting of 2 GM kernels and 18 non-GM kernels, groups consisting of 1 GM kernel and 19 non-GM kernels, and groups consisting of 20 non-GM kernels. These groups were named A, B, and C groups, respectively. As a set of blind samples for a laboratory, 6 A groups, 6 B groups, and 6 C groups were sorted at random and numbered from 1 to 18. A set of blind samples was provided to each of 12 laboratories. In each laboratory, the blind samples were analyzed in one experiment according to the testing protocol.

## RESULTS AND DISCUSSION

**Design of the Testing Protocol.** To perform group testing for maize grains in a practical manner, it is essential to develop an easy-to-use, high-throughput, and cost-effective sample pretreatment and GM maize screening assay for groups of kernels. A series of immunoassays for individual GM traits might be a good candidate, but a protein-based methodology limits the range of detectable GM events. We designed a testing method comprising a sample pretreatment step in which a group of maize kernels is ground in a lysis buffer with a household food processor and a subsequent PCR assay step in which the lysed sample is directly analyzed as a DNA template. We experimentally adjusted testing conditions, and the testing protocol was fixed as described in the Materials and Methods section. An overview of the testing protocol is shown in Figure 2. In this testing protocol, we fixed the number of kernels in a group to 20. This was because our preliminary investigation indicated that a group consisting of 20 kernels was easy to handle and gave stable PCR assay results. For sample preparation, we recommend using a grain counter plate to make small pools efficiently without intentional bias. For the sample pretreatment step, we used a household food processor that permitted sequential grinding of many samples just by changing glass vessels. The use of a household food processor promises a high-throughput treatment with a

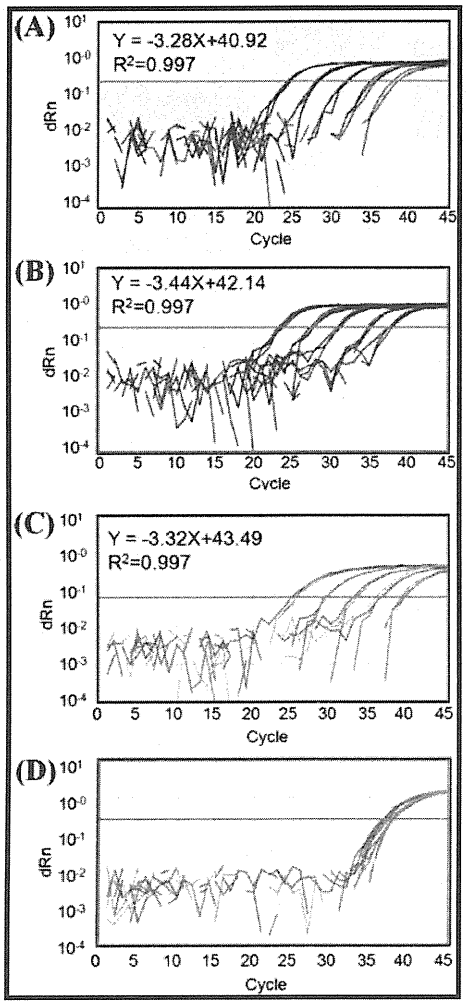


**Figure 3.** DNA amplification lines and parameters of their regression lines. Dilution series of plasmid DNAs were analyzed in triplicate. (A) pP35S series in the GM maize screening assay, (B) pTNOS series in the GM maize screening assay, (C) pSSIIb series in the experimental control assay, and (D) IPC detection results in the experimental control assay with the pSSIIb series. Regression lines were calculated from the mean values of triplicate analyses, and their parameters are shown in plots A–C.

minimum investment for grinding instruments. In addition, sample grinding in a lysis buffer does not require handling of dry flour, which simplifies the method and reduces the chance of contamination. For the PCR assay step, we designed two qualitative duplex real-time PCR assays, namely, GM maize screening and experimental control assays, using Ampdirect technology as a PCR reagent, which reduces the influence of PCR inhibitors. For the GM maize screening assay, the P35S and TNOS regions were selected as targets, because commercially available GM events have at least one, if not both, of these regions as part of their recombinant DNAs. TaqMan probes both of P35S and TNOS were labeled with FAM as a reporter dye, because P35S and TNOS detections were not necessarily distinguished. Meanwhile, we developed an experimental control assay to individually detect both SSIIb and 40 copies of pART, as the endogenous reference DNA and as an IPC, respectively. TaqMan probes for SSIIb and IPC detections were labeled with HEX and FAM, allowing us to distinguish between SSIIb and IPC. The experimental control assay was designed to confirm that the reaction mixture contained sufficient DNA extraction without PCR inhibition. To avoid detecting very tiny amounts of contamination, in terms of analytical robustness, we decided that DNA amplifications with Ct values of up to 40 were positive.

**Evaluation of PCR Assays.** We evaluated analytical performances of the PCR assay step. To do so, we prepared plasmid DNAs, each of which had a single target sequence for P35S, TNOS, SSIIb, and IPC detection. We confirmed the amplification linearity by using dilution series of plasmids except for pART (Figure 3). Detection results for P35S, TNOS, and SSIIb showed high coefficient values (>0.990). IPC detections were successfully obtained, irrespective of predominant SSIIb amplification in the same reaction mixture. Then, we confirmed the detection specificity with genomic DNAs from commercially distributed GM maize events and non-GM crops (Figure 4). P35S and/or TNOS regions were detected for all GM maize events, and these Ct values roughly corresponded to the numbers of copies of the P35S and/or TNOS regions in each event. Meanwhile, for non-GM maize, soy, wheat, barley, and rice, nonspecific detection was not observed, as expected. Although the specificity evaluation was carried out using only the single-GM-event samples, the results suggested that the GM stacked events derived from the single events would be detected in the developed assays. By analyzing the plasmid dilution series, we confirmed that the detection sensitivity of our method was high enough to detect 40 copies of target DNAs (Table 1). Then, we compared PCR inhibitions between P35S, TNOS, and IPC detections. We selected SDS, NaCl, EDTA, and crude maize extracts as PCR inhibitors that exist in PCR mixtures. Under the coexistence of these inhibitors at various concentrations, positive and negative detections were counted both in the GM maize screening assay with the pP35S or pTNOS plasmid and in the experimental control assay with the pART plasmid (Table 2). The results indicated that the IPC detection was sensitive to PCR inhibitors as well as P35S and TNOS detections when at least 40 copies of the P35S or TNOS regions were included in a reaction mixture. We also evaluated the PCR assays on the 7500 real-time PCR instrument, and the results are available in the Supporting Information. There were no large differences in results between the 7900HT and 7500 real-time PCR instruments.

**Analysis of Simulated Samples According to the Testing Protocol.** We performed analyses with the simulated groups of a maize sample that included one GM kernel among 20 kernels (Figure 5). As the GM maize kernel, we used F1-generation seeds of the MON810 event, which has the lowest number of copies of the target of the GM maize screening assay. No false negative result was observed in the GM maize screening assay, suggesting that the testing protocol had the capacity to detect at least one GM kernel in a group. Homogeneity of Ct-value variances of SSIIb detection between different non-GM materials was confirmed by Bartlett's test ($\alpha = 0.05$), and one-way analysis of variance (ANOVA) ($\alpha = 0.05$) of the data showed no significant differences ($p = 0.08$). This indicated that stable DNA extraction was achieved, irrespective of maize materials. In these results, the numbers of copies of SSIIb from 20 kernels were calculated as being between 5200 and 21000 copies (mean value of all results, 15000 copies) based on the calibration curve of the pSSIIb plasmid dilution series. This suggested that, if there was one kernel of GM maize in a group, at lease 5200/20 copies of P35S and/or TNOS regions would be expected to be present in a


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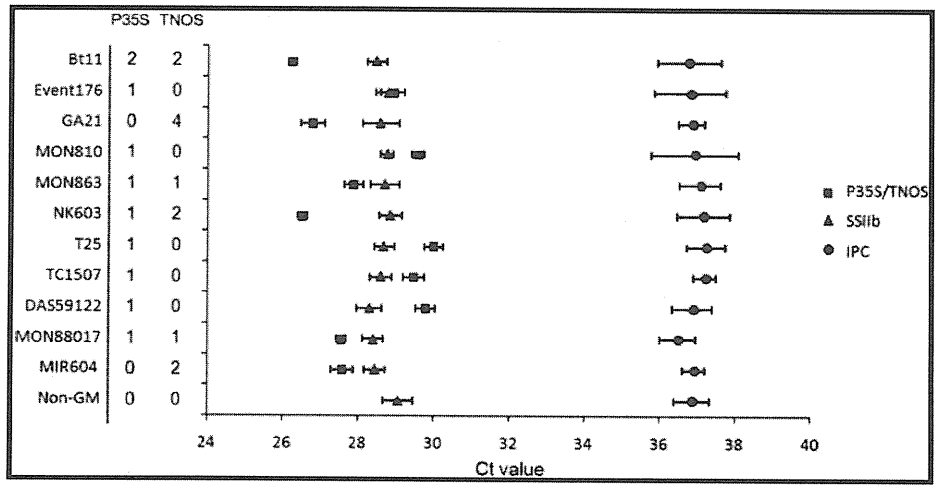


**Figure 4.** Specificity evaluation of PCR assays. DNA samples from GM events and non-GM maize were subjected to PCR assays ($n = 6$). The means of Ct values ± standard deviations are shown in the graphs. The numbers of P35S and TNOS regions in each GM-event haploid genome are summarized beside the names of the GM events.

**Table 1. Sensitivity Evaluation of PCR Assays**

| detection | theoretical number of plasmid copies | number of positives | positive rate (%) |
|---|---|---|---|
| P35S | 40 | 21/21 | 100 |
| | 20 | 21/21 | 100 |
| | 10 | 16/21 | 76 |
| | 0 | 0/21 | 0 |
| TNOS | 40 | 21/21 | 100 |
| | 20 | 21/21 | 100 |
| | 10 | 20/21 | 95 |
| | 0 | 0/21 | 0 |
| SSIIb | 40 | 21/21 | 100 |
| | 20 | 18/21 | 86 |
| | 10 | 12/21 | 57 |
| | 0 | 0/21 | 0 |

**Table 2. Comparative Analyses of PCR Inhibition between IPC, P35S, and TNOS Detections**

| inhibitor | final concentration | IPC detection | P35S detection | TNOS detection |
|---|---|---|---|---|
| SDS | 0.01% | 3/3 | 3/3 | 3/3 |
| | 0.05% | 3/3 | 3/3 | 3/3 |
| | 0.5% | 0/3 | 0/3 | 0/3 |
| NaCl | 2 mM | 3/3 | 3/3 | 3/3 |
| | 10 mM | 3/3 | 3/3 | 3/3 |
| | 100 mM | 0/3 | 0/3 | 0/3 |
| EDTA | 0.1 mM | 3/3 | 3/3 | 3/3 |
| | 0.2 mM | 3/3 | 3/3 | 3/3 |
| | 1 mM | 0/3 | 0/3 | 0/3 |
| crude maize extract | 1/1000 | 3/3 | 3/3 | 3/3 |
| | 1/500 | 2/3 | 3/3 | 3/3 |
| | 1/10 | 0/3 | 0/3 | 0/3 |

reaction mixture, and thus the IPC detection designed to have 40 copies of plasmid DNA as a template would be capable of checking PCR inhibition in the GM maize screening assay.

**Evaluation of Robustness in the Sample Pretreatment Step.** We evaluated the robustness of the sample pretreatment step with groups of non-GM kernels by slightly changing pretreatment conditions ($n = 6$ per condition). The modified conditions were the grinding time (10 s, 15 s, 20 s, or 25 s), the lysis time (5 min, 10 min, or 20 min), and the lysis temperature (15, 20, or 25 °C). The Ct values of SSIIb and IPC detections in the experimental control assay were evaluated (Figure 6). The homogeneity of Ct-value variances between conditions was confirmed by Bartlett's test ($\alpha = 0.05$), and then Ct values were analyzed by one-way ANOVA ($\alpha = 0.05$). Ct values under various lysis times and temperatures did not significantly differ. Meanwhile, Ct values of SSIIb detection under the various grinding times showed significant differences, suggesting that the amount of extracted DNA was influenced by the grinding time. We concluded that the sample pretreatment step was sufficiently robust in terms of the lysis time and temperature, but that the grinding time should be strictly controlled.

**Collaborative Trial for Method Validation.** A collaborative trial was carried out according to the procedure described in a previous report and guideline.[16,17] The results for the individual groups are available in the Supporting Information. All of the results showed the expected positive/negative determinations corresponding to the presence/absence of GM kernel(s) in each group, except for a result that was rejected because of unsuccessful IPC detection. The results indicated that the method accurately detected the presence of GM and the absence of cross-contamination between groups. After removal of the result rejected because of the unsuccessful IPC detection, the false-negative rates were calculated separately for the A and B groups, and the false-positive rate was calculated for the C groups (Table 3). Both false-negative rates were 0%, which fulfilled the criterion for the limit of detection for qualitative GMO detection methods as described in the ISO standard regarding GMO analysis.[18] In addition, the Ct values of the detections were found to be stable even under inter-laboratory evaluation (Figure 7). Thus, the testing method was validated to have sufficient performance for the reliable detection of one GM maize kernel in a group.


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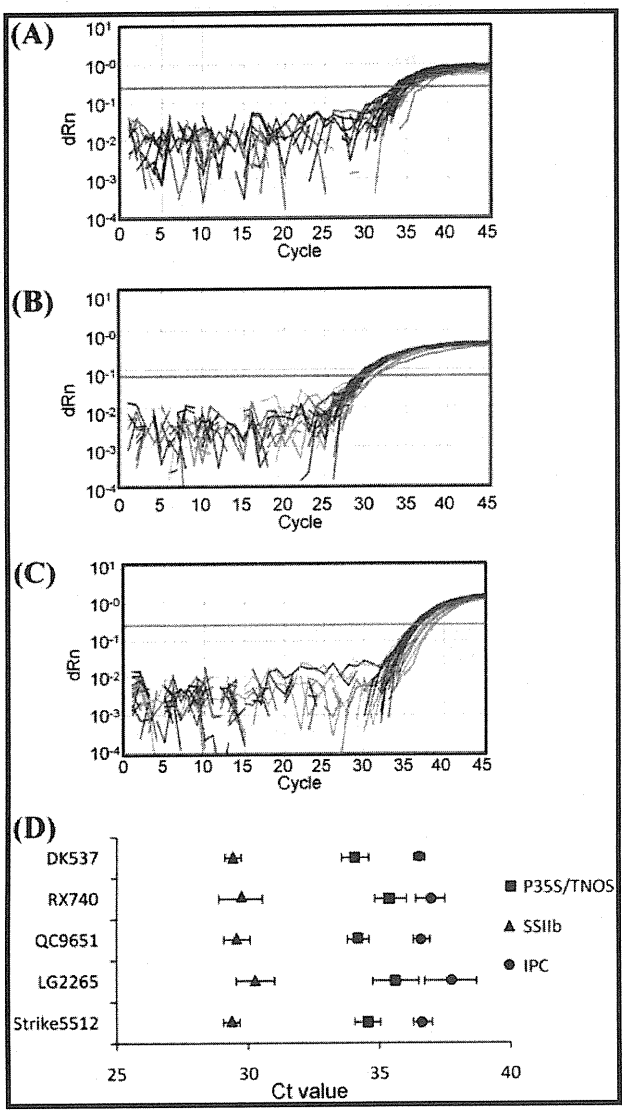


**Figure 5.** Testing results for the simulated samples including a MON810 kernel mixed in with five kinds of non-GM maize materials. (A) Amplification lines in the GM maize screening assay, (B) amplification lines of SSIIb detection in the experimental control assay, (C) amplification lines of IPC detection in the experimental control assay, and (D) summary of Ct value data for each non-GM background (means ± standard deviations, $n = 6$).

**Practical Use of the Group Testing Based on the Developed Method.** In summary, we have described an easy-to-use analytical method for group testing. This method was efficient enough to analyze 18 groups within 3 h at a low cost. Although our method harnesses two targets, namely, P35S and TNOS, to cover the commercially distributed GM maize events so far, this might become insufficient as new GMO events become available. There have been some reports describing highly multiplexing real-time PCRs for qualitative GMO detection.[19−22] The availability of the PCRs described in these studies suggests that it will be possible to update our method to test for new GMOs as they enter the market.

To practice group testing, it is indispensable to first determine the appropriate testing conditions, that is, the number of kernels per group (group size), the number of groups, and the maximum

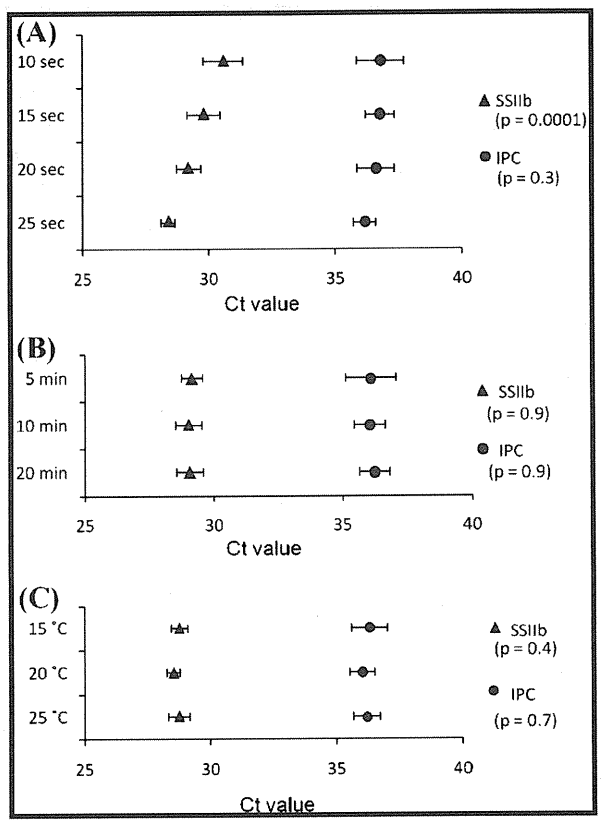


**Figure 6.** Robustness evaluation for the pretreatment step by simulated sample analyses. Effects by (A) grinding time, (B) lysis time, and (C) lysis temperature. Data are shown as means of Ct values ± standard deviations ($n = 6$). $p$ values given by one-way ANOVA ($\alpha = 0.05$) are shown under the graph legends.

**Table 3. Summary of Results in the Interlaboratory Study**

| group | number of rejections | false-positive results | false-positive rate (%) | false-negative results | false-negative rate (%) |
|---|---|---|---|---|---|
| A | 1/72 | – | – | 0/71 | 0 |
| B | 0/72 | – | – | 0/72 | 0 |
| C | 0/72 | 0/72 | 0 | – | – |

number of GM-positive groups for acceptance. Statistical calculation programs previously reported, such as Seedcalc, facilitate the determination of the optimal testing conditions depending on the analyst's purpose.[9,12] In our method, the group size was fixed at 20; however, the other parameters could be freely chosen. We confirmed that, even when the group size was fixed at 20, the testing conditions suitable for various threshold levels of GMO content such as 0.9%, 3%, and 5% could be selected by using the already existing calculation programs.

As an official method in Japan, the single-kernel-based method has already been used to determine whether the GMO content in a bulk maize sample exceeds 5%. The testing procedure requires analysis of 90 kernels for the first screening. If there are 3 or more GM kernels in the first 90 kernels tested, another set of 90 kernels must be tested. If the total number of GM kernels in the two tests (180 kernels) is 9 or less, then the GMO content of the bulk


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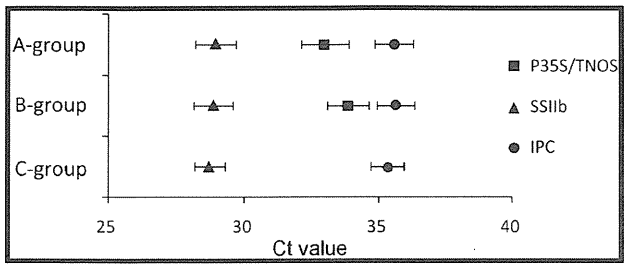


**Figure 7.** Summary of Ct value data in the interlaboratory study. The definitions of the groups A–C are given in the Materials and Methods section. Data are shown as the means ± standard deviations ($n$ = 71 for the A group and $n$ = 72 for the B and C groups).

sample is below 5% and is acceptable.[23] Based on the operating characteristic curve calculated by the Seedcalc program, we can design group testing that has approximately the same accuracy of judgment as the single-kernel-based method. The designed sampling plan is as follows: A group contains 20 maize kernels, and 10 groups are analyzed for the first screening. If there are 7 or more GM-positive groups in the first screening, another set of 10 groups will be tested. If the total number of GM-positive groups in the two tests (20 groups) combined is 12 or less, the GMO content of the bulk sample is determined to be below 5%. A comparison of operating characteristic curves between the single-kernel-based method and our group testing is provided in the Supporting Information. The slope of an operating characteristic curve represents the uncertainty of judgment that is caused by the sampling, and an analyst should take it into consideration. The introduction of group testing using our method will significantly decrease time and cost for inspection.

Furthermore, calculation programs permit the estimation of a GMO content value with confidence intervals from the testing results. For example, when 8 groups are determined as GM-positive in the testing of 20 groups containing 20 kernels per group, the GMO content will be estimated as 2.52% and its two-sided confidence interval will be between 1.06% and 4.97% at the 95% confidence level. In this manner, one can obtain quantitative information on the GMO content of the bulk sample based on the qualitative testing results and the established statistics.

We believe that group testing is a useful measure for weight/weight GMO content evaluation in maize grains, irrespective of increasing GM stacked events. Certainly, the strategy limits a sample to only seeds or grains and is not applicable to processed foods. However, group testing would contribute to the assured segregation of GM and non-GM maize through the production and transportation systems.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Nucleotide sequence information on PCR amplicons, results of analytical performance evaluation on a 7500 real-time PCR instrument, results of interlaboratory studies, and comparative analysis of the single-kernel-based method and the designed group testing. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ ABBREVIATIONS USED

ANOVA, analysis of variance; BHQ1, black hole quencher 1; EDTA, ethylenediaminetetraacetic acid; EU, European Union; GM, genetically modified; GMO, genetically modified organism; HEX, hexachlorofluorescein; IPC, internal positive control; PCR, polymerase chain reaction; NaCl, sodium chloride; SDS, sodium dodecyl sulfate; TNOS, NOS terminator region; SSIIb, starch synthase IIb gene of *Zea mays*; P35S, 35S promoter region; Ct, threshold cycle; UV, ultraviolet; FAM, 6-carboxyfluorescein.

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# 検査機関の信頼性確保に関する研究

平成 23 年度

研究成果の刊行物・別刷

学会発表

# 冷凍餃子を試料とした加工食品中の農薬分析における技能試験

○起橋雅浩、中山裕紀子、内田耕太郎、永吉晴奈、山口貴弘、柿本健作、尾花裕孝

大阪府立公衆衛生研究所

【目的】平成20年初頭に冷凍餃子への農薬混入事件が発覚したため、加工食品に対する不安が急激に増大し、これを受けて加工食品に対する農薬分析の需要が喚起された。しかし、複数食材が含まれる加工食品に対して、生鮮食品に準じた検査方法で農薬分析が行える範囲は限定的である。そこで、地方衛生研究所9機関を対象に加工食品を用いた技能試験を実施し、加工食品中の残留農薬分析への適応状況を検証した。

【方法】農薬を添加した技能試験用試料を2種類調製した。これら試料は同一農薬を添加し、その添加濃度には4：5の違いをもたせた。参加機関には添加農薬候補25種類を示し、添加農薬と添加濃度を通知せず試験を行った。分析法は1例を示したが、各機関で最適な分析法を使用することとし、測定機器としてGC-MS、LC-MSの使用を依頼した。なお試験期間を試料発送後2ヶ月とした。

試料調製：冷凍餃子（味の素冷凍食品株式会社製;252 g）を半解凍後フードプロセッサーで細切均一化し、撹拌機（ケンミックス・アイコーKM-800）で混合し、ブランク試料、添加試料2種をそれぞれ約4 kgずつ調製した。農薬の添加に際しては、添加用混合溶液（ジメトエート；120 μg/mL、クロルピリホス；110 μg/mL、ジエトフェンカルブ；70 μg/mL、クレソキシムメチル；90 μg/mL、イミダクロプリド；160 μg/mL：林純薬工業製）を4mLまたは5mLと、合成着色料の黄色4号または青色2号を添加し、濃度を黄：70～160 ppb、青：87.5～200 ppbとした。合成着色料は試料の識別および均一化の指標とした。これらを約200 gずつアルミ製シール袋に入れて密封した。試料毎に容器6個から12試料を分析し、一元配置分散分析により均一である事を確認した。2ヶ月後に容器1個から8試料を分析し、試験期間中農薬濃度が安定であったことを確認した。

評価方法：機関ごとに2種の試料（黄、青）の測定値和と差を求め、機関間Zスコア（ZB）と機関内Zスコア（ZW）を算出した。このZB、ZWを用いて散布図を作成し、ユーデンプロットの手法による複合評価を行った。ZB=ZW=0を中心とする半径|Z|=2の円内を良好、半径|Z|=3の円外を不良とし、２つの円の間を疑わしい、と判定した。

【結果】GC-MSでは7機関、LC-MS/MSでは5機関で、測定できた全ての農薬が「良好」と判定された。全機関を通じて7項目が「疑わしい」と評価されたが、それはのべ評価項目数（農薬×検出器×機関：80項目）の約9％であり、参加機関は高い精度を示した。

【考察】全機関とも誤検出は無く、報告値も160農薬（農薬×試料×検出器×機関）中150農薬は回収率として70～120％であり、全体でも60～122％と良好な結果であった。

本研究は厚生労働科学研究費補助金（食品の安心・安全確保推進研究事業）および文部科学省科学研究費補助金により実施した。

# 原材料に分別可能な加工食品試料を用いた農薬分析技能試験

○福井直樹、小阪田正和、高取聡、北川陽子、柿本葉、中辻直人、中山裕紀子、起橋雅浩、尾花裕孝

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【目的】平成20年初頭の有機リン系農薬が混入した冷凍餃子の喫食による健康被害の発生を契機に、加工食品に対する残留農薬分析の需要が高まった。加工食品の残留農薬分析に関して、大きく二点の課題が挙げられる。一点目は、分析法の構築である。加工食品は、複雑な食材が混在する、または、煩雑な加工工程を経た複合食品であるため、食品の特性に応じた分析法の検討が必要である。二点目は、食品衛生法の食品規格適合性の判断である。加工食品は、個別残留基準の設定されたものを除き、原則として一律基準0.01 ppmが適用される。ただし、加工食品を構成する食品原料が食品衛生法の食品規格に適合している場合には、一律基準超過によらず適合するものとみなされる。したがって、最終製品の加工食品の分析値のみで、食品規格への適合性の判断を行うのは不適当である。分析法については、様々な機関で、多くの分析法が構築されている。その一方、加工食品の食品規格適合性の判断まで想定した技能試験は行われていない。そこで、分別技能を加味した技能試験の実施を目的として、原材料ごとに分別可能な技能試験用加工食品試料を新たに開発した。

【方法】技能試験用加工食品試料は、原材料ごとに分別が可能であることを前提として、模擬ポークビーンズ（原材料：大豆、トマトピューレ、ロースハム）を開発した。これは、①分別に適するような原材料で構成、②特定の原材料に農薬の局在化が可能、③原材料間で農薬の移行が微量、④技能試験参加機関へ配布する時点で、試料が均一化（渾然一体化）していない特性を有している。試料を、原材料の分別ができない程度まで混和した状態で、繰り返し容器から採取した場合には、そこに含まれる農薬量は均一である一方、原材料の分別ができる状態で繰り返し容器から採取した場合には、そこに含まれる原材料の比率が異なるために農薬量が不均一であり、加工食品の食品規格適合性の判断を考察するうえで有用な試料であると考えられた。開発した試料に9種類の農薬を添加し、地方衛生研究所9機関を対象に、現在、技能試験を実施中である。

【結果及び考察】LC-MS/MSを用いて、模擬ポークビーンズおよび分別した原材料ごとに均一化して分析を行った。調製直後に容器間における均一性試験を行い、調製後約2ヶ月後に安定性試験を行った。均一性試験で、一元配置分散分析を行った結果、危険率5％で、容器間で農薬量に有意差なしと判定された。また、安定性試験の結果、調製直後に対して、9種類の農薬の残存率は、模擬ポークビーンズ全体で97～105％、農薬を添加した原材料で98～106％と良好な結果であった。

【謝辞】本研究は平成23年度厚生労働科学研究費補助金（食品の安心･安全確保推進研究事業）及び平成22、23年度科学研究費補助金（基盤研究C）の助成を受けて実施した。

# 高速溶媒抽出－LC/UV法によるシクロピアゾン酸の分析

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【目的】シクロピアゾン酸(CPA)は，*Aspergillus*属や*Penicillium*属の菌が産生するカビ毒の一種で，産生菌種はピーナッツ，とうもろこし，卵および飼料中などから検出されている。CPAの生理活性としては，ラット肝臓GOT，GPTおよびγ-GTP活性の上昇をもたらし，骨格筋の筋小胞体$Ca^{2+}$-ATPaseを特異的に阻害し，共役する$Ca^{2+}$の能動輸送を抑制する作用を有している。これまでに家畜や動物の中毒事例，更にヒトにおいても中毒の疑いがある事例が報告されている。他方，食品汚染として，アフラトキシンとの同時汚染の発生も報告されている。CPA摂取による健康被害が懸念されることから，食品汚染実態の把握および迅速な分析法確立が求められている。

すでに，発酵食品の一種である液状調味料(めんつゆ)を試料として，*Penicillium*属のカビを接種・培養して，その培養上清や菌体からCPAを検出した[1,2]。

今回，農産物の中でも輸入の依存度が高く，CPA汚染が危惧されるピーナッツと乾燥とうもろこしを選択して，高速溶媒抽出(ASE)法とLC/UVを用いた，簡便・迅速なCPA分析法を検討した。

【方法】(1) 試料溶液の調製；ASE装置にはDIONEX ASE-300を用いた。抽出セルの底部に円筒ろ紙および珪藻土約1 gを積層した後，粉砕した試料2.5 gに珪藻土3.0 gを加えて混和したものを充填した。ASE条件として，抽出溶媒，抽出温度および抽出サイクルなどを種々検討した。試料抽出液の一定量を減圧乾固し，希メタノール／水で再溶解したものをOasis®HLBカートリッジ(30 mg, 1 cc)を用いて精製し，試験溶液とした。

(2) CPA測定；LC装置にはHITACHI 655A-12 & L-500 LC Controllerを用いた。検出器にはShimadzu SPD-6AVを用い，測定波長は220nmとした。LCの分離カラムにはDIONEX Acclaim® Mixed-Mode WAX-1 (4.6 mm i.d. × 150 mm, 5 μm)を採用し，移動相にはアセトニトリル：25 mMリン酸緩衝液(pH6.0)=(7:3)を用い，流速は1 mL/minとした。

【結果および考察】農産物(ピーナッツ，乾燥とうもろこし)中のCPA定量分析法の構築に関して，ASE操作の至適条件を検討した。その結果，抽出溶媒として50%メタノール，抽出温度として25℃（室温），抽出サイクル数は1回の抽出操作で十分であることが分かった。添加回収試験を行ったところ，ピーナッツおよび乾燥トウモロコシの両試料とも，CPA溶出付近には妨害ピークのない良好なクロマトグラムが得られ，平均回収率は約80%，相対標準偏差は6 %未満であった。

また，実試料として輸入品のピーナッツ，乾燥トウモロコシおよびピスタチオの分析を行ったところ，いずれの試料からもCPAは検出されなかった。

1) 斉藤ら，日本薬学会第129年会(2009)
2) 佐々木ら，日本薬学会第131年会（2011）

ELISA による液状調味料中に含まれるシクロピアゾン酸の分析

Analysis of cyclopiazonic acid contaminated in liquid seasoning by ELISA

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【目的】シクロピアゾン酸（CPA）はマイコトキシンの一種であり、*Penicillium* 属や *Aspergillus* 属が産生するカビ毒である。CPA は動物実験において嘔吐、下痢、中枢神経系の抑制などの中毒症状や、乳汁への移行が報告されている。検出例としてはピーナッツやトウモロコシ、動物用飼料がある。また、調味料（めんつゆ）のような加工食品からも検出されている。CPA 汚染食品による健康被害を防止するために、CPA の迅速、簡便かつ高感度な分析法が求められている。本研究では、食品分析法として液状試料のめんつゆを測定対象とした ELISA による CPA 分析法の構築を試みた。また、めんつゆの前処理法を検討した。

【方法】ELISA では間接競合法を採用した。CPA と KLH(Keyhole limpet hemocyanin)結合体を固相化抗原とし、第 1 抗体には CPA ウサギ抗血清、第 2 抗体には HRP 標識ヤギ抗ウサギ IgG（H+L）を使用した。酵素基質には TMB、反応停止液には希硫酸溶液を使用した。めんつゆの前処理法の検討においては、原液の希釈のみ、有機溶媒による液液抽出、および液液抽出+固相抽出処理液を用いて、それぞれ作成したマトリックス検量線を比較・評価した。

【結果および考察】ELISA による CPA 分析では、標準品において 1～100 ppb の範囲で良好な検量線を得ることができた。また、めんつゆの前処理法においては、液液抽出+固相抽出はもちろん、液液抽出および原液の希釈でも、それぞれ作成したマトリックス検量線は、マトリックスが存在しない CPA 標準品の検量線と良好に一致した。このことから、ELISA におけるめんつゆの簡便な前処理法として原液の希釈法が採用できると考えられた。本研究により ELISA による CPA 分析法の構築と、めんつゆのより簡便な前処理方法を構築することができた。