of GLA was previously prepared from 18β-GLA and its derivatives were intensively synthesized and investigated for various pharmaceutical activities, including anticancer activity[9]. The isomerization from 18β-epimer to 18α-epimer proceeded under an alkaline condition, which implies a possibility of contamination of 18α-isomer in GA reagents. Therefore, this study attempted to confirm the entire structure of GA, including GLA, using NMR.

To confirm the aglycone GLA structure, the detailed structure of GLA was analyzed by 2D NMR experiments. The ^{1}H-^{13}C HMQC experiment correlated all proton signals with the corresponding 21 carbon atoms. As shown in Fig. 2, the cross peaks observed in the COSY experiment gave five spin-spin systems (H1-H3, H5-H7, H15-H16, H18-H19, and H21-H22). The ^{1}H-^{13}C HMBC correlations of seven singlet methyl signals (H23, H24, H25, H26, H27, H28, and H29) allowed the connection of these methyl groups to adjacent quaternary carbons (C4, C8, C10, C14, C17, and C20). Subsequently, the quaternary carbons were connected to the spin-spin systems by the HMBC correlations to yield two six-membered rings, namely the A and E rings (Fig. 2). The HMBC correlations from a singlet methine signal (H9) to C8, C10, and C11 revealed a bridge connection of two quaternary carbons of C8 and C10 *via* C9 to yield B rings, and further indicated the ketone (C11) to be located adjacent to C9 (Fig. 2). The HMBC correlations from a singlet olefin signal (H12) to the ketone C11 and to a quaternary olefinic carbon (C13) constructed the connection from C11 to C13. This connection was further extended to the quaternary carbon (C14) by the HMBC correlation of the methyl signal (C27) to C13 to yield a 6/6/6 ring system, namely the A, B, and C rings. The HMBC correlation from H12 to C18 showed a connection between C13 and C18 to make ring D. Finally, a carboxylic acid (C30) was connected to the quaternary carbon (C20) on the E ring by the HMBC correlations from the adjacent methyl signal (H29) to C30. Thus, the planar structure of GLA was confirmed to be a triterpene structure containing five rings (Fig. 2). Subsequently, the stereochemistry of GLA was elucidated from the NOESY correlations and coupling constants (Fig. 3). The NOESY correlations around the A and B rings (H1a/H3, H3/H5, H1a/H5, H1a/H9, and H5/H9) indicated axial orientations of all these protons, which means that the hydroxyl group at C3 has a β-orientation. The equatorial position of the C23 methyl group was confirmed by a NOESY correlation observed between H3 and H23. The large coupling constant ($J_{H18,H19\alpha}$ = 13.5 Hz) indicated an axial orientation at C18 on the E ring. The NOESY correlations between H18 and H28 showed the *cis*-form of H18 and the methyl group (C28). In addition to these data, the NOESY correlations between H12 and H18 indicated the β-orientation of H18. The NOESY correlation between H29 and H16 showed that the carboxylic acid (C30) had an axial orientation, as with H18. These results confirmed that the aglycone of GA was 18β-glycyrrhezinic acid (18β-GLA).

To confirm the structure of the glucuronic acids (GlcA1 and GlcA2), the structure of GlcA1 and GlcA2 were analyzed by 2D NMR experiments and from the *J* values from their ^{1}H NMR spectra. The spin-spin systems starting from the anomeric proton (H1′) at δ_H 4.51 to H5′ at δ_H 3.76 in the GlcA1 unit was assigned by the COSY experiment (Fig. 2). The presence of a carboxylic acid of GlcA1 was confirmed by the HMBC correlation from H-5′ to a carboxyl carbon (C6′) at δ_C 172.6 (Table 1). The signal of H1′ was split into a doublet and showed a coupling constant ($J_{H1',H2'}$) of 7.5 Hz (Table 1). Generally, anomeric configurations are assigned from the magnitude of $J_{1,2}$ with values of 7-9 Hz for the diaxial coupling associated with a β-anomers, while 2-4 Hz is indicative of the equatorial-axial coupling of α-anomers[10]. Furthermore, the coupling constants of other oxymethin protons, such as $J_{H2',H3'}$, $J_{H3',H4'}$, and $J_{H4',H5'}$, were observed to be around 9.5 Hz (Table 1). These values were sufficiently large to assure axial orientations for the five protons (H1′ ~ H5′), allowing a chair form of a pyranosyl ring of GlcA1 (Fig. 3). This interpretation was also supported by the NOESY correlations for H1′/H3′, H1′/H5′, H3′/H5′, and H2′/H4′ (Fig. 3). Thus, the glycosidic bond at C1′ in the glucuronopyranosyl structure of GlcA1 is oriented equatorially and this result definitely indicated that GlcA1 is β-form, not α-form. The glycosidic linkage from the anomeric proton to the aglycone GLA was confirmed by the HMBC correlations from H1′ to C3 (Fig. 2). The structure of glucuronic acid, GlcA2, was also confirmed in a similar manner to GlcA1. An anomeric proton (H1″) observed at δ_H 4.62 also had a large coupling constant (7.7 Hz), confirming the β-form of GlcA2, as with GlcA1 (Fig. 4). The inter-glycosidic linkage was confirmed to be a β1 → 2 link by the HMBC correlation from H1″ to C2′ at δ_C 84.0 of GlcA1 (Fig. 2). The chirality of GlcA units were determined to be both D-form, because previous studies indicated that both units were D-form and furthermore the presence of L-GlcA has not been reported from any natural sources. Based on the foregoing evidence, we concluded that the structure of GA is 3β-hydroxy-11-oxo-18βH-olean-12-en-30-oic acid 3-*O*-[β-D-glucuronopyranosyl-(1 → 2)-β-D-glucuronopyranoside].

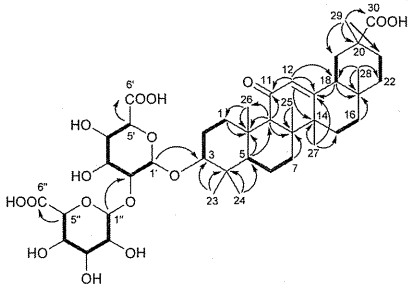

Fig. 2. Key correlations of ^{1}H-^{1}H COSY (bold lines) and ^{1}H-^{13}C HMBC (arrows) of GA

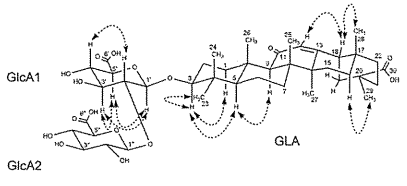

Fig. 3. Structure of GA and NOESY correlations

In six other commercial GA or GA ammonium salt reagents, the configurations of the two GlcA units were both β-form, as observed with the GA from Wako (Fig. 4). Two anomeric protons of GlcA1 and GlcA2 showed identical coupling constants in each GA or GA ammonium salt, although there were slight differences in chemical shifts between GA and GA ammonium salts (Fig. 4).

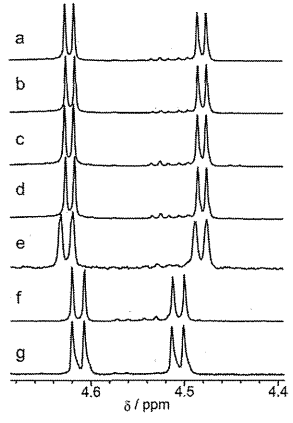

Fig. 4. ^{1}H NMR spectra (600 MHz) around anomeric proton signals of GlcA1 and GlcA2 of seven GA reagents in CD_3OD

Five spectra (a ~ e) are GA ammonium salts, while the remaining two spectra (f and g) are GA.

Ⅳ Discussion

The present NMR studies demonstrated that GlcA1 and GlcA2 were both β-form in the seven GA and GA ammonium salt reagents purchased from seven sources. These results clearly indicated that the supplier information on GA structure is incorrect. We have little information as to why this misinterpretation of the GA structure has occurred. In 1950, Lythgoe and Trippett first identified two hexuronic acids in GA as GlcA units[11]. Furthermore, they proposed that the stereochemistry of GlcA units was one β-link as the internal glycosidic bond and the other as an α-link; this was determined by comparison of $[\alpha]_D$ values between a permethylate derivative of GlcA units prepared from GA and authentic glycosidic compounds. This interpretation was generally accepted, and the α-configuration of GlcA1 in GA was taken as correct. After approximately 40 years, however, Khalilov *et al.* revealed the configuration of GlcA1 to be β-form based on an ^{13}C-NMR study of GA purified from natural sources[12]. Report of the revised structure was subsequently followed by an advanced NMR study on GA[13]. During research on natural products in *Glycyrrhiza* plants, GA was often isolated as a by-product and its structure was elucidated[7, 8]. These reports also showed that the GlcA1 configuration of the isolated GA is β-form. Furthermore, the X-ray crystal structure of GA dipotassium salt was recently analyzed to evaluate the coordination system of potassium ions to GA, which subsequently indicated GlcA1 to be β-form[14, 15]. Accordingly, recent research has determined the GlcA1 configuration in GA to be β-form. Nevertheless, commercial reagent catalogues and chemical databases, including package inserts for drugs, designated GlcA1 in GA as α-configuration, which has led to confusion in the research areas of analytical chemistry and biochemistry.

GA is one of the most well known and successful natural sweeteners, and is also used as a phytomedicine. Moreover, numerous biochemical and chemical studies dealing with GA have been reported. Yet, surprisingly, its incorrect structure continues to be used in commercial catalogue product information. Furthermore, since the incorrect catalogue information is likely to be recognized as the standard structure of the compound, this might lead to misinterpretation of research results. We believe that this study definitively clarifies this misinformation, and we urge the rapid revision of the incorrect structure of GA in commercial catalogues and other literature.

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論　　文

グリチルリチン酸に含まれるグルクロン酸の立体化学の確認

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概　要

グリチルリチン酸（GA）は 2 つのグルクロン酸（GlcA1 と GlcA2）を含むトリテルペン型サポニンである。GA はカンゾウ属（*Glycyrrhiza*）の植物の根に含まれている。生薬として、また食品に添加する天然甘味料として長く用いられてきた。精製された GA は分析用の標準品または生化学試薬として複数の試薬会社から入手が可能である。試薬会社のカタログには、GlcA1 の立体化学はα型で、一方の GlcA2 はβ型と記されている。Chemical Abstract においても、GlcA1 はα型で GlcA2 はβ型とされている。しかしながら近年の研究では、2 つの GlcA はともにβ型との報告が続いている。この混乱を解決すべく、複数の試薬会社から高純度に精製された GA または GA 塩の試薬を入手し、1 次元および 2 次元 NMR によって構造を詳細に解析した。その結果、2 つの GlcA はともにβ型であることが確認された。

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A method for the detection of shrimp/prawn and crab DNAs to identify allergens in dried seaweed products

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Abstract

Crustacean protein (tropomyosin) has frequently been detected in processed foods containing seaweed. In Japanese regulations for the labeling of allergenic food ingredients, the PCR method for detecting extracted shrimp/prawn and crab DNA is stipulated to discriminate shrimp/prawn and crab in processed foods. It has, however, been difficult to extract shrimp/prawn and crab DNA in processed foods including seaweed. We modified the DNA extraction protocol of the DNeasy *mericon* Food kit, and compared the yield and purity of DNA extracted from dried seaweed powder containing 1, 5, 10, 100, or 10,000 μg/g of freeze-dried edible shrimp/prawn or crab using various commercially available DNA extraction kits. The improved DNA extraction method provided sufficient yield and purity of extracted DNA suitable for the detection of specific DNA using the PCR method. To directly evaluate the applicability of the DNA extraction method, we employed PCR amplification with primers (PyrbcL01–5'/PyrbcL01–3') designed for the detection of the *Pyropia yezoensis rbcL* gene. The primer pair could generate amplicons from several commercial nori food products and dried seaweed powder containing shrimp/prawn or crab. The limit of detection for shrimp/prawn or crab DNA extracted by the improved DNA extraction method is 1 μg per g dried seaweed powder. In conclusion, we showed that the improved method is simple, rapid and highly sensitive, and can be used to detect shrimp/prawn and crab DNA in dried seaweed food products.

Keywords : allergen, crustacean, DNA extraction method, dried seaweed product, PCR

I Introduction

The Japanese Ministry of Health, Labour, and Welfare (MHLW) stipulated the allergen labeling system by amending the Food Sanitation Law in April 2001. In particular, the labeling of egg, milk, wheat, buckwheat, and peanut ingredients in any commercial processed food became mandatory in April 2002 in response to individuals with food allergies. Therefore, the MHLW has prescribed official Japanese methods for determining allergens to validate the labeling of food products.

The labeling of shrimp/prawn and crab became mandatory in June 2008, and the enzyme-linked immunosorbent assay (ELISA) methods for quantitative screening and PCR for qualitative confirmation were announced as the official methods for the detection of shrimp/prawn and crab[1]. Two commercially available ELISA kits for screening were validated according to international validation protocols[2] and were used to validate the labeling for shrimp/prawn and crab[3]. However, because of the high amino acid sequence homology between shrimp/prawn and crab[4], these ELISA kits failed to distinguish between shrimp/prawn and crab tropomyosin. Furthermore, these ELISA kits can detect tropomyosin derived from other crustaceans and insects not encompassed by the food labeling regulation. In addition, PCR is commonly used to identify either shrimp/prawn or crab contamination and to exclude false positives.

Crustacean protein was frequently detected in a recent survey of processed food products primarily containing seaweed (seaweed products)[5]. Since many crustaceans such as the skeleton shrimp (*Pseudoprotella phasma*) inhabit the same river and sea environments where seaweed is harvested, the contamination of seaweed products with crustacean protein is thought to occur as a result of bycatch. A definitive confirmation test for crustacean protein contamination of seaweed products using PCR has been impeded by the difficulties of extracting DNA. Although several reports show the successful detection of crustacean contamination in young sardines (shirasu), dried young sardines (chirimenjyako) and fish paste, confirmation of crustacean contamination in seaweed products has been problematic[6-8].

Seaweed is a generic name for algae used as a food ingredient, and includes red algae, green algae, and cyanobacteria (blue–green algae). Seaweed is widely consumed as a raw and processed food, and contains proteins, carbohydrates, minerals, dietary fiber, and vitamins[9]. Therefore, seaweed products are long-standing popular foods in coastal countries such as Japan[10]. However, the intake of seaweed products might induce an allergic response in some patients with an allergy to crustacean because of bycatch contamination. Thus, to protect the health of consumers and guarantee their right to information, the detection of crustacean in seaweed is necessary to prevent responses in those allergic to crustacean.

In this study, model seaweed processed food samples containing 1, 5, 10, 100, or 10,000 μg/g of shrimp/prawn or crab were examined using an improved DNA extraction method, and the limits of detection (LODs) for shrimp/prawn and crab DNA were determined using qualitative PCR. Subsequently, the applicability of the DNA extraction method was confirmed in commercial dried sheets of seaweed (nori food products).

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II Materials and Methods

1. Samples

The nori food products were purchased at a supermarket in Chiba, Japan and ground using a mill mixer (IMF-700G; Iwatani, Co., Ltd, Tokyo, Japan) to prepare powdered nori model samples containing shrimp/prawn or crab. Qualitative analysis using the FA test Immunochromato-crustacean "Nissui" (LOD, 1.0 μg/g; Nissui Pharmaceutical Co., Ltd, Ibaraki, Japan) confirmed the absence of crustacean protein in the nori powder. Frozen black tiger prawn (*Penaeus monodon*) and frozen boiled hair crab (*Erimacrus isenbeckii*) were purchased at a supermarket in Chiba, Japan for use as standard materials. After shell removal, freeze-drying and grinding, powdered black tiger prawn and hair crab were prepared from edible body parts (muscle) as standard shrimp/prawn or crab materials. To investigate the sensitivity of the established method, samples were powdered using a mill mixer and prepared to contain 1, 5, 10, 100, or 10,000 μg/g of freeze-dried shrimp/prawn or crab per g dried nori powder.

2. ELISA

To detect crustacean tropomyosin, the commercially available ELISA kit FA test for EIA-crustacean (N kit; Nissui Pharmaceutical) and Crustacean (M kit; Maruha Nichiro Holdings Inc., Ibaraki, Japan) were used according to the manufacturer's instructions and the official method notified by Consumer Affairs Agency, Government of Japan[1].

3. Pretreatment of samples

The moisture content of nori food products is generally < 10%[11], and absorption of the DNA extraction buffer into samples often inhibits DNA extraction from foods such as dried seaweed products. Thus, nori food products tend to completely absorb water under all DNA extraction methods except for the cetyl trimethyl ammonium bromide (CTAB)-based method described in the following section 4. In these procedures, samples (1.0 g) were completely mixed with 10 mL of ultra-pure distilled water (Life Technologies, Carlsbad, CA, USA) by inversion in 50 mL centrifuge tubes. Mixtures were then centrifuged at 3,000 × g for 10 min and supernatants were carefully removed. Finally, precipitated samples were used as water-absorbed samples for subsequent DNA extraction.

4. Extraction of DNA

DNA was extracted using a DNeasy Plant mini kit (DPM method; Qiagen, Hilden, Germany), Genomic-tip 20/G kit (GT method; Qiagen), the DNeasy *mericon* Food kit (DMF method; Qiagen), and the CTAB-based method. The DPM method was performed according to the manufacturer's instructions with slight modifications. Briefly, water-absorbed samples were mixed with 10 mL of AP1 buffer at 65°C and 10 μL of RNase A (17,500 U; Qiagen) and then incubated at 65°C for 15 min. Subsequently, 3,250 μL of AP2 buffer was added, and the mixtures were incubated at room temperature for 10 min. The solutions were centrifuged at 5,000 × g for 5 min and supernatants were obtained and centrifuged again at 10,000 × g for 5 min. The resulting supernatants were then transferred to a QIAshredder Spin Column and centrifuged at 10,000 × g for 2 min. The eluates (3 mL) were mixed with 4.5 mL of AP3/Ethanol buffer and transferred to DNeasy Mini Spin Columns. The columns were washed twice with 500 μL of AW/Ethanol buffer by centrifugation, and DNAs were finally eluted with 100 μL of distilled water.

The GT method was performed according to the manufacturer's instructions with slight modifications. Briefly, 20 mL of G2 buffer and 200 μL of α-amylase (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) were added to pretreated samples, mixed completely by inverting in 50 mL centrifuge

tubes, and incubated at 37°C for 1 h. Subsequently, 100 µL of Proteinase K (> 600 mAU/mL; Qiagen) and 20 µL of RNase A were added, and the mixtures were incubated at 50°C for 2 h. After centrifugation, supernatants were transferred to 15 mL centrifuge tubes and then loaded on a Genomic-tip equilibrated with 1.0 mL of QBT buffer. The column was washed three times with a total of 6 mL of QC buffer, and the bound DNA was twice eluted with a total of 2 mL of QF buffer at 65°C. DNA was precipitated by the addition of 2 µL of Ethatinmate (Wako Pure Chemical Ind., Ltd., Osaka, Japan) and 1.4 mL of isopropanol followed by centrifugation and 70% ethanol wash, and the DNA was finally dissolved in 100 µL of distilled water at 65°C.

The DMF method was performed according to the manufacturer's instructions with slight modifications. Briefly, water-absorbed samples were mixed with 10 mL of Food Lysis buffer and 25 µL of Proteinase K and incubated at 60°C for 30 min. The mixtures were then cooled on ice to room temperature. After centrifugation at 2,500 × g for 5 min, 700 µL aliquots of supernatant were mixed with 500 µL of chloroform in 1.5 mL microtubes and vigorously vortexed. The mixtures were then centrifuged, and 450 µL of the supernatant (aqueous phase) and 1.8 mL of PB buffer were mixed in 15 mL centrifuge tubes. The mixtures were then transferred to QIAquick Spin Columns, and the columns were washed with 500 µL of AW2 buffer. The columns were centrifuged at 17,900 × g for 1 min until all residual AW2 buffer was drained; after the addition of 100 µL of EB buffer, the QIAquick Spin Columns were kept at room temperature for 1 min. Finally, the DNA was eluted into 1.5 mL microtubes by centrifugation. The modified DNA extraction method was designated as the DMF-mSFP method.

The CTAB-based method was performed according to the procedures described in the Japanese official standard methods[1].

5. Spectrophotometric evaluation of extracted DNA

The concentration and quality of extracted DNA were evaluated by measuring ultraviolet (UV) absorption using a Bio-Spec mini spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). DNA purity was estimated according to ratios of absorbance at 260 nm and 230 nm (A260/230), and 260 nm and 280 nm (A260/280); absorption at 320 nm was subtracted as background.

6. PCR analysis and agarose gel electrophoresis

For universal detection, the AN1–5'/AN2–5'/AN–3'[12] primer pair for detecting common animals and CP03–5'/CP03–3'[13], Plant01–5'/Plant01–3'[14] or Placon5/Placon3 primer pairs (Promega KK, Tokyo, Japan) for common plant DNA were used. The universal detection for seaweed was performed with the PyrbcL01–5' (GGTCCTGCAACTGGATTGAT)/PyrbcL01–3' (AGGAAATCAAGACCGCCTTT) primer pair, which was designed using published sequences of the *Pyropia yezoensis* chloroplast ribulose-1, 5-bisphosphate carboxylase (*rbcL*) gene (GenBank: AB243204) using Primer3 free online software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/, last accessed March 2014). The specific detection of shrimp/prawn and crab was performed with ShH12–05'/ShH13–03' and CrH16–05'/CrH11–03' primer pairs, respectively, as previously described[15, 16]. All PCR primer pairs except for Placon5/Placon3 were synthesized and purified by FASMAC Co., Ltd. (Kanagawa, Japan) using reverse-phase columns. Specific primers were purchased from FASMAC for the detection of Akiami paste and mantis shrimp. PCR was performed using PCR AmpliTaq Gold PCR Master Mix (AmpliTaq reagent; Life Technologies) and Ampdirect plus (Ampdirect reagent; Shimadzu). PCR reactions with the plant primer pairs CP03–5'/CP03–3' and Plant01–5'/Plant01–3' were performed using AmpliTaq reagent in a total volume of 25 µL, in 0.2 mL reaction tubes containing 50 ng of template DNA, 1 × PCR buffer II, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.2 µmol/L forward and reverse primers, and 0.625 units of Taq DNA polymerase. PCR conditions were as follows: 10 min of preheating at 95°C and 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, followed by 7 min at 72°C. Another PCR reaction for plants was performed with Placon5/Placon3 according to the manufacturer's instructions. To detect animal and crab DNA, $MgCl_2$ concentrations in the PCR reactions were 3.0 mmol/L and 2.0 mmol/L, respectively, as described in the official method[1]. To detect shrimp/prawn, the concentration of ShH12–05'/ShH13–03' primers in PCR reactions was 0.3 µmol/L each. PCR conditions for animal and crab DNA amplifications were as follows: 10 min of preheating at 95°C and 40 cycles of 30 s at 95°C, 30 s at 50°C or 54°C for animal or crab, respectively, and 30 s at 72°C, followed by 7 min at 72°C. PCR conditions for shrimp/prawn DNA amplifications were as follows: 10 min of preheating at 95°C and 45 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C, followed by 7 min at 72°C. PCR reactions for the detection of all targets were also performed using Ampdirect reagent in a total volume of 25 µL, in 0.2 mL reaction tubes containing 50 ng template DNA, 1 × Ampdirect plus, 0.4 µmol/L forward and reverse primers and 0.625 units of Taq DNA polymerase. PCR conditions were as for the AmpliTaq reagent, and all PCR reactions were performed using a Veriti thermal cycler (Life Technologies). After PCR amplification, the products (7.5 µL) were mixed with 6 × loading buffer (1.5 µL; Wako Pure Chemical Ind., Ltd.) and electrophoresed at a constant voltage (100 V) on 4% agarose gels in 0.5 × TBE buffer (44.5 mmol/L Tris, 44.5 mmol/L boric acid, and 0.1 mmol/L EDTA; pH 8.3) using a Mupid-exU (Advance Co., Ltd., Tokyo, Japan). After electrophoresis, gels were stained in 0.5 × TBE buffer containing 0.5 µg/mL ethidium bromide for 20 min and destained in 0.5 × TBE buffer for 20 min. The gels were photographed using a FAS-III Model-TM20 (Toyobo Co., Ltd., Osaka, Japan).

Ⅲ Results and Discussion

1. Design of PCR primers for *P. yezoensis* DNA

Preliminary data showed poor DNA extraction efficiency of commercial nori food products using the three DNA extraction methods of the Japanese official standard methods, due to the high viscosity of supernatants during cell lysis and the absorption of DNA extraction buffers into samples. The GT method is frequently used for DNA extraction to confirm the accuracy of ingredient labels on foodstuffs[17, 18], and provides sufficient quantities of pure DNA for PCR reactions. However, the transfer and elution through the column is time consuming, and the DNA extracted from commercial nori food products failed to amplify using any primer pairs for universal detection of plants (CP03–5'/CP03–3', Plant01–5'/Plant01–3', and Placon5/Placon3 primer pairs; data not shown).

To avoid false-negatives, the applicability of extracted DNA in PCR should be confirmed. Major seaweed species in nori food products include *P. yezoensis*, *Pyropia tenera*, and *Pyropia pseudolinearis*; in Japan, *P. yezoensis* f. *narawaensis* is the most popularly consumed[19]. Therefore, we designed the PCR primers PyrbcL01–5'/PyrbcL01–3' (152 bp) from the *P. yezoensis rbcL* gene. The primer pairs designed from the genome DNA sequence of *P. yezoensis* were able to generate amplicons from several commercial nori food products (data not shown). Furthermore, extracted DNAs obtained from powdered nori model samples containing 1, 5, 10, 100, or 10,000 µg/g of freeze-dried shrimp/prawn or crab were successfully amplified (Fig. 1).

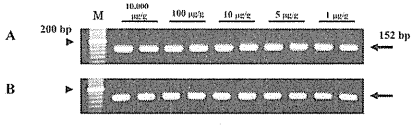

Fig. 1. Agarose gel electrophoresis of PCR products *(rbcL)* using the PyrbcL01–5'/PyrbcL01–3' primer pair from samples containing 1, 5, 10, 100, or 10,000 µg/g of shrimp (A) or crab (B) in powdered nori products
Arrows and arrowheads indicate the expected PCR amplification products for *P. yezoensis* chloroplast *rbcL* (152 bp) and the position of 200 bp in 20 bp ladder size standard, respectively. Lanes M, 20 bp ladder size standard (These bands were in the range of 20 – 1,000 bp at 20 bp intervals.).

Thus, PCR using the PyrbcL01–5'/PyrbcL01–3' primer pair can be used to validate extracted DNA obtained from nori food products.

2. Protocol optimization of the DNeasy *mericon* Food (DMF) kit

The CTAB-based method is widely used to extract total DNA from food materials and can effectively remove substances that inhibit PCR, such as certain proteins and polysaccharides[20]. However, this method has a number of disadvantages, including complex procedures and low DNA yields. Nori food products contain substantial amounts of characteristic polysaccharides such as alginate, fucoidan, carrageenan, and agarose. In particular, the sulfated polysaccharide porphyran is regarded as a strong PCR inhibitor in plants[21] and warrants use of the CTAB-based method for seaweed products.

Therefore, we optimized the total DNA extraction procedure using a DNeasy *mericon* Food (DMF) kit, which is based on the CTAB-based method[22]. The standard protocol (SP) and the small fragment protocol (SFP) are described in the manufacturer's instructions, and sample weights required for these protocols are 2.0 g and 200 mg, respectively. The SFP procedure of the DMF kit is designed for effective extraction of total DNA from processed foods. However, the DNA extracted from processed foods has typically been subjected to extensive thermal treatments, high pressure, irradiation, pH changes and drying, thus resulting in highly fragmented DNA[23]. Consequently, the SFP procedure uses column-binding conditions that are optimized for maximal recovery of short DNA fragments (shorter than 100–200 base pairs).

We then compared the six procedures shown in Table 1. Since the presence of crustacean in nori food products is irregular, the use of small amounts of a given sample might be limiting. For this reason, under all conditions, total DNA was extracted from 1 g samples. Ten milliliters of Food Lysis Buffer were added to water-absorbed samples in SP, SFP, and mSFP (modified SFP). Twenty milliliters of Food Lysis Buffer were added to non-water-absorbed samples in SP2, SFP2, and mSFP2 (modified SFP2) procedures, and samples were thoroughly agitated and mixed to produce sufficient volumes of supernatant. The resulting yield and purity of extracted DNA are shown in Table 1.

Regardless of the water absorption pretreatment, the mSFP and mSFP2 procedures gave the best yield and purity of extracted DNA compared with the SP (SP2) and SFP (SFP2) procedures. Pretreatment increased the DNA yields of all procedures. Furthermore, we considered the SFP procedure to be a simple step. These data show that the pretreatment facilitates DNA extraction from dried foods such as nori food products. To further increase in the yield of extracted DNA,

the mixture volume of the supernatant and PB buffer were varied 450 μL and 1.8 mL, respectively. In the following experiment, the modified procedure of mSFP of the DMF kit (DMF-mSFP method) was used to prepare high quality and quantity total DNA from nori food products.

3. DNA extraction from powdered nori samples containing shrimp/prawn or crab

The yield and purity of DNA extracted from powdered nori model samples spiked with five different concentrations of shrimp/prawn or crab were evaluated using the DPM, GT, CTAB, and DMF-mSFP extraction methods. The DNA was twice extracted from each sample. The yield and purity of DNA extracted from the shrimp/prawn and crab model samples are shown in Tables 2 and 3, respectively.

The highest yield of DNA extracted from the shrimp/prawn model samples was achieved with the GT method. In contrast, the CTAB-based method produced the lowest yield,

Table 1. Conditions of DNA extraction and spectrophotometric analysis of extracted DNAs

Protocol	Condition[a)]				Extracted DNA	
	Food Lysis Buffer (mL)	Supernatant (μL)	Buffer PB (μL)	Elution (μL)	DNA (μg)	A260/280
SP	10	350	350	150	1.38	1.89
SFP	10	250	1000	100	2.06	2.01
mSFP	10	350	1400	100	3.13	1.98
SP2	20	350	350	150	0.47	3.00
SFP2	20	250	1000	100	0.95	1.76
mSFP2	20	350	1400	100	1.92	1.98

a) DNA extractions were carried out by DMF, method.
SP, SFP and mSFP were treated for water absorption of samples with DNase free water, whereas SP2, SFP2 and mSFP2 were not.
SP(SP2) and SFP(SFP2) indicate standard protocol and small fragment protocol in DNeasy *mericon* Food Handbook, respectively.
mSFP(mSFP2) varied in volume of supernatant as compare to SFP(SFP2).

Table 2. Spectrophotometric analysis of DNAs extracted from model foods (nori containing shrimp)

Shrimp Conc. (μg/g)	DPM method[a)]			GT method[b)]			DMF-mSFP method[c)]			CTAB method[d)]		
	DNA (μg)	Ratio		DNA (μg)	Ratio		DNA (μg)	Ratio		DNA (μg)	Ratio	
		A260/280	A260/230		A260/280	A260/230		A260/280	A260/230		A260/280	A260/230
10,000	4.8	1.85	9.29	19.7	1.89	2.17	5.9	1.93	11.56	1.6	1.67	0.74
	2.3	1.75	-2.00	22.3	1.87	1.92	6.8	1.93	13.27	1.9	1.70	0.89
100	3.9	1.89	-9.79	20.7	1.88	2.03	5.7	1.93	6.57	2.8	1.61	0.55
	3.1	1.86	-18.48	21.7	1.86	1.87	7.3	1.95	8.68	1.7	1.72	0.93
10	2.9	1.90	-6.79	23.4	1.86	1.95	8.8	1.97	5.94	1.9	1.71	0.89
	3.3	1.86	10.50	22.6	1.85	1.82	8.3	1.97	5.98	1.5	1.78	0.98
5	4.1	1.89	37.70	21.3	1.86	1.88	7.3	1.95	8.08	2.9	1.70	0.64
	2.2	1.82	-2.54	19.5	1.86	1.86	7.5	1.96	7.62	1.5	1.69	0.89
1	2.6	1.78	-3.75	21.4	1.87	1.91	6.9	1.89	10.11	1.7	1.72	0.70
	2.9	1.78	18.11	24.5	1.85	1.88	7.0	1.96	15.35	1.8	1.71	1.02

a) Modified method using DNeasy Plant Mini kit (Qiagen, Hilden, Germany)
b) Modified method using Genomic-tip 20/G (Qiagen)
c) Modified method using mSFP2 protocol of DNeasy *mericon* Food kit (Qiagen) as shown in Table 1
d) The Japanese official method described in reference No.1

Table 3. Spectrophotometric analysis of DNAs extracted from model foods (nori containing crab)

Crab Conc. (μg/g)	DPM method[a)]			GT method[b)]			DMF-mSFP method[c)]			CTAB method[d)]		
	DNA (μg)	Ratio		DNA (μg)	Ratio		DNA (μg)	Ratio		DNA (μg)	Ratio	
		A260/280	A260/230		A260/280	A260/230		A260/280	A260/230		A260/280	A260/230
10,000	4.5	1.89	5.49	23.8	1.83	1.67	7.2	1.90	6.12	2.6	1.77	0.77
	3.6	1.79	3.59	23.4	1.83	1.75	6.7	1.88	5.86	1.7	1.66	1.11
100	4.4	1.85	4.26	22.9	1.85	1.86	7.0	1.90	5.85	1.8	1.79	0.66
	3.7	1.76	1.82	23.7	1.84	1.75	8.4	1.95	4.98	2.2	1.71	1.00
10	3.8	1.81	1.90	27.5	1.84	1.78	9.3	1.91	3.95	2.1	1.78	0.82
	3.8	1.87	7.86	27.2	1.83	1.75	8.8	1.95	6.19	2.0	1.74	1.09
5	4.4	1.82	6.52	27.4	1.83	1.76	8.8	1.97	5.50	1.7	1.79	0.76
	4.8	1.81	-49.06	27.2	1.84	1.74	6.9	1.89	5.69	1.4	1.69	1.13
1	3.9	1.93	32.00	20.1	1.86	2.01	6.1	1.94	12.90	1.5	1.74	0.77
	3.8	1.86	10.20	21.0	1.86	2.04	7.3	1.95	7.33	2.0	1.82	1.17

Abbreviations and symbols are as in Table 2.

due to the use of aliquots of lysates from starting samples. The DNA extraction using the DMF-mSFP method resulted in the second highest yield of DNA.

The contaminants were assessed according to absorption ratios of A260/280 and A260/230 respectively, which are widely used to evaluate DNA quality for PCR amplification. According to the official method, the A260/280 and A260/230 absorption ratios of recommended DNA quality for the following PCR detection are 1.2 – 2.5 and >2.0, respectively[1)]. The A260/280 absorption ratio of DNA extracted by all methods fell within the range 1.6 – 2.0; in contrast, only the A260/230 absorption ratio of DNA extracted by the DMF-mSFP method are in the range of recommended DNA quality. These results suggest that the DMF-mSFP method effectively remove certain polysaccharides (Table 2). The yield and purity of DNA extracted from the crab model samples showed similar tendencies to those of the shrimp/prawn model samples (Table 3).

Since seaweed generally contains large amounts of polysaccharides and is highly viscous, DNA extraction using the GT method, which involves gravity-flow chromatography, is time consuming; similarly, the CTAB-based method is time consuming as it involves multiple steps for removing certain proteins and polysaccharides[1)]. In contrast, the DPM and DMF-mSFP methods were performed using silica membrane-type spin columns, allowing the rapid extraction by simultaneous procedures from multiple samples. However, the DPM method gave low-purity DNA, judging from the A260/230 values. According to these examinations, the results suggest that the DMF-mSFP method is superior to the other methods for extracting total DNA from nori food products.

In the official method for allergenic substance testing[1)], PCR for the detection of common animal or plant DNA is required to assess the validity of extracted DNA and to avoid false-negative results. To directly evaluate the applicability of DNA extracted by the DMF-mSFP method, PCR with three kinds of universal plant primer pairs (CP03–5'/CP03–3', Plant01–5'/Plant01–3' or Placon5/Placon3) for the detection of common plant DNA was performed using the DNA extracted from the shrimp/prawn or crab model samples and AmpliTaq reagent. The PCR generated no plant specific products (data not shown). In addition, the PCR with the universal animal primer pairs (AN1–5'/AN2–5'/AN–3') for the detection of common animal DNA was performed using the DNA extracted from both model samples. Several amplification products (ranging from 40 bp to 600 bp) in the PCR test for shrimp/prawn model samples were obtained (data not shown). By contrast, no amplification products in the PCR test for model samples containing crab were obtained (data not shown). The similar PCR test using animal specific primer pairs and Ampdirect reagent also gave several non-specific products (data not shown).

The primer pair for detecting common animal DNA is designed from a highly conserved region of the 16S rRNA gene of mitochondrial DNA[12)]. However, this region carries various nucleotide insertions and deletions among animal species[12)], and the specific amplification product generated using the universal animal primer pair should be 370–470 bp in length. Accordingly, we considered that many amplification products generated in the PCR test using the universal animal primer pair cannot be used to evaluate the validity of extracted DNA.

Therefore, we evaluated the validity of extracted DNA using PCR amplification with primers (PyrbcL01–5'/PyrbcL01–3') designed for the detection of the *P. yezoensis rbcL* gene and Ampdirect reagent (Fig. 1). The region of the *P. yezoensis rbcL* gene was clearly amplified using DNA extracted from the shrimp/prawn or crab model samples.

These results demonstrated the superiority of the DMF-mSFP method for detecting shrimp/prawn and crab DNA in nori food products.

4. Sensitivity of shrimp/prawn and crab DNA detection

The LODs for the specific detection of shrimp/prawn and crab DNA extracted from the powdered nori model samples containing 1, 5, 10, 100, or 10,000 μg/g of freeze-dried shrimp/prawn or crab powder per g dried nori powder were determined (Fig. 2).

With the AmpliTaq reagent, the LODs for the specific detection of shrimp/prawn and crab DNA were 5 μg/g and 100 μg/g, respectively. However, use of the Ampdirect reagent led to LODs of 1 μg/g in both specific detections. Although LODs for the specific detection of shrimp/prawn DNA using AmpliTaq reagent were similar to those using Ampdirect reagent, LODs of crab DNA between PCR using AmpliTaq reagent and Ampdirect reagent differed greatly (Fig. 2). We speculate that these differences might be attributed to the neutralization of PCR inhibitors[24, 25)], or the high amplification efficiency of Taq DNA polymerase in the Ampdirect reagent.

5. Specific detection of shrimp/prawn or crab DNA from commercial dried seaweed products

To investigate the applicability of detection methods for shrimp/prawn and crab DNA in commercial nori food products, we employed eight commercial nori food products that did not declare shrimp/prawn and crab contents on their label. Concentrations of crustacean tropomyosin for the nori food products were determined using two ELISA kits (Table 4).

As shown in Table 4, the crustacean protein concentrations in the nori food products were 0.3 – 8.8 μg/g and 1.3 – 8.0 μg/g using N and M kits, respectively. Furthermore, no sample contained over 10 μg/g crustacean protein concentration. The

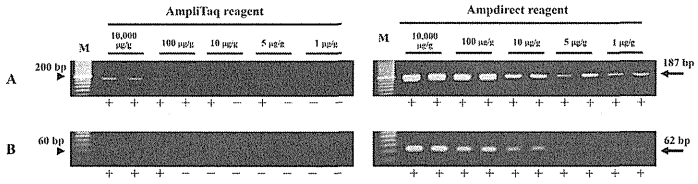

Fig. 2. Sensitivity of the specific detection method for shrimp (A) or crab (B) in powder nori products using two different PCR reagents

Arrows and arrowheads indicate the expected PCR amplification products and the position in 20 bp ladder size standard, respectively. AmpliTaq reagent, AmpliTaqGold (Life Technologies). Ampdirect reagent, Ampdirect plus (Shimadzu). Lanes M, 20 bp ladder size standard (These bands were in the range of 20 – 1,000 bp at 20 bp intervals.). "+" and "–" mean the lanes in which the amplified band was detected and not, respectively.

Table 4. Detection of crustacean proteins and DNA (crab and shrimp) in dry seaweed products and spectrophotometric analysis of extracted DNA by DMF

Sample No.	ELISA (μg/g)		PCR			Extracted DNA		
	N Kit [a]	M Kit [b]	Control [c] (Seaweed)	Shrimp [d]	Crab [e]	DNA (μg)	Ratio A260/280	Ratio A260/230
1	0.4	1.3	+	+	+	31	2.19	3.53
2	0.8	2.1	+	+	+	13	2.00	4.89
3	7.6	8.0	+	–	–	95	2.21	2.80
4	0.5	1.4	+	–	–	22	2.03	3.36
5	8.8	6.8	+	+	+	15	2.09	4.51
6	4.2	4.2	+	+	+	60	2.19	2.78
7	5.8	7.4	+	–	–	27	2.20	3.29
8	0.3	1.9	+	–	–	22	2.03	3.21

a) FA test EIA-crustacean (Nissui Pharmaceutical Co., Ltd, Ibaraki, Japan)
b) Crustacean kit (Maruha Nichiro Holdings, Inc., Ibaraki, Japan)
c) To evaluate the validity of DNA extracted from seaweed products for the PCR
d) For specific detection of shrimp using the ShH12–05'/ShH13–03' primer pair
e) For specific detection of crab using the CrH16–05'/CrH11–03' primer pair

nori food products were then subjected to DNA extraction followed by control (PyrbcL)-PCR, shrimp-PCR, and crab-PCR. The DNA was simply and rapidly extracted from all samples using the DMF-mSFP method, with yields of 13 – 95 μg and A260/280 and A260/230 adsorption ratios of 2.00 – 2.21 and 2.78 – 4.89, respectively (Table 4). All extracted DNA was in the range of recommended DNA yield and quality described in the Japanese official method. PCR with the PyrbcL01–5'/PyrbcL01–3' primer pair was then performed to validate extracted DNA using the Ampdirect PCR reagent and specific amplification products were observed. These results suggested that PCR inhibitors in the extracted DNA did not influence the PCR reactions (Fig. 3).

In subsequent shrimp-PCR and crab-PCR, samples 1, 2, 5, and 6 were positive in both reactions. In a previous study, shrimp-PCR gave false-negative results with the Akiami paste shrimp (*Acetes japonicus*), whereas crab-PCR gave false-positive results with the mantis shrimp (*Oratosquilla oratoria*)[15]. Therefore, to avoid false results, the detection of Akiami paste and mantis shrimp were examined in samples 3, 4, 7, and 8, and samples 1, 2, 5, and 6, respectively, using specific Akiami paste and mantis shrimp primers. Consequently, no amplification products were observed in all examined samples (data not shown), suggesting that samples 1, 2, 5, and 6 are mixed with both shrimp/prawn and crab.

The official PCR methods for shrimp/prawn and crab taxonomically detect *Penaeoidea*, *Sergestidae* family of the suborder *Dendrobranchiata* and infraorder *Caridea*, *Astacidae*, *Achelata* of the suborder *Pleocyemata*, and infraorder *Brachyura* and the *Lithodidae* family of the infraorder *Anomura*[15], respectively. The amplification regions of both PCR protocols were on the mitochondrial 16S rRNA gene, allowing the detection of all species of these groups. Using the developed DNA extraction method for the detection of shrimp/prawn and crab DNA in the commercial nori food products enabled the detection of DNA of shrimp/prawn and

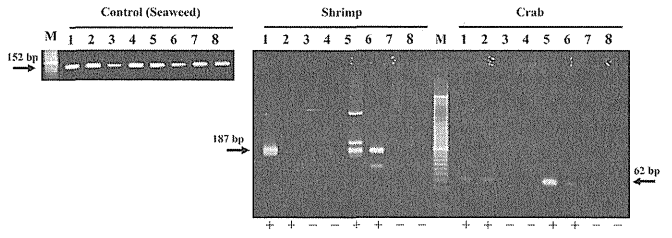

Fig. 3. Investigation of commercial seaweed products

Control (seaweed) evaluation of the validity of DNA extracted from seaweed products using PCR amplification with the PyrbcL01–5'/PyrbcL01–3' primer pair. Arrows indicate expected PCR amplification products. The lane numbers correspond to the sample numbers from Table 4. Lane M, 20 bp ladder size standard (These bands were in the range of 20 – 1,000 bp at 20 bp intervals.).

crab, with a detection limit of 1 μg/g in both model samples. However, samples 3, 4, 7, and 8 were negative in both PCR tests, even though according to the ELISA results, crustacean protein was present at greater than 1 μg/g in those samples. These results suggest that samples 3, 4, 7, and 8 contain crustaceans of the *Amphipoda*, *Mysida*, and *Euphausiacea* families, which are not subject to mandatory labeling for shrimp and crab.

The present DNA extraction methods are applicable to the identification of shrimp/prawn or crab DNA in commercial nori food products. Further studies are required to investigate discrepancies between the results of ELISA and PCR methods.

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論　　文

特定原材料検査における乾燥海苔製品中のえび・かに DNA 検出法の検討

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キーワード：特定原材料、甲殻類、DNA 抽出方法、乾燥海苔製品、ポリメラーゼ連鎖反応

概　要

甲殻類タンパク質（トロポミオシン）は、海苔を含む加工食品で高頻度に検出されている。アレルギー物質の表示に関する日本の規制では、抽出されたエビとカニの DNA を検出するための PCR 法が、加工食品中のエビとカニを区別するために規定されている。海苔を含有した加工食品では、エビとカニの DNA を抽出することが難しい。我々は、DNeasy *mericon* Food kit の DNA 抽出プロトコールを改良し、凍結乾燥したエビとカニの可食部を 1 ～ 10,000 μg/g 含む乾燥海苔粉末から抽出された DNA を用いて、いくつかの市販の DNA 抽出キットと収量・精製度の比較検討を行った。その結果、改良した DNA 抽出方法は、PCR 法を用いた特異 DNA の検出に適した十分な DNA の収量、精製度を示した。抽出 DNA の適用性を直接評価するために、スサビノリ *rbcL* 遺伝子の塩基配列からプライマーペアを作成した。このプライマーペアは、いくつかの市販の海苔製品や、エビまたはカニを含んだ乾燥海苔粉末から増幅産物を生成することが可能であった。改良した DNA 抽出方法により抽出されたエビとカニの DNA の検出下限値は、海苔乾燥粉末 1 g あたり、それぞれ 1 μg であった。また、市販の海苔製品においても良好な適用性を示した。改良した方法は、簡便、迅速、高感度であり、乾燥海苔製品において、エビとカニの DNA を検出するために利用可能であった。

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ORIGINAL PAPER

A novel trait-specific real-time PCR method enables quantification of genetically modified (GM) maize content in ground grain samples containing stacked GM maize

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Abstract Stacked genetically modified (GM) maize is increasingly produced; thereby, current event-specific quantitative real-time polymerase chain reaction (qPCR) methods have led to the overestimation of GM organism (GMO) content compared with the actual weight/weight percentage of GM organism in maize samples. We developed a feasible qPCR method in which the GMO content is calculated based on the quantification of two herbicide-tolerant trait genes, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (*cp4epsps*) and phosphinothricin *N*-acetyl-transferase from *Streptomyces viridochromogenes* (*pat*) to quantify the GMO content in ground grain samples containing stacked GM maize. The GMO contents of two genes were quantified using a plasmid calibrant and summed for quantification of total GMO content. The trait-specific method revealed lower biases for examination of test samples containing stacked GM maize compared with the event-specific method. Our results clearly show that the trait-specific method is not only simple and cost-effective, but also useful in quantifying the GMO content in ground grain samples containing stacked GM maize, which are expected to be major events in the near future. The developed method would be the only feasible way to conduct the quantification of GMO content in the ground maize samples containing stacked GM maize for the verification of the labeling regulation.

Keywords Genetically modified maize · qPCR · Trait-specific method · Stacked GM maize

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Introduction

In recent years, an increasing number of genetically modified (GM) crops have been developed using recombinant DNA technology and are widely cultivated as sources of food and feed in many countries. GM crops have generally been assessed and authorized for use as food by administrative authorities. However, the use of GM crops for food remains controversial among consumers in many countries. Labeling of GM foods allows consumers to make informed food choices. Therefore, many countries have mandated the labeling of foods containing a specified threshold level of GM crops (0.9 % in the European Union, 3 % in Korea and 5 % in Japan) [1]. To monitor the content of GM crops such as maize [2–8], soybean [2–4, 8–10] and other crops [11–13] in foods, in general, the quantitative real-time polymerase chain reaction (qPCR) has been used. In several countries including Japan, the regulatory threshold levels of GMO content are evaluated on a weight/weight (w/w) basis and are calculated based on the GM event-specific DNAs to taxon-specific DNA ratio measured using qPCR.

Recently, the production of stacked GM maize grains, with two or more GM events for enhanced production efficiency [14], has been increasing worldwide. The GMO content of maize samples containing stacked GM maize is generally overestimated when determined using qPCR methods, as compared to the actual w/w percentage of GM maize, because a kernel of stacked GM maize contains the GM-specific DNAs in proportion to the number of GM events. To avoid overestimation, we developed an individual kernel detection method that involves multiplex real-time PCR using the extracted DNA from individual ground maize kernels [15–18]. This detection system has already been implemented in Japan as an official GM maize detection method [19]. Moreover, a GMO content evaluation method based on group testing strategy [20–22] was recently developed [23]. In this method, GMO content is statistically evaluated based on qualitative PCR for multiple small portions, consisting of 20 maize kernels. However, these methods are not applicable to ground grain samples such as corn grits, corn flour and corn meal. Moreover, both methods are time consuming and require additional equipment with large sample numbers. A simpler, time-saving and cost-effective method is required for roughly quantifying GMO content in maize samples containing stacked GM maize.

We previously determined the GM maize content on a kernel basis and the events of GM maize kernels in non-identity-preserved (IP) maize samples imported from the USA in 2005 and 2009 using an individual kernel detection system [24, 25]. The main GM maize events detected in the non-IP maize samples in 2009 were MON88017, MON810 × MON88017, NK603, MON810, TC1507 × DAS59122, MON810 × NK603, TC1507, DAS59122 and MON863. With the exception of single GM maize events of MON810 and MON863, these GM maize events contain a herbicide-tolerant trait gene encoding either 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (*cp4epsps*) or phosphinothricin *N*-acetyl-transferase from *Streptomyces viridochromogenes* (*pat*) (Supplementary Table S1). Moreover, many other stacked GM maize events detected in the non-IP maize samples in 2009 also contain *cp4epsps* or *pat*. Thus, we hypothesized that the GMO content in ground maize samples containing stacked GM maize might be quantified from the sum of *pat* and *cp4epsps* contents. This hypothetical method, termed the trait-specific method, might be applicable to ground samples, whereas the individual kernel detection method and the group testing method are applicable only to kernel samples. Moreover, the hypothetical method for the determination of trait-specific gene might be also applicable to kernel samples, with the advantages of easy sample preparation and enhanced cost-effectiveness. To date, *pat* or *cp4epsps* is used in most GM lines as an herbicide-tolerant trait gene. In this study, we developed a trait-specific method that can quantitate GMO content by measuring only *cp4epsps* and *pat* using qPCR and demonstrated that the developed method is appropriate for approximate quantification of GMO content in ground grain samples containing stacked GM maize by evaluating the performance of the developed method by quantitating five test samples in comparison to the event-specific method.

Materials and methods

Maize materials

The MON88017, MON810, MON863, NK603, MON 88017 × MON810, MON810 × NK603 and non-GM maize seeds were kindly provided by Monsanto Co. (St. Louis, MO). Seeds of TC1507 and DAS59122 were kindly provided by Pioneer Hi-Bred International (Johnston, IA). The 5 % MON810 certified reference material (CRM), 5 % NK603 CRM and 10 % TC1507 CRM were purchased from Sigma-Aldrich (St. Louis, MO).

DNA extraction

The maize seeds were ground using a Mixer Mill MM200 (Retsch, Haan, Germany). Genomic DNA was extracted and purified from 1 g of ground maize powder using a DNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, with the following modifications. Five milliliters of AP1 buffer (QIAGEN) and 10 μL of 100 mg/mL RNase (QIAGEN) were added to the sample and vortexed thoroughly and then incubated at 65 °C for 1 h. The mixture was incubated at 65 °C for another 1 h after the addition of 200 μL of Proteinase K (QIAGEN). During incubation, the mixture was mixed several times by vortexing the tubes. After incubation, 1.8 mL of AP2 buffer (QIAGEN) was added to the mixture and vortexed and then incubated on ice for 15 min. The mixture was centrifuged at 2,300×*g* for 15 min at room temperature in a swing-out rotor, and the supernatant was applied to a QIAshredder Maxi spin column. The column was centrifuged at 2,300×*g* for 5 min at room temperature, and 5.1 mL of AP3/E buffer (QIAGEN) was added to 3.4 mL of flow-through solution, followed by vortexing thoroughly. The mixture was applied to a DNeasy Maxi spin column, followed by centrifugation at 2,300×*g* for 5 min at room temperature. The column was washed with 12 mL of AW buffer (QIAGEN) and then centrifuged at 2,300×*g* for 15 min at room temperature. To elute the DNA, 1 mL of pre-warmed distilled water (65 °C)

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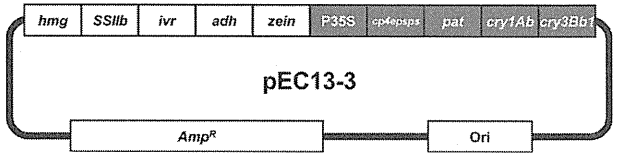

Fig. 1 Schematic diagram of pEC13-3 integrating ten fragments (*hmg*, *SSIIb*, *ivr*, *adh*, *zein*, P35S, *cp4epsps*, *pat*, *cry1Ab* and *cry3Bb1*). Amp^R, ampicillin resistance gene. Ori, origin of replication

was added to the column. After incubation at room temperature for 5 min, the column was centrifuged at 2,300×*g* for 10 min at room temperature. An equal amount of isopropyl alcohol was added to the eluted solution, and the mixture was mixed thoroughly. After incubation at room temperature for 5 min, the mixture was centrifuged at 12,000×*g* at 4 °C for 15 min. The pellet was rinsed with 500 μL of 70 % (v/v) ethanol and centrifuged at 12,000×*g* at 4 °C for 3 min. The supernatant was discarded and the precipitate was dried. The DNA was dissolved in 100 μL of distilled water.

DNA concentrations were determined by measuring UV absorption at 260 nm with a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Samples were diluted to 20 ng/μL with sterile distilled water. The extracts (600 ng) were analyzed by electrophoresis on a 1.0 % agarose gel containing Midori Green Advanced DNA strain (NIPPON Genetics, Tokyo, Japan).

Preparation of plasmid DNA

To quantitate the GM maize content, we prepared plasmid DNA as a calibrant. Ten targeted DNA fragments consisting of five reference genes [high mobility group protein (*hmg*, AJ131373), starch synthase IIb (*SSIIb*, NM_001111410), invertase A (*ivr*, ZMU16123), alcohol dehydrogenase 1 (*adh*, X04050) and delta zein protein (*zein*, FJ557103)] and five GM genes [cauliflower mosaic virus 35S promoter (P35S, AB863197), *cp4epsps* (AY125353), *pat* (DQ156557) and two Bt toxins (*cry1Ab*; AY326434 and *cry3Bb1* [26])] were incorporated (as shown in Fig. 1) by PCR as described previously [2] using appropriate primers. The resultant fragment was ligated into pUC19, and its sequence was confirmed by nucleotide sequence analyses and designated as pEC13-3. The cells of *Escherichia coli* DH5α were transformed using pEC13-3. The plasmid was extracted with a Plasmid Mega Kit (QIAGEN) and purified by ultracentrifugation with cesium chloride. The purified pEC13-3 was cut by *Nde*I, and the resultant linearized plasmid DNA was purified again by ultracentrifugation with cesium chloride. The copy number of purified pEC13-3 was estimated as that of *SSIIb* by qPCR as described previously [2], and plasmid DNA was diluted with 5 ng/μL ColE1 plasmid solution in tris-ethylenediaminetetraacetic acid buffer (Nippon Gene, Tokyo, Japan) to 20, 125, 1,500, 20,000 and 250,000 copies per 2.5 μL.

Table 1 The GM maize event and content (%) of test samples prepared by mixing the ground samples of several GM maize and non-GM maize

GM maize event	Sample number				
	1	2	3	4	5
MON88017	2.0	1.3	1.3	1.5	–
MON810 × MON88017	–	1.2	1.3	1.5	3.0
NK603	1.0	0.6	0.7	–	–
MON810	0.9	0.5	0.7	–	–
TC1507 × DAS59122	–	0.4	0.5	1.0	1.0
MON810 × NK603	–	0.4	0.5	1.0	1.0
TC1507	0.6	0.3	–	–	–
DAS59122	0.3	0.2	–	–	–
MON863	0.2	0.1	–	–	–
Total	5.0	5.0	5.0	5.0	5.0

Preparation of test samples

To prepare the five test samples, the ground grain samples of several GM maize events were mixed with ground non-GM maize at 5 % (w/w) GMO content (Table 1). Genomic DNA was extracted from each test sample in three parallels and diluted to 20 ng/μL with sterile distilled water.

qPCR assay

qPCR assay was performed by trait- and event-specific qPCR methods using an ABI PRISM™ 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA). For the trait-specific method, five sets of primer pairs and probe (*hmg* [27–30], *SSIIb* [31], *ivr* [32], *adh* [32] and *zein* [32]) were identical with those in previous reports. Two sets of primer pairs and probe (*cp4epsps* and *pat*) were designed using Primer Express software (Life Technologies) (Table 2). These sets of primer pairs and probe were evaluated for PCR efficiency and linearity of calibration curves for each gene in pEC13-3. Trait-specific quantification was performed by quantitating *cp4epsps* and *pat* in each DNA extracted in three parallels. A 25 μL volume of the reaction mixture contained 2.5 μL of template DNA, 12.5 μL of TaqMan® Universal PCR Master Mix (Life Technologies), 0.5 μM of each primer and

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Table 2 Primers and probes used for qPCR assay

Target gene	Accession number	Primer or probe name	Primer or probe sequence (5′–3′)	Amplicon size (bp)
reference gene				
hmg	AJ131373	hmg-F	TTGGACTAGAAATCTCGTGCTGA	79
		hmg-R	GCTACATAGGGAGCCTTGTCCT	
		hmg-P	FAM-CAATCCACACAAACGCACGCGTA-TAMRA	
SSIIb	NM_001111410	SSIIb3-5′	CCAATCCTTTGACATCTGCTCC	114
		SSIIb3-3′	GATCAGCTTTGGGTCCGGA	
		SSIIb-P	FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMRA	
ivr	ZMU16123	ivr-F	CGCTCTGTACAAGCGTGC	135
		ivr-R	GCAAAGTGTTGTGCTTGGACC	
		ivr-P	FAM-CACGTGAGAATTTCCGTCTACTCGAGCCT-TAMRA	
adh	X04050	adh-F	CGTCGTTTCCCATCTCTTCCTCC	103
		adh-R	CCACTCCGAGACCCTCAGTC	
		adh-P	FAM-AATCAGGGCTCATTTTCTCGCTCCTCA-TAMRA	
zein	FJ557103	zein-F	GCCATTGGGTACCATGAACC	104
		zein-R	AGGCCAACAGTTGCTGCAG	
		zein-P	FAM-AGCTTGATGGCGTGTCCGTCCCT-TAMRA	
trait gene				
cp4epsps	AY125353	cp4epsps-F	TTCACGGTGCAAGCAGCC	82
		cp4epsps-R	GACTTGTCGCCGGGAATG	
		cp4epsps-P	FAM-CGCAACCGCCCGCAAATCC-TAMRA	
pat	DQ156557	pat-F	GGCCTTCCAAACGATCCAT	96
		pat-R	CCATCCACCATGCTTGTATCC	
		pat-P	FAM-ATGAGGCTTTGGGATACACAGCCCG-TAMRA	

0.2 μM of probe. PCR conditions were as follows: 2 min at 50 °C, 95 °C for 10 min followed by 45 cycles of 30 s at 95 °C and 1 min at 59 °C. Standard curves were calibrated using the five concentrations of plasmid DNAs, such as 20, 125, 1,500, 20,000 and 250,000 copies per reaction. The no-template control containing 5 ng/μL ColE1 plasmid was also prepared as the negative control for analysis. For event-specific quantification, event-specific sequences of MON88017, MON810, MON863, NK603, TC1507 and DAS 59122 were quantitated in each DNA extracted in three parallels according to the methods reported by the European Commission's Joint Research Centre [27–30, 33, 34]. Standard curves were calibrated by using genomic DNA extracted from 5 % MON810 CRM, 5 % NK603 CRM and 10 % TC1507 CRM, and 10 % MON88017, 5 % DAS59122 and 10 % MON863 prepared from ground powders of GM maize and non-GM maize. Triplicate reactions for each DNA extracted in three parallels were conducted using trait- and event-specific qPCR.

Data analysis

For trait-specific quantification, the baseline was set to cycles 3 through 15 and the ΔRn threshold for plotting quantification cycle (C_q) values was set to 0.2 during exponential amplification. The PCR efficiency (E, %) of reference genes was calculated using the slope of the standard curve according to the following formula:

$$\text{PCR efficiency } (E, \%) = \left[10^{(-1/\text{slope})} - 1\right] \times 100 \quad (1)$$

The ratio of the copy number of *hmg* and trait gene (*cp4epsps* and *pat*) in GM maize seeds, defined as the conversion factor (C_f), was calculated using the following formula:

$$C_f = \frac{\text{copy number of trait gene in the DNA extracted from GM maize seeds}}{\text{copy number of } hmg \text{ in the DNA extracted from GM maize seeds}} \quad (2)$$

To prevent overestimation of GMO content, we used the corrected C_f value calculated using following formula:

$$\text{Corrected } C_f = \sum \left(C_{f(\text{trait})} \times x\right) \quad (3)$$

Table 3 The corrected C_f calculated from the C_f value and the rate of each GM maize event containing *cp4epsps* or *pat*

GM maize event	*cp4epsps*		*pat*	
	C_f	x[a]	C_f	x[a]
MON88017	0.30	0.38	–	–
MON810 × MON88017	0.28	0.34	–	–
NK603	0.72	0.18	–	–
MON810	–	–	–	–
TC1507 × DAS59122	–	–	0.61	0.42
MON810 × NK603	0.71	0.10	–	–
TC1507	–	–	0.30	0.38
DAS59122	–	–	0.29	0.20
MON863	–	–	–	–
Corrected C_f[b]	0.41		0.43	

[a] x is the rate of a GM maize event in all main GM maize events containing *cp4epsps* or *pat*, which was calculated using the data of GMO content in the non-IP maize sample imported from the USA in 2009. The sum of x of all main GM maize events containing *cp4epsps* or *pat* was 1.00

[b] The corrected C_f was calculated from the sum of the product of each C_f and x as follows: for *cp4epsps*, the corrected $C_{f(cp4epsps)} = C_{f(MON88017)}\ (0.30) \times x_{(MON88017)}\ (0.38) + C_{f(MON810 \times MON88017)}\ (0.28) \times x_{(MON810 \times MON88017)}\ (0.34) + C_{f(NK603)}\ (0.72) \times x_{(NK603)}\ (0.18) + C_{f(MON810 \times NK603)}\ (0.71) \times x_{(MON810 \times NK603)}\ (0.10) = 0.41$; for *pat*, the corrected $C_{f(pat)} = C_{f(TC1507 \times DAS59122)}\ (0.61) \times x_{(TC1507 \times DAS59122)}\ (0.42) + C_{f(TC1507)}\ (0.30) \times x_{(TC1507)}\ (0.38) + C_{f(DAS59122)}\ (0.29) \times x_{(DAS59122)}\ (0.20) = 0.43$

where $C_{f(trait)}$ is each C_f value for *cp4epsps* or *pat* in a GM maize event containing *cp4epsps* or *pat* and x is the rate of a GM maize event in all main GM maize events containing *cp4epsps* or *pat*, which was calculated using the data of GMO content in the non-IP maize sample imported from the USA in 2009 [25] (Table 3). The corrected C_f was calculated by summing the product of $C_{f(trait)}$ and × ($C_{f(trait)}$ × x) for each GM maize event. The GMO content (%) of a sample for each trait gene was calculated using the following formula:

$$\text{GMO content (\%)} = \frac{\text{copy number of trait gene in the DNA extracted from a sample}}{\text{copy number of } \textit{hmg} \text{ in the DNA extracted from a sample} \times \text{corrected } C_f} \times 100 \quad (4)$$

Total GMO content was calculated by summing the GMO content for *cp4epsps* and *pat*.

For event-specific quantification, the baseline was set to cycles 3 through 15 and the ΔRn threshold for plotting C_q values was set to 0.1–0.5 during exponential amplification. The GMO content (%) of a sample for each GM sequence was calculated using the following formula:

$$\text{GMO content (\%)} = \frac{\text{copy number of event-specific sequence in the DNA extracted from a sample}}{\text{copy number of endogenous gene in the DNA extracted from a sample}} \times 100 \quad (5)$$

Table 4 The PCR efficiencies (E) and linearity (R^2) of calibration curves for five reference genes and two herbicide-tolerant trait genes in pEC13-3

Target gene	E		R^2	
	Mean	RSD (%)	Mean	RSD (%)
Reference gene				
Hmg	100.7	2.2	0.9990	0.08
SSIIb	98.7	2.3	0.9980	0.10
Ivr	96.7	2.4	0.9960	0.39
Adh	96.7	3.2	0.9961	0.13
Zein	97.0	2.0	0.9989	0.06
Trait gene				
Cp4epsps	96.7	3.7	0.9992	0.05
Pat	94.7	3.0	0.9992	0.03

Total GMO content was calculated by summing the GMO content for each event-specific sequence.

Results and discussion

Construction of plasmid DNA

Plasmid pEC13-3 was constructed by tandem integration of ten PCR products amplified from five reference genes (*hmg*, *SSIIb*, *ivr*, *adh* and *zein*) and five GM genes (P35S, *cp4epsps*, *pat*, *cry1Ab* and *cry3Bb1*) (Fig. 1) as a calibrant. The purified pEC13-3 was diluted to 20, 125, 1,500, 20,000 and 250,000 copies per 2.5 µL, equivalent to 0.12, 0.72, 8.6, 115 and 1,440 ng of genomic DNA of F1 GM maize seed, respectively, based on the genome size of maize (the diploid DNA content per nucleus, 5.75 pg/2C) [35]. The dilution series was sufficient to quantitate GM maize content from 0.23 to 100 % in 50 ng of genomic DNA.

The PCR efficiencies of reference genes in pEC13-3 were calculated by the designated qPCR system (Table 4). Among the five reference genes, *hmg* showed the highest PCR efficiency [E = 100.7 %, relative standard deviation (RSD) = 2.2 %], followed by *SSIIb* (98.7 %), *zein* (97.0 %), *adh* (96.7 %) and *ivr* (96.7 %), with RSD ranging from 2.0 % to 3.4 %. Moreover, the standard curve of *hmg* showed greater linearity (R^2 = 0.9990, RSD = 0.08 %) than that of the other reference genes (0.9960–0.9989), with RSD ranging from 0.06 to 0.39 %. The relative copy number of *hmg* in some GM maize events was compared to that of *SSIIb*, which is a standard reference gene of maize used in Japanese regulation (Supplementary Table S2), where that of *hmg* or *SSIIb* in the non-GM maize was 1.00. The resulting data of quadruplicate reactions showed that the mean of the relative copy numbers of *hmg* (1.21, RSD = 0.6–12.5 %) was lower than that of *SSIIb* (1.36, RSD = 4.1–9.6 %). The estimated copy number of *SSIIb* in GM maize was higher than non-GM maize as compared to that of *hmg*. These results suggest that *hmg* is the most appropriate reference gene for the designated qPCR system in this method. On the other hand, the PCR efficiencies of the two herbicide-tolerant trait genes, *cp4epsps* and *pat* (96.7 and 94.7 %, respectively), were comparable to the reference genes (96.7–100.7 %), and both standard curves of the two genes showed excellent linearity (R^2 = 0.9992) (Table 4).

Measurement of conversion factor

To determine the C_f value required for the trait-specific qPCR method, the copy number of *hmg* and the trait gene in the genomic DNA extracted from GM maize seed were each measured. All experiments were repeated three times, and the mean values were set as the C_f value (Table 3). The C_f value from whole seed [$C_{f(seed)}$] should be a mean value between the C_f value from embryo [$C_{f(emb)}$] and that from endosperm [$C_{f(endo)}$], because the DNA amounts derived from embryo and endosperm are equivalent in each seed [36]. In F1 hybrid maize having a single copy of the trait gene per maize genome, the ideal $C_{f(emb)}$ is theoretically expected to be 0.5, and on the other hand, the ideal $C_{f(endo)}$ should be 0.33 or 0.67 in paternally or maternally derived GM maize events, respectively [31]. As a result, the ideal $C_{f(seed)}$ should be 0.42 or 0.59. In this study, the experimental C_f values for trait genes in GM maize events having a single copy of transgene per genome were 0.28–0.30. The C_f values for *cp4epsps* in NK603 and MON810 × NK603 having two copies of *cp4epsps* per genome [37] were 0.72 and 0.71, respectively. In TC1507 × DAS59122 having two copies of *pat* per genome, the C_f value for *pat* was 0.61. The discrepancy between ideal and experimental values has been previously reported [2, 31] and may be due to differences in PCR efficiencies resulting from the amount of non-targeted sequences in plasmid and genomic DNA [2] or due to differences in the efficiency of DNA extraction between reference and trait genes [31].

GM maize imported to Japan contains not only single GM maize events having a single copy of the trait gene per genome, but also stacked GM maize events having multiple copies of the trait gene per genome as previously reported [25]. The corrected C_f values for each trait gene were required to calculate GMO contents in maize samples containing GM maize events having multiple copies of the trait gene per genome, because GMO content is overestimated using the C_f value calculated from each GM maize event having a single copy of the trait gene per genome. Therefore, the corrected C_f value for *cp4epsps* (0.41), which was calculated based on each C_f value in GM maize events having *cp4epsps* and the rate of a GM maize event determined from a non-IP maize sample imported from the USA in 2009, was used (Table 3). Similarly, the corrected C_f value for *pat* (0.43) was used (Table 3).

GMO content in test samples

To approximately quantitate the GMO content in five test samples (Table 1), which were prepared by mixing the ground grain samples of several GM maize and non-GM maize at 5 % (w/w) GMO content (Table 1), we extracted genomic DNA from each test sample in three parallels without degradation of DNA (Supplementary Fig. S1) and performed qPCR assays for trait- and event-specific methods (Table 5). The trait-specific method results showed lower RSD (1.3–17.1 %) as compared to those of the event-specific method (4.1–45.2 %), which is consistent with previously reported validation studies [28, 38–42]. Moreover, in the trait-specific method, the biases against theoretical values, which are calculated on the basis of the copy number of the trait gene per genome, were positive values for *cp4epsps* (3.6–18.6 %) and negative values for *pat* (−14.8 to −34.2 %). This suggests that the experimental values of GMO contents for *cp4epsps* were higher than the theoretical values, whereas those for *pat* were lower than the theoretical values. On the other hand, the absolute values of bias against theoretical value for the event-specific method were much higher (4.5–108.6 %) than those for the trait-specific method (3.6–34.2 %). In particular, the absolute values of bias in TC1507 (19.0–108.6 %) were the highest, followed by DAS59122 (50.3–63.3 %), MON863 (50.0–60.0 %), MON810 (13.0–39.9 %), NK603 (10.9–34.1 %) and MON88017 (4.5–16.5 %). These tendencies toward high biases were also shown in the previously reported validation studies [28, 38–42], where the biases were lower than those in this study. In the absolute values of total bias against theoretical value, there was little difference between the trait-specific method (1.2–6.8 %) and the event-specific method (3.6–21.8 %). These results demonstrate that the trait-specific method has higher repeatability and lower bias for each reaction, although the two methods show similar bias overall.

Test sample #1 contained only single GM maize events. Samples #2, #3 and #4 contained both single and stacked GM maize events, and the highest content of stacked GM maize events was in sample #4, followed by samples #3

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Table 5 The theoretical value and experimental value (mean) of GMO content, relative standard deviation (RSD) and bias for trait- and event-specific methods

	Sample number				
	1	2	3	4	5
Trait-specific method					
cp4epsps					
Theoretical value (%)[a]	4.0	4.5	5.0	5.0	5.0
Experimental value (mean, %)	4.3	5.0	5.2	5.9	5.2
RSD (%)	13.9	1.9	13.2	17.1	11.1
Bias of experimental value against theoretical value (%)	8.5	11.9	3.6	18.6	4.1
pat					
Theoretical value (%)	0.9	1.3	1.0	2.0	2.0
Experimental value (mean, %)	0.8	1.0	0.7	1.5	1.3
RSD (%)	1.3	3.9	4.6	14.8	12.6
Bias of experimental value against theoretical value (%)	−14.8	−25.7	−25.0	−26.8	−34.2
Total					
Theoretical value (%)	4.9	5.8	6.0	7.0	7.0
Experimental value (mean, %)	5.1	6.0	5.9	7.4	6.5
RSD (%)	11.6	2.1	11.9	16.4	9.4
Bias of experimental value against theoretical value (%)	4.2	3.5	−1.2	5.6	−6.8
Bias of theoretical value against 5 % (%)[b]	−2.0	16.0	20.0	40.0	40.0
Bias of experimental value against 5 % (%)[c]	2.1	20.0	18.6	47.9	30.5
Event-specific method					
MON88017					
Theoretical value (%)	2.0	2.5	2.6	3.0	3.0
Experimental value (mean, %)	2.3	2.4	2.7	3.2	3.4
RSD (%)	20.2	45.2	10.5	17.1	19.9
Bias of experimental value against theoretical value (%)	16.5	−4.7	4.5	7.6	14.1
MON810					
Theoretical value (%)	0.9	2.1	2.5	2.5	4.0
Experimental value (mean, %)	0.5	1.4	1.8	1.9	3.5
RSD (%)	14.1	6.9	14.6	10.7	7.6
Bias of experimental value against theoretical value (%)	−39.9	−34.8	−28.9	−22.6	−13.0
NK603					
Theoretical value (%)	1.0	1.0	1.2	1.0	1.0
Experimental value (mean, %)	1.2	1.1	1.5	1.3	1.2
RSD (%)	7.3	8.1	10.3	4.4	5.2
Bias of experimental value against theoretical value (%)	16.0	10.9	21.9	34.1	20.2
TC1507					
Theoretical value (%)	0.6	0.7	0.5	1.0	1.0
Experimental value (mean, %)	1.3	0.8	0.3	0.7	0.7
RSD (%)	44.0	18.4	6.9	7.1	8.0
Bias of experimental value against theoretical value (%)	108.6	19.0	−33.2	−27.8	−28.5
DAS59122					
Theoretical value (%)	0.3	0.6	0.5	1.0	1.0
Experimental value (mean, %)	0.5	0.9	0.8	1.6	1.6
RSD (%)	9.8	4.1	10.3	7.2	8.3
Bias of experimental value against theoretical value (%)	63.2	51.2	50.3	56.8	63.3
MON863					
Theoretical value (%)	0.2	0.1	0.0	0.0	0.0
Experimental value (mean, %)	0.3	0.2	0.0	0.0	0.0
RSD (%)	17.5	4.7	–	–	–
Bias of experimental value against theoretical value (%)	60.0	50.0	–	–	–
Total					
Theoretical value (%)	5.0	7.0	7.3	8.5	10.0
Experimental value (mean, %)	6.1	6.7	7.0	8.8	10.5
RSD (%)	4.8	14.8	4.3	4.9	4.2
Bias of experimental value against theoretical value (%)	21.8	−3.6	−3.5	3.4	4.5
Bias of theoretical value against 5 % (%)	0.0	40.0	46.0	70.0	100.0
Bias of experimental value against 5 % (%)	21.8	35.0	40.8	75.9	109.1

[a] This value is calculated based on the copy number of transgene per genome

[b] Bias of theoretical value against 5 % GMO content on a w/w basis

[c] Bias of experimental value against 5 % GMO content on a w/w basis

and #2. Test sample #5 contained only stacked GM maize events. In regard to bias of the experimental value against 5 % (w/w) GMO content, the trait-specific method (2.1–47.9 %) showed lower bias than the event-specific method (21.8–109.1 %); specifically, the difference in bias between the two methods was largest in test sample #5 (30.5 and 109.1 % for trait- and event-specific methods, respectively). These results suggest that the overestimation of the event-specific method was higher than that of the trait-specific method in proportion to the content of the stacked GM maize event. Moreover, these results are consistent with the bias of theoretical value against 5 % (w/w) GMO content (−2.0–40.0 % for the trait-specific method and 0–100.0 % for the event-specific method). These results suggest that the difference in bias between the two methods was attributed not only to the accuracy of the method, resulting from the difference in calibrant, PCR efficiency and so on, but also to the principle of the method. The uncertainty in GMO content determined by the trait- and event-specific methods of the sample containing stacked GM maize events is largely attributed to sample characteristics, such as the content and the kind of stacked GM maize events. Theoretically, the uncertainty in GMO content determined by the trait-specific method is expected to be smaller than that determined by the event-specific method, because the overestimation of the trait-specific method is smaller than that of the event-specific method. This theory was substantiated by this study. Each test sample was designed according to the distribution of GM maize events in the US market in the past, present and future. Test sample #1, containing only single GM maize events, was modeled on GM maize events planted in the past. Test samples #2, #3 and #4, containing single and stacked GM maize grains, were modeled on GM maize events planted in the present, and sample #5, containing only stacked GM maize grains, was modeled on GM maize events to be planted in the future. The results of this study suggest that the trait-specific method has higher trueness (GMO content on a w/w basis) than the event-specific method, especially for the GM maize events planted in the present and future.

A drawback of the trait-specific method, if any, is the possible oversight of single GM maize events having no *cp4epsps* and *pat*, such as MON810 and MON863 (Supplementary Table S1). The National Agricultural Statistics Service (NASS) reported that the percentage of stacked GM maize events has consecutively increased from 1 % in 2000 to 71 % in 2013 in the USA [43]. Indeed, our previous studies showed that the percentage of stacked GM maize events in non-IP samples imported to Japan from the USA increased from 12 % in 2005 [24] to 35 % in 2009 [25]. Judging from these data, almost all GM maize is composed of stacked GM maize events, indicating that no single GM maize events will be distributed commercially in the future. In this situation, the potential oversight of single GM events having no *cp4epsps* and *pat* can be ignored. The content of single GM maize events having no *cp4epsps* and *pat* would be measured from non-IP maize samples imported into japan in future using the individual kernel detection method [15]. Furthermore, the corrected C_f for *cp4epsps* or *pat* would need to be consecutively updated by monitoring the GM maize events in non-IP maize samples imported into Japan, because the rates of the GM maize events would be predicted to vary every year. In addition, the trait-specific method can prove GMO content, but not the existence of stacked GM maize events in maize sample, whereas the individual kernel detection method enables identification of stacked GM maize event in a maize kernel by combining with the event-specific method [17, 18, 44].

In countries evaluating GMO content on a w/w basis, current event-specific method has the potential to lead to an excess of the regulatory threshold levels of GMO content in the ground maize samples containing stacked GM maize events, even though the actual GMO content is lower than the regulatory threshold levels. We believe that the proposed trait-specific method would be the only feasible way to solve this problem and would be useful not only for the countries importing maize and requiring the verification of the labeling regulation on a w/w basis, such as Japan and Korea, but also for the countries exporting maize, such as the USA and Brazil.

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Conclusion

In this study, we developed a trait-specific method that can quantitate GMO content by measuring only *cp4epsps* and *pat* using qPCR. This method overcomes the drawback associated with event-specific methods, in which the GMO content of stacked GM maize samples is greatly overestimated. The developed trait-specific method would be the only feasible way to conduct the quantification of GMO content in the ground maize samples containing stacked GM maize, which will increasingly be found in the future, for the verification of the labeling regulation.

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Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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