Appendix Inspection Methods for Foods Containing Allergens

Introduction

This inspection method is composed of the most reliable methods at present for the purpose of scientifically verifying the labeling system of specified ingredients, etc. It is practically impossible to apply this inspection method to all eligible foods that are distributed as food raw materials, additives and processed foods. Please note that further application examples will be accumulated, and the current inspection methods will be revised.

The measurement result of the total protein content of the specified ingredient by this inspection method does not always accurately match the actual content because by food processing, specified ingredient components may be changed or degraded, or efficiency of extraction from food may vary.

1. Inspection Principles and Sample Preparation Methods

1.1. Inspection Principles

In this inspection, since all processed foods are assumed to be inspection samples, the measurement results vary depending on the description of sample. Principles for reducing the variation are described below.

- Inspection shall be performed by a package of food.
- Sample is considered as an edible portion where non-edible portion of inspection sample is removed.
- Since it is considered that specified ingredients in samples are heterogeneously distributed, perform a homogenization before being subjected to inspection.
- Homogenized samples are considered as a prepared sample.
- Regardless of the properties of solid or liquid, collect a certain amount of the prepared sample to be inspected by using gravimetry.
- Perform all inspections, including sample preparation, at locations where there is no air movement and there is little variability in temperature and humidity.
- Clean the mill, food processor or weighing apparatus with neutral detergent, etc., and then soak them in alkaline detergent overnight in order to perform a micro-measurement. Otherwise, sonicate these apparatuses for 30 minutes using an ultrasonic cleaning machine.
- Perform sample preparation and inspection in a partitioned space to prevent contaminating.

1.2. Sample Preparation Method

The whole edible part contained in one packaged food is considered as a sample. Then, the whole amount of the sample is ground thoroughly with a mill or a food processor, etc.*, and mixed homogeneously to make the prepared sample.

* Use Ace Homogenizer AM-11 (NISSEI Corporation), Retsch GM200 (Retsch GmbH), or equipment from which equivalent results are obtained.

NOTE)

[1] Instant foods (cup noodles, cup soups, etc.) include foods whose contents (soups, seasoning, noodles, etc.) are packaged individually. For instant foods with such packaged forms, the whole is considered as one packaged unit. All of the packaged foods are mixed and homogenized, therefore the homogenized mixture is considered as a prepared sample.

[2] For boxed lunch consisting of a variety of foods such as a popular type of Japanese bento, Makunouchi bento, the whole boxed lunch is considered as one packaged unit. All of the foods (rice, side dishes, and individually-packaged seasoning etc.) are

mixed and homogenized, and the mixture should be considered as a prepared sample.

2. Inspection Method for Specified Ingredients

Inspection method for specified ingredients shall meet the following requirements.

- For the quantitative inspection method, the recovery rate should range between 50% and 150%, inclusive, and the reproducibility should be 25% or less in the inter-laboratory validation performed with five or more samples in eight or more laboratories. (The sample in which specified ingredient protein levels are 10 μg/g should be included.)
- For the qualitative inspection method, the positive rate for samples containing specified ingredient protein should be 90% or more, and the negative rate for blank samples should be 90% or more in the inter-laboratory validation performed with five or more samples in six or more laboratories. Presentation of data demonstrating higher specificity than the quantitative inspection method is needed. It is desirable that the sample in which specified ingredient protein levels are 10 μg/g should be included.
- The results of these inter-laboratory validations, and false-positive and false-negative data should be attached to the instructions and published.
- The evaluation of the inspection methods should be in accordance with the “Guideline for Evaluating Inspection Methods for Foods Containing Allergens” attached as Appendix 4.

2.1. Quantitative Inspection Method

2.1.1. Overview of Quantitative Inspection Method

It is a method to quantitatively detect proteins derived from specified ingredients in foods. Generally, ELISA assay measuring antigen-antibody reaction is used.

Quantitative inspection method other than ELISA assay may be used, but in these cases, the performance of the used method should be equivalent to or greater than that of the above ELISA assay.

The inspection should be performed according to the procedures described in the instructions for each inspection, including reagents and precautions.

2.1.2. Judgement of Results of Quantitative Inspection Method

For samples with 10 μg or more protein content derived from specified ingredients per 1 g of foods collected, it is judged that there is a possibility that amount of specified ingredients contained in the samples is not trace amount.

(However, in the case of shrimp and crab, such samples cannot be distinguished and are detected collectively as crustacean.)

When the obtained value falls within the range of 8-12 μg/g as a result of the first measurement, perform the procedure with the same prepared sample again, and then perform a second measurement. Judge the measurement result based on the average of values obtained in the first time and the second time. If it is not possible to collect a second sample from the prepared sample, obtain another inspection sample to inspect it.

When using ELISA assay, caution should be exercised with respect to the following points:

- Repeat the subsequent procedures after ELISA if CV value of the three wells measure sets is 20% or higher in the result obtained using ELISA assay.
- Calculate the concentration of specified ingredient-derived protein in each well from the calibration curve obtained by fitting a 4-factor logistic regression to the measured

value obtained from the reference solution with each concentration. Multiply the calculated value by the dilution ratio determined for each inspection to calculate the amount of specified ingredient-derived protein per weight of foods collected.

2.2. Qualitative Inspection Method

2.2.1. Overview of Qualitative Inspection Method

The qualitative inspection method includes Western blot, PCR, Real-time PCR, and nucleic acid chromatography. In general, for egg and milk, Western blot is used. On the other hand, generally, PCR is used for shrimp and crab, PCR or real-time PCR is used for wheat, buckwheat and peanut, real-time PCR or nucleic acid chromatography is used for walnut.

Qualitative inspection method other than Western blot, PCR, real-time PCR, or nucleic acid chromatography may be used, but in these cases, the performance of the used method should be equivalent to or greater than the performances of the above inspection methods.

The inspection should be performed according to the procedures described in the instructions for each inspection, including reagents and precautions.

2.2.2. Western Blot

In Western blot, if clear bands around the molecular weight (apparent molecular weight in S DS-PAGE: Ovalbumin M.W. 50,000; Ovomucoid M.W. 38,000; Casein M.W. 33,000-35,000; β-Lactoglobulin M.W. 18,400) of proteins from specified ingredients are detected, the specified ingredient is considered positive. Determine with reference to the band position of the standard reference solution, as appropriate. Check whether the reference solution (1 μg/mL) of egg or milk to be inspected as positive control is detected. If the reference solution (1 μg/mL) is not detected, the inspection is considered unsuitable, and the inspection shall be repeated from the sample preparation. It is judged that there is a contamination exceeding trace amounts of each specified ingredient (egg, milk) if the inspection results are positive by using either antibody, Ovalbumin or Ovomucoid for measuring egg protein, or Casein or β-Lactoglobulin for measuring milk protein.

2.2.3. PCR

Extract DNA from foods according to DNA Extraction and Purification Method (2.2.3.2.) and perform the qualitative PCR described below using the obtained DNA sample solution. Perform two replicated DNA extractions in parallel for one prepared sample, and perform all subsequent steps to PCR amplicon confirmation independently in parallel for the two extracted DNA.

2.2.3.1. Sample Preparation

Samples are prepared according to 1.1. and 1.2.

However, in the case of a sample which is difficult to homogenize by simple grinding using mixer, mill or the like, add water having the same weight as that of the sample in the homogenization process to perform the process sufficiently. Thereafter, a sample which has been subjected to a freeze-drying process and a grinding process again is considered as a prepared sample. When a sample is liquid, the sample which has been subjected to homogenization using mixer, mill or the like, and then subjected to a freeze-drying process and a grinding process using mixer, mill or the like again is considered as a prepared sample.

2.2.3.2. DNA Extraction and Purification

A surfactant cetyltrimethylammonium bromide (CTAB) method for extracting and purifying DNA using a CTAB and a phenol/chloroform mixed solution has a wide scope of applications, and is a very excellent method capable of obtaining a highly pure DNA in which a PCR inhibitor hardly remains, but a hazardous reagent such as chloroform and a complicated purification operation are required. In contrast, DNA can be extracted and purified relatively simply by using a commercially available DNA extraction kit. Commercially available DNA extraction kits include silica gel membrane based kits, ion-exchange based kits, etc. Since each of these kits has its own characteristics, DNA should be extracted using methods suitable for the inspection samples. This section describes the purification methods using CTAB method, the silica gel membrane based kit (QIAGEN DNeasy Plant Mini) and the ion-exchange based kit (QIAGEN Genomic-Tip 20/G).

Unless otherwise specified, all of the water used for extracting and purifying DNA shall be the ultra pure water that was purified to 17MΩ/cm with Milli-Q system, etc. from RO water passing through reverse osmosis membrane or distilled water, and autoclaved at 121°C for not less than 20 minutes.

2.2.3.2.1. Silica Gel Membrane based Kit[*1]

Weigh 2g of prepared sample to put in a polypropylene centrifuge tube (50 mL volume)[*2], and add 10mL of AP1 buffer solution and 10μL of RNase A 10 previously warmed to 65°C to the centrifuge tube. Then, mix vigorously with a vortex mixer so that no sample lumps remain, and warm the centrifuge tube at 65°C for 15 minutes. Invert the centrifuge tube several times and stir the sample during warming. After warming process, add 3250 μL of AP2 buffer solution and allow the mixture to stand at room temperature for 5 minutes, and then centrifuge at 3000 × g for 5 minutes at room temperature. After completion of centrifugation, immediately transfer the supernatant to another centrifuge tube. Then, load the separately-collected supernatant onto a QIAshredder spin column and centrifuge the supernatant at 10,000 × g for 2 min at room temperature. Transfer the resulting eluate to a fresh polypropylene centrifuge tube (15 mL volume). At this time, the volume per loading is 500 μL. Repeat loading several times until loading 3 mL of obtained supernatant is completed. To the finally obtained eluate, add a mixture of AP3 buffer solution and ethanol [*3]whose volume is 1.5 times as much as the volume of the eluate, and stir the mixture by a vortex mixer for 10 seconds to obtain a solution. Load 500 μL of the obtained solution into a mini spin column, and centrifuge at 10,000 × g for 1 minute at room temperature to discard the eluate. Then, load another 500 μL of the remaining solution onto the same mini spin column and centrifuge it under the same conditions. The eluate is discarded. Repeat the same procedures until all of the solution is gone. Then, load 500 μL of AW buffer solution onto the column and centrifuge it at 10,000 × g for 1 minute at room temperature. Discard the resulting eluate and repeat the same procedure once more. After discarding the eluate, centrifuge at 10,000 × g or higher for 15 minutes at room temperature to dry the mini spin column. After drying, transfer the mini spin column to the centrifuge tube that comes with the kit. Add 50 μL of water previously warmed to 65 °C, allow the solution to stand for 5 minutes, and then centrifuge at 10,000 × g for 1 minute at room temperature to elute DNA. Perform the same elution procedure once more, and combine the resulting eluates to make DNA sample stock solution (total 100 μL).

*1 This method is mainly applicable to inspection samples that are little-

processed (flour, buckwheat flour, peanut ground, and equivalent processed foods). It should be kept in mind that the degree of refinement of DNA is low in inspection samples that are greatly processed and have a high content of sugar and oil and fat ingredients, and that an adequate quantity may not be extracted even as a DNA quantity. In case that DNA is not extracted from prepared sample by this method, try to extract DNA using the ion-exchange based kit method described in 2.2.3.2.2.

*2 Preparation and collecting of samples shall be performed according to the method described in 2.2.3.1.

*3 A mixture of AP3 buffer solution and ethanol
A mixture of AP3 buffer solution and ethanol is considered as solution for which AP3 buffer solution and ethanol (96-100 %) is mixed in a ratio of 1:2 (V/V).

2.2.3.2.2. Ion-Exchange based Kit[*1]

Weigh 2g of prepared sample to put in a polypropylene centrifuge tube (50 mL volume)[*2]. Add G2 buffer[*3] 7.5 mL to the same centrifuge tube and mix vigorously with a vortex mixer. After mixing, add 7.5 mL of G2 buffer solution additionally and 200 μL of α-amylase[*4] (1 mg/mL) to mix again with a vortex mixer. After mixing, warm at 37°C for 1 hour. During warming, invert the centrifuge tube several times and stir the sample. After warming, add 100 μL of Proteinase K[*5] and 20 μL of RNase A and mix with a vortex mixer, and warm at 50°C for 2 hours. During warming, invert the centrifuge tube several times and stir the sample. Then, centrifuge under low temperature (4°C) at 3000 × g or higher for 15 minutes. Transfer the obtained supernatant after centrifuging to a polypropylene centrifuge tube (15 mL volume). After transferring, centrifuge more lightly to remove any suspended residue in the solution. During this centrifugation procedure, equilibrate QIAGEN Genomic-Tip 20/G by using 1mL of QBT buffer solution[*3]. After completing centrifugation, load the supernatant by 2mL in installments on the equilibrated QIAGEN Genomic-Tip 20/G. After completing the loading procedure for the entire volume of supernatant, load 2mL of QC buffer solution[*3] on tip and wash it. Repeat the same cleaning procedure three times in total, and then transfer the tip to a new polypropylene centrifuge tube (15 mL volume). After completing the washing procedure, add 1mL of QF buffer solution*3 previously warmed to 50°C to the tip and elute DNA. Perform the same elution procedure for the same tip once more. Add isopropyl alcohol whose volume is 0.7 times the volume of the obtained eluate to 2mL of obtained eluate and mix well. Centrifuge at a low temperature (4°C) for 15 minutes at 10,000 × g or higher. Remove only the supernatant while taking care not to remove the precipitate[*6]. Add 1mL of 70% ethanol to the centrifuge tube after removing the supernatant. Centrifuge at a low temperature (4°C) for 5 minutes at 10,000 × g or higher. Discard supernatant, and dry the remaining precipitate by vacuum drying for about 5 minutes using an aspirator. At this time, be careful not to completely dry it. Confirm that the precipitate has dried, add 100 μL of water, warm at 65 °C for 5 minutes, and melt DNA by pipetting to make a DNA sample stock solution.

*1 This method is applicable to inspection samples with a high degree of processing, which are mainly subjected to processing such as sugar addition, oil treatment, heat mixing, and fermentation. If DNA is not extracted from prepared sample by this method, attempt to extract DNA using the silica gel membrane based kit described in 2.2.3.2.1.

*2 Preparation and collecting of samples shall be performed according to the method described in 2.2.3.1.
*3 G2 buffer solution, QBT buffer solution, QC buffer solution, and QF buffer solution come with the kit, but can be prepared according to the kit instructions if not available.
*4 Use solutions of SIGMA Inc. (Cat. No. A-6380) or the equivalents in potency.
*5 Use solutions of QIAGEN Inc. (Cat. No. 19133) or the equivalents in potency.
*6 This precipitate is the extracted DNA. Depending on the inspection sample, an extremely small amount of DNA is extracted, therefore it may be impossible to visually observe the precipitate. However, the procedure should be performed with attention to the fact that there is a precipitate at the bottom of the centrifuge tube.

2.2.3.2.3. CTAB Method[*1]

Weigh 2g of prepared sample to put in a polypropylene centrifuge tube (50 mL volume), add 15 mL of CTAB buffer solution[*2] to the centrifuge tube, and mix using a homogenizer. Add 30 mL of CTAB buffer solution to wash the edge of the centrifuge tube as well as the tip of the homogenizer, mix by inverting the tube, and warm at 55 °C for 30 minutes. After warming, stir the solution, and weigh 600 µL of the homogenous solution to put in a microcentrifuge tube (1.5 mL volume). Then, add 500 µL of phenol/chloroform mixed solution[*3] to the collected solution, mix by inverting the tube, and suspend lightly with a vortex mixer. After suspending, centrifuge at 7500 × g for 15 minutes under room temperature conditions, and transfer the separated water layer (upper layer) to a new microcentrifuge tube. In this case, care should be taken to avoid touching the middle layer. Add 500 µL of chloroform/isoamyl alcohol mixed solution[*4] to the separately-collected water layer, mix by inverting the tube, and suspend lightly with a vortex mixer again. After suspending, centrifuge the suspended solution at 7,500 × g under room temperature for 15 minutes, and transfer the separated water layer (upper layer) to a new microcentrifuge tube. Add an equal volume of isopropyl alcohol (room temperature) to the separately-collected solutions, mix by inverting the tube, and centrifuge at 7,500 × g under room temperature for 15 minutes. Discard the supernatant by decantation, taking the precipitate into consideration. Then, add 500 µL of 70 % ethanol from the tube wall surface gently, followed by centrifugation at 7500 × g for 1 minute under room temperature. After centrifugation, aspirate ethanol as much as possible without touching the precipitate. To dry the precipitate remaining in the centrifuge tube, perform a vacuum drying treatment by using an aspirator for 2 to 3 minutes. Be careful not to dry it completely during the drying treatment. Add 50 µL of TE buffer solution*5 and mix well. Allow the mixture to stand at room temperature for 15 minutes. During this time, mix by inverting the tube several times to make sure to dissolve the precipitate completely. Add 5µL of RNase A to the obtained solution and warm at 37 °C for 30 minutes. Then, add 200 µL of CTAB buffer solution to the warmed solution, then, add 250 µL of chloroform/isoamyl alcohol mixture, mix by inverting the tube, and suspend lightly with a vortex mixer. After suspending, centrifuge at 7500 × g for 15 minutes under room temperature, and transfer the separated water layer (upper layer) to a new microcentrifuge tube. In this case, collect the water layer separately without touching the middle layer. Add 200 µL of isopropyl alcohol to the separately-collected solutions, mix by

inverting the tube. After mixing by inverting the tube, centrifuge for 10 minutes at 7,500 × g under room temperature, and discard the supernatant by decantation, paying attention to the precipitation. Then, add 200 µL of 70 % ethanol gently from the tube wall surface, followed by centrifugation for one minute at 7500 × g under room temperature conditions. After centrifugation, aspirate ethanol as much as possible and be careful not to touch the precipitate. To dry the precipitate remaining in the centrifuge tube, perform a vacuum drying treatment by using an aspirator for 2 to 3 minutes. Be careful not to dry it completely during the drying treatment. Add 50 µL of water, mix, and allow the mixture to stand at room temperature for 15 minutes. During this time, mix by inverting the tube several times to make sure to dissolve the precipitate. The completely dissolved mixture is considered as DNA sample stock solution.

*1 Use this method when a sufficient quantity of DNA cannot be extracted even as the inspection with the silica gel membrane based kit and the ion-exchange based kit are performed and the quantity is determined according to the method described in 2.2.3.2.4.

*2 CTAB Buffer Solution
Weigh 8 mL of 0.5 mM EDTA (pH 8.0), 20 mL of 1 M Tris/hydrochloric acid (pH 8.0) and 56 mL of 5 M NaCl aqueous solution to put in a beaker, mix them, and then add water to make about 150 mL of solution. Add 4 g of Cetyltrimethylammonium bromide (CTAB) while stirring in this solution to dissolve the CTAB completely. Add more water to make 200 mL of solution in total. The autoclaved solution is considered as CTAB buffer solution.

*3 Phenol/Chloroform Mixed Solution
A mixture of 1 M Tris/hydrochloric acid (pH 8.0) saturated phenol and chloroform/isoamyl alcohol in a ratio of 1:1 (v/v) is considered as phenol/chloroform mixed solution.

*4 Chloroform/Isoamyl Alcohol Mixed Solution
A mixture of chloroform and isoamyl alcohol in a ratio of 24:1 (v/v) is considered as chloroform/isoamyl alcohol mixed solution.

*5 TE Buffer Solution
Solution that is prepared with water so that the final concentration of Tris/hydrochloric acid (pH 8.0) and EDTA (pH 8.0) is 10 mM and 1mM respectively is considered as TE buffer solution.

2.2.3.2.4. Check of Purity of DNA and Quantity Determination

Collect 5µL of DNA sample stock solution, add 45 µL of TE buffer solution to make 50 µL of solution, and measure the ultra-violet absorption spectrum over 200-320 nm. At this time, record the absorbances of 230 nm, 260 nm and 280 nm (O.D. 230, O.D. 260 and O.D. 280*). Then, calculate the DNA concentration by considering 1 (the value of O.D. 260) as 50 ng/µL DNA. Moreover, calculate the ratio of O.D. 260 / O.D. 280, confirm that the ratio is 1.2-2.5. If the absorbance ratio does not reach 1.2, repeat the extraction.

In absorbance measurement of extracted DNA by using any of the three DNA extraction methods described in 2.2.3.2., when extraction of a substantial amount of DNA is not confirmed for the value of O.D.260, or when the quality of DNA sample stock solution meeting the above conditions is not confirmed, the extraction procedure should be performed using other extraction methods.

As described in 2.2.3.3.2., although DNA sample solution is prepared at a concentration of 20 ng/µL in principle, depending on the inspection sample, DNA

may be poorly extracted and DNA sample solution may fail to be prepared at concentration of 20 ng/μL. In such cases, prepare the sample solution at concentration closest to 20 ng/μL. The prepared solution is considered as DNA sample solution. As a rule, the absorbance ratio of O.D. 260/O.D. 280 should be within the range of 1.2-2.5. If the DNA described above is not extracted even after three extraction methods, DNA sample solution should be prepared using DNA sample stock solution having the value closest to the principle absorbance ratio range of 1.2-2.5 in O.D. 260/O.D. 280, and PCR amplificaion should be performed.

* O.D. 230 is absorbance derived from low molecular weight compounds such as sugar, phenols, and the like, and calculate the ratio of O.D. 260/O.D. 230. When this ratio is less than 2.0, PCR reaction may not be successfully finished due to the impact of the above contaminants. O.D. 260 is considered as absorbance from DNA and O.D. 280 is considered as absorbance from impurities such as protein.

2.2.3.3. Qualitative PCR

In the qualitative PCR, the target sequence regions contained in the extracted DNA are amplified by performing polymerase chain reaction (PCR)* using oligonucleotides called primers, and the amplicons are separated and stained with electrophoresis to detect. This method enables specific detection of specified ingredients of interest, and determines the presence or absence of specified ingredients in the inspection sample based on the presence or absence of amplicons.

* In PCR, the target sequence region can be amplified if the template DNA is present even in a very small amounts. Therefore, careful attention should be paid to the contamination of DNA (particularly PCR amplicons) in actual experimental procedures and in the preservation of daily experimental settings. Since DNA is also degraded by DNA degrading enzymes secreted from the human skin surface, contamination of these enzymes must be prevented. Considering these points, use tubes and tips that are autoclaved at 121°C for not less than 20 minutes immediately before use and are disposable. For tips, use of disposable tips with sterile filter is also effective in preventing unintended contamination of DNA.

Moreover, unless otherwise specified, all of the water used in the qualitative PCR shall be ultra pure water that was purified to 17MΩ/cm with Milli-Q system etc. from RO water passing through reverse osmosis membrane or distilled water, and autoclaved at 121°C for not less than 20 minutes.

2.2.3.3.1. PCR Amplification

The specified ingredients that can be detected by the qualitative PCR are 5 species: peanut, wheat, buckwheat, shrimp, and crab. The conditions of PCR amplification differ for each of them. Among PCR amplification conditions described in 2.2.3.3.2. to 2.2.3.3.6., the inspection should be performed using PCR condition appropriate for the specified ingredient species to be detected. In each inspection, each of DNA for which two replicated DNA extractions are performed in parallel for one prepared sample is prepared to have the specified concentration, and then used as a template DNA for PCR. First, perform a PCR amplification by using primer pairs for detecting plant DNA*1*3 or primer pairs for detecting animal DNA*2*3, and then determine the result in light of the judgement

cases described in 2.2.3.5., and perform the second PCR amplification based on the judgement result by using the primer pairs for detecting each specified ingredient.

1 Primer pairs and amplified band lengths for detecting plant DNA are as follows:

Primer Pairs for Detecting Plant DNA

F-primer (CP03-5') : 5'-CGG ACG AGA ATA AAG ATA GAG T-3'

R-primer (CP03-3') : 5'-TTT TGG GGA TAG AGG GAC TTG A-3'

Amplified Band Length

124 bp

Used equipment, preparation method of reaction solution, and PCR reaction conditions are the same as PCR amplification for detecting peanut in 2.2.3.3.2.

2 Primer pairs, amplified band lengths and reaction conditions* for detecting animal DNA are as follows:

Primer Pairs for Detecting Animal DNA

F-primer

AN1-5': 5'-TGA CCG TGC GAA GGT AGC-3'

AN2-5': 5'-TAA CTG TGC TAA GGT AGC-3'

Mix AN1-5' and AN2-5' in a ratio of 1:1 to use.

R-primer (AN-3') : 5'-CTT AAT TCA ACA TCG AGG TC-3'

Amplified Band Length

370-470 bp

Prepare the reaction solution in the reaction sample tube for PCR according to the procedures below. Add 2.5 µL of DNA sample solution* (50 ng as DNA) that is prepared so that its concentration becomes 20 ng/µL to the solution containing 1x PCR buffer solution*, 0.20 mM dNTP, 3.0 mM magnesium chloride, 0.625 units Taq DNA polymerase*, and 0.2 µM of 5' primer and 0.2 µM of 3' primer to make 25 µL of reaction solution in total. Then, place the reaction sample tube in PCR amplifier*.The reaction conditions are as follows: Keep the temperature at 95 °C for 10 minutes to initiate the reaction. Then, perform PCR amplification for 40 cycles for which 1 cycle is 95 °C for 0.5 minutes, 50 °C for 0.5 minutes, and 72 °C for 0.5 minutes. Then, keep the temperature at 72°C for 7 minutes for a final reaction, and store at 4°C. The obtained reaction solution is considered as PCR amplification solution.As blank reaction solution for PCR reaction, make sure to prepare the other solutions simultaneously that no primer pair is added and that no DNA sample solution is added.

See "PCR amplification for detecting peanut" described in 2.2.3.3.2. for PCR buffer solution, Taq DNA polymerase, DNA sample solution, and PCR amplifier.

*3 Primer pairs for detecting plant DNA and Primer pairs for detecting animal DNA are designed to widely detect plant DNA and animal DNA respectively. For this reason, as target genes, we select genes that are widely distributed in the plant or animal kingdoms and that are highly, but not completely, conserved, and there may be sequence insertions and/or deletions in genes of plants or animals. Therefore, caution should be exercised as there may be a little difference in the amplified band length depending on the inspection sample. Select a primer pair for detecting plant DNA or a primer pair for

detecting animal DNA depending on the properties of the ingredients of the inspection sample.

2.2.3.3.2. PCR Amplification for Detecting Peanut

Prepare the reaction solution in the reaction sample tube for PCR according to the procedures below. Add 2.5 μL of DNA sample solution*4 (50 ng as DNA) that is prepared so that its concentration becomes 20 ng/μL to the solution containing 1x PCR buffer solution*1, 0.20 mM dNTP, 1.5 mM magnesium chloride, 0.2 μM of 5' primer and 0.2 μM of 3' primer*2, and 0.625 units Taq DNA polymerase*3 to make 25 μL of reaction solution in total. Then, place the reaction sample tube in PCR amplifier*5. The reaction conditions are as follows: Keep the temperature at 95 °C for 10 minutes to initiate the reaction. Then, perform PCR amplification for 40 cycles for which 1 cycle is 95 °C for 0.5 minutes, 60 °C for 0.5 minutes, and 72 °C for 0.5 minutes. Then, keep the temperature at 72°C for 7 minutes for a final reaction, and store at 4°C. The obtained reaction solution is considered as a PCR amplified reaction solution. As blank reaction solution for PCR reaction, make sure to prepare the other solutions simultaneously that no primer pair is added and that no DNA sample solution is added. For the inspection procedure, perform PCR amplification with primer pairs for detecting plant DNA and then confirm that DNA with quality required for PCR amplification was extracted. Then, perform PCR amplification using primer pairs for detecting peanut according to the judgement cases described in 2.2.3.5.

*1 PCR Buffer Solution

Use PCR buffer II (Thermo Fisher Scientific Inc.), or buffer solution for which equivalent results are obtained.

*2 Primer pairs and amplified band lengths for detecting peanut are as follows:

Primer Pairs for Detecting

F-primer (agg04-5') : 5'-CGA AGG AAA CCC CGC AAT AAA T-3'

R-primer (agg05-3') : 5'-CGA CGC TAT TTA CCT TGT TGA G-3'

Amplified Band Length

95 bp

*3 Taq DNA Polymerases

Use AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific Inc.), or polymerase for which equivalent results are obtained.

*4 Although DNA sample solution should be prepared at a concentration of 20 ng/μL in principle, depending on the inspection sample, DNA may be poorly extracted and DNA sample solution may be prepared only at concentration of 20 ng/μL or lower. In such cases, prepare the sample solution at maximum concentration closest to the principle. The prepared solution is considered as DNA sample solution.

*5 PCR Amplifier

Use GeneAmp PCR System 9600, 9700 (Thermo Fisher Scientific Inc.), or system for which equivalent results are obtained.

2.2.3.3.3. PCR Amplification for Detecting Buckwheat

Used equipment, preparation method of reaction solution, and PCR reaction conditions are the same as PCR amplification for detecting peanut in 2.2.3.3.2. In addition, the reaction solution composition is also the same except that the 5′ and 3′ primers* are changed to a primer pair for buckwheat detection.

* Primer pairs, amplified band lengths for detecting buckwheat are as follows:
Primer Pairs for Detecting
F-primer (FAG19-5') :5'-AAC GCC ATA ACC AGC CCG ATT-3'
R-primer (FAG22-3') :5'-CCT CCT GCC TCC CAT TCT TC-3'
Amplified Band Length
127 bp

2.2.3.3.4. PCR Amplification for Detecting Wheat

Used equipment, preparation method of reaction solution, and PCR reaction conditions are the same as PCR amplification for detection of peanut in 2.2.3.3.2. In addition, the reaction solution composition is also the same except that the 5′ and 3′ primers* are changed to a primer pair for wheat detection.

* Primer pairs, amplified band lengths for detecting wheat are as follows:
Primer Pairs for Detecting
F-primer (Wtr01-5') :5'-CAT CAC AAT CAA CTT ATG GTG G-3'
R-primer (Wtr10-3') :5'-TTT GGG AGT TGA GAC GGG TTA-3'
Amplified Band Length
141 bp

2.2.3.3.5. PCR Amplification for Detecting Shrimp *1

Prepare the reaction solution in the reaction sample tube for PCR according to the procedures below. Add 2.5 µL of DNA sample solution*5 (50 ng of DNA) that is prepared so that its concentration becomes 20 ng/µL to the solution containing 1x PCR buffer solution*2, 0.20 mM dNTP, 1.5 mM magnesium chloride, 0.625 units Taq DNA polymerase*4 and 0.3 µM 5' and 3' primers*3, and to make 25 µL of reaction solution in total. Then, place the reaction sample tube in PCR amplifier*6. The reaction conditions are as follows: Keep the temperature at 95°C for 10 minutes to initiate the reaction. Then, perform PCR amplification for 45 cycles for which 1 cycle is 95 °C for 1 minute, 56 °C for 1 minute, and 72 °C for 1 minute. Then, keep the temperature at 72°C for 7 minutes for a final reaction, and store at 4°C. The obtained reaction solution is considered as PCR amplification solution. As blank reaction solution for PCR reaction, make sure to prepare the other solutions simultaneously that no primer pair is added and that no DNA sample solution is added. For the inspection procedure, perform PCR amplification with primer pairs for detecting plant DNA or animal DNA and then confirm that DNA with quality required for PCR amplification was extracted. Then, according to the judgement cases described in 2.2.3.5., perform PCR amplification using primer pairs for detecting shrimp.

*1 Chinese mitten crab, Dungeness crab, Japanese giant crab, Red snow crab, Deepwater red crab, and Swimming crab have been confirmed that amplicons may be detected in PCR amplifications for detecting shrimp. If it is unclear whether the obtained PCR amplicon is derived from shrimp or crab, the PCR amplicon should be subjected to the following restriction-enzyme treatments for determination.

Mix 17 µL of PCR amplification solution, 2 µL of restriction enzyme 10×M buffer*, and 1 µL of restriction enzyme HaeIII*, and treat the mixture at 37 °C for 16 hours. The obtained reaction solution is analyzed by agarose gel electrophoresis in 2.2.3.4. to confirm the restriction enzyme digested fragment from the shrimp.

For restriction enzyme 10×M buffer and a restriction enzyme HaeIII, use

enzymes manufactured by Takara Bio Inc. or enzymes for which equivalent results are obtained.

Length of Fragment Processed by Restriction Enzyme
149 bp

However, because primer pairs for detecting DNA of shrimp are designed to detect DNA of various shrimp belonging to the decapoda of crustacea, there may be sequence insertions and/or deletions in some species of shrimp. Therefore, caution should be exercised as there may be a little difference in the length of fragment processed by restriction enzyme depending on the inspection sample.

*2 PCR Buffer Solution
Use PCR buffer II (Thermo Fisher Scientific Inc.), or solution for which equivalent results are obtained.

*3 Primer pairs, amplified band lengths for detecting shrimp are as follows:
Primer Pairs for Detecting
F-primer (ShH12-05') :
5'-TTA TAT AAA GTC TRG CCT GCC-3'
For ShH12-05', the eighth base from the 3' end is synthesized as mixed base (R) of A and G.
R-primer (ShH13-03') :
ShH13-03'-1: 5'-GTC CCT CTA GAA CAT TTA AGC CTT TTC-3'
ShH13-03'-2: 5'-GTC CCT TTA TAC TAT TTA AGC CTT TTC-3'
ShH13-03'-3: 5'-GTC CCC CCA AAT TAT TTA AGC CTT TTC-3'
ShH13-03'-1, ShH13-03'-2 and ShH13-03'-3 are mixed in a 1:1:1 ratio to use the mixture.
Amplified Band Length
187 bp

Primer pairs for detecting DNA of shrimp are designed to detect DNA of various shrimp belonging to the decapoda of crustacean. Therefore, there may be sequence insertions and/or deletions in some species of shrimp. Therefore, caution should be exercised as there may be a little difference in the amplified band length depending on the inspection sample.

*4 Taq DNA Polymerases
Use AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific Inc.), or polymerase for which equivalent results are obtained.

*5 Although DNA sample solution should be prepared at a concentration of 20 ng/μL in principle, depending on the inspection sample, DNA may be poorly extracted and DNA sample solution may be prepared only at concentration of 20 ng/μL or lower. In such cases, prepare the sample solution at maximum concentration closest to the principle. The prepared solution is considered as DNA sample solution.

*6 PCR Amplifier
Use GeneAmp PCR System 9600, 9700, Veriti Thermal Cycler (Thermo Fisher Scientific Inc.), or system for which equivalent results are obtained. When GeneAmp PCR System 9700 and Veriti thermal cyclers are used, the amplification should be performed in 9600 Emulation Mode.

2.2.3.3.6. PCR Amplification for Detecting Crab

Prepare the reaction solution in the reaction sample tube for PCR according to the procedures below. Add 2.5 μL of DNA sample solution[*4] (50 ng as DNA) that

is prepared so that its concentration becomes to 20 ng/μL to the solution containing 1x PCR buffer solution[*1], 0.20 mM dNTP, 2.0 mM magnesium chloride, 0.2 μM of 5' primer and 0.2 μM of 3' primer[*2], and 0.625 units Taq DNA polymerase[*3] to make 25 μL of reaction solution in total. Then, place the reaction sample tube in PCR amplifier[*5]. The reaction conditions are as follows: Keep the temperature at 95 °C for 10 minutes to initiate the reaction. Then, perform PCR amplification for 40 cycles for which 1 cycle is 95 °C for 0.5 minutes, 54 °C for 0.5 minutes, and 72 °C for 0.5 minutes. Next, store at 4°C, and the obtained reaction solution is considered as PCR amplification solution. As blank reaction solution for PCR reaction, make sure to prepare the other solutions simultaneously that no primer pair is added and that no DNA sample solution is added. For the inspection procedure, perform PCR amplification with primer pairs for detecting plant DNA or animal DNA and then confirm that DNA with quality required for PCR amplification was extracted. Then, according to the judgement cases described in 2.2.3.5., perform PCR amplification using primer pairs for detecting crab.

*1 PCR Buffer Solution
Use PCR buffer II (Thermo Fisher Scientific Inc.), or solution for which equivalent results are obtained.

*2 Primer pairs, amplified band lengths for detecting crab are as follows:
Primer Pairs for Detecting
F-primer (CrH16-05') :
CrH16-05'-1: 5'-GCG TTA TTT TTT TTG AGA GTT CWT ATC GTA-3'
CrH16-05'-2: 5'-GCG TAA TTT TTT CTG AGA GTT CTT ATC ATA-3'
CrH16-05'-3: 5'-GCG TTA TTT TTT TTA AGA GTA CWT ATC GTA-3'
CrH16-05'-4: 5'-GCG TTA TTT CTT TTG AGA GCT CAT ATC GTA -3'
For CrH16-05'-1 and CrH16-05'-3, the eighth base from the 3' end is synthesized as mixed base (W) of A and T.
CrH16-05'-1, CrH16-05'-2, CrH16-05'-3 and CrH16-05'-4 are mixed in a 10:1:6:3 ratio to use the mixture.
R-primer (CrH11-03') :5'-TTT AAT TCA ACA TCG AGG TCG CAA AGT-3'
Amplified Band Length
62 bp

*3 Taq DNA Polymerases
Use AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific Inc.), or polymerase for which equivalent results are obtained.

*4 Although DNA sample solution should be prepared at a concentration of 20 ng/μL in principle, depending on the inspection sample, DNA may be poorly extracted and DNA sample solution may be prepared only at concentration of 20 ng/μL or lower. In such cases, prepare the sample solution at maximum concentration closest to the principle. The prepared solution is considered as DNA sample solution.

*5 PCR Amplifier
Use GeneAmp PCR System 9600, 9700, Veriti Thermal Cycler(Thermo Fisher Scientific Inc.), or system for which equivalent results are obtained. When GeneAmp PCR System 9700 and Veriti thermal cyclers are used, the amplification should be performed in 9600 Emulation Mode.

2.2.3.4. Agarose Gel Electrophoresis

Analyze PCR amplicon by agarose gel electrophoresis to confirm DNA amplified

bands.

2.2.3.4.1. Preparation of Agarose Gel

Weigh the required amount of agarose, add TAE buffer solution[*1] and heat to dissolve the agarose. Then, add 5 μL of ethidium bromide solution (10 mg/mL)[*2] per 100 mL of gel. When the gel has cooled to around 50 °C, pour the gel into a gel maker, and the gel is sufficiently cooled at room temperature and solidified to prepare a gel[*3]. It is desirable to use the gel immediately, but it can be stored for several days since the gel is immersed in a buffer solution. Since concentration of gel should be determined according to length of DNA to be migrated, the gel concentration (2.0-4.0%) should be determined according to the band length of the target product to be migrated. (It is appropriate to use agarose gel with 2.5-4.0% concentration for detection of specified ingredient.)

*1 TAE Buffer Solution

Prepare TAE buffer solution using distilled water so that the final concentrations of Tris-acetic acid and EDTA are 40 mM and 1 mM, respectively.

*2 Ethidium Bromide

It is a fluorescent reagent that enters between strands of double-stranded DNA and has potent carcinogenic and toxic effects. Always wear gloves and mask during handling.

*3 Prestaining

Here, we describe the prestaining method. No ethidium bromide solution may be added at this stage, and after electrophoresis, staining may be performed according to the post-staining method described in 2.2.3.4.3. (In cases that the expected amplified band lengths are short, it is desirable to perform post-staining to facilitate visualization.)

2.2.3.4.2. Electrophoresis

Place the gel in an electrophoresis chamber filled with TAE buffer solution. Mix 7.5μL of PCR amplification solution and an appropriate volume of gel loading buffer solution, and inject the mixture into the wells of the gel. Note that if it takes a long time to inject to the wells, DNA will diffuse and the results will be unclear. Next, perform electrophoresis at a 100 V constant voltage. When BPB contained in the gel loading buffer solution pervades about 2/3 of the gel, stop the electrophoresis.

2.2.3.4.3. Coloration of the Gel (Poststaining)

If prestaining is performed, the procedure described in this section is not necessary.

Transfer the gel, in which electrophoresis has been performed, into a container containing enough TAE buffer solution to allow the gel to soak. Then, add 5 μL of ethidium bromide solution (10 mg/mL) per 100 mL of buffer solution. Place container on a shaker and stain the mixed solution for about 20 minutes with light shaking. After that, transfer the stained gel to a container containing only TAE buffer solution, and perform destaining while shaking lightly for about 20 minutes.

2.2.3.4.4. Gel Image Analysis

Place a cling film[*] on the stages inside the gel image analyzer, and place the gel that has completed electrophoresis and staining procedures on the cling film

and irradiate it with UV light (312 nm). Check the electrophoretic pattern on the monitor of the gel image analyzer. Determine the presence or absence of bands of interest compared to DNA molecular weight standard markers. If the corresponding PCR amplified band is detected in the blank reaction solution, the result after DNA extraction is invalidated, and redo the experiment. The electrophoresis results are stored as image data.

* Cling Film

  If the film is not made of polyvinylidene chloride, ultraviolet rays are absorbed and an image may not be obtained. Therefore, caution should be exercised.

2.2.3.5. Judging the Results

2.2.3.5.1. Judging the Inspection Results for Peanut

DNA for which two replicated DNA extractions are performed in parallel for one prepared sample is prepared to have the specified concentration, and then used as a template DNA to perform PCR. First, perform the first PCR amplification using a primer pair for detecting plant DNA. If PCR amplified bands of 124 bp are detected in both cases that any of the two DNA sample solutions is used (sample number 1 in judgement case of primer pair for detecting plant DNA described below), it is judged that DNA with quality required for PCR amplification was extracted in both sample solutions, and then PCR amplification using a primer pair for detecting peanut is performed for each sample solution. If the second PCR amplification with primer pairs for detecting peanut results in the detection of PCR amplified bands of 95 bp in both or any of DNA sample solutions, the inspection sample will be judged as positive for peanut (sample numbers 1 and 2 in judgement case of primer pairs for detection described below). If no PCR amplified band is detected in any of the two PCR sample solutions (sample numbers 2 and 3 in judgement case of primer pairs for detecting plant DNA described below) as a result of the first PCR amplification using the primer pair for detecting plant DNA, discontinue the inspection using such sample solution and perform a second PCR amplification using the primer pair for detection with only the sample solution for which PCR amplified band is obtained as a template. Consequently, if PCR amplified band of 95 bp is detected, the inspection sample is judged to be positive for peanut. In addition, as sample number 4 in judgement cases of primer pair for detecting plant DNA below shows, if PCR amplified bands were not obtained from both two DNA sample solutions when the first PCR amplification was performed using primer pairs for detecting plant DNA, it is judged that DNA with the required quality for PCR amplification had not been extracted, and the extraction method other than DNA extraction method used previously described in 2.2.3.2. should be attempted. If similar results are obtained when using the three DNA extraction methods described in 2.2.3.2., it is judged that DNA extraction from the inspection sample in question is not possible and DNA cannot be detected by PCR. Judgement cases are described below.

Judgement Case of Primer Pairs for Detecting Plant DNA

	Sample No.	1	2	3	4
Extraction 1		+	+	-	-
Extraction 2		+	-	+	-

	Case 1	Case 2	Case 3

+ : Detected amplified band , - : Not detected amplified band

Case 1: PCR amplification with the primer pair for detection is performed in two DNA sample solutions.

Case 2: PCR amplification with the primer pair for detection is performed in only DNA sample solution for which amplified band is obtained.

Case 3: It is judged that DNA extraction by this method is difficult, and the optimization of DNA extraction method is attempted. If only the same results (PCR amplified bands were not obtained) are obtained after attempting three DNA extraction methods, it is judged that DNA extraction from the inspection sample in question is not possible and DNA cannot be detected by PCR.

Judgement Case of Primer Pair for Detection

	Sample No.	1	2	3
Extraction 1		+	+	-
Extraction 2		+	-	-
Judgement		Positive	Positive	Negative

+ : Detected amplified band , - : Not detected amplified band

When the optimal extraction method is not selected for the inspection sample, as described in 2.2.3.2., it is difficult to extract DNA that can serve as a template for PCR in both quantity and quality. DNA sample solution to be subjected to PCR should be extracted and purified by the optimal extraction method, and the DNA sample solution shall meet the standard described in 2.2.3.2.4. in principle.

2.2.3.5.2. Judging the Inspection Results for Buckwheat

If PCR amplified band of 124 bp is detected in the lane using the primer pair for detecting plant DNA, and PCR amplified band of 127 bp is detected in the lane using the primer pair for detecting buckwheat, the inspection sample is judged to be positive for buckwheat. The procedure for judging the results, judgement case, and precautions are the same as those for judging the inspection results for peanut described in 2.2.3.5.1.

2.2.3.5.3. Judging the Inspection Results for Wheat

If PCR amplified band of 124 bp is detected in the lane using the primer pair for detecting plant DNA, and PCR amplified band of 141 bp is detected in the lane using the primer pair for detecting wheat, the inspection sample is judged to be positive for wheat. The procedure for judging the results, judgement case, and precautions are the same as for judging the inspection results for peanut described in 2.2.3.5.1.

2.2.3.5.4. Judging the Inspection Results for Shrimp

If PCR amplified band of 124 bp or PCR amplified band ranging from 370 to 470 bp is detected in the lane using the primer pair for detecting plant DNA or animal DNA, and PCR amplified band of 187 bp is detected in the lane using the primer pair for detecting shrimp, the inspection sample is judged to be positive for shrimp. However, for PCR amplification using primer pairs for detecting shrimp, false positive results may be shown in Chinese mitten crab, Dungeness crab,

Japanese giant crab, Red snow crab, Deepwater red crab, and Swimming crab according to previous studies. Therefore, if it is unclear whether the resulting PCR amplicon is derived from shrimp or crab, perform restriction enzyme digestion of PCR amplicon as described in 2.2.3.3.5. and confirm the enzyme digestion fragments (149 bp) of the shrimp-derived PCR amplicon*. The procedure for judging the results, judgement case, and precautions are the same as those for judging the inspection results for peanut described in 2.2.3.5.1.

* Restriction Enzyme Digestion

It is confirmed that false positive results are shown for Chinese mitten crab even after treatment with restriction enzyme digestion.

2.2.3.5.5. Judging the Inspection Results for Crab

If PCR amplified band of 124 bp or PCR amplified band ranging from 370 to 470 bp is detected in the lane using the primer pair for detecting plant DNA or animal DNA, and PCR amplified band of 62 bp is detected in the lane using the primer pair for detecting crab, the inspection sample is judged to be positive for crab*. The procedure for judging the results, judgement case, and precautions are the same as those for judging the inspection results for peanut described in 2.2.3.5.1.

* Type of Crab for which False Positive Results are shown.

It is confirmed that false positive results are shown for Japanese mantis shrimp. In addition, it is confirmed that there are some kinds of shrimps for which false positive results are shown.

2.2.4. Real-time PCR

Extract DNA from foods according to the DNA extraction and purification method of PCR (2.2.3.2.) and perform the qualitative real-time PCR described below using the obtained DNA sample solution.

Meanwhile, perform two replicated DNA extraction in parallel for one prepared sample, and perform all subsequent steps to real-time PCR amplification confirmation independently in parallel for the two extracted DNA.

2.2.4.1. Sample Preparation

Preparation of samples shall be performed according to the sample preparation for PCR described in 2.2.3.1.

2.2.4.2. DNA Extraction and Purification

DNA extraction and purification shall be performed according to the DNA extraction and purification for PCR described in 2.2.3.2.

2.2.4.3. Qualitative Real-time PCR

Real-time PCR is a method for real-time monitoring of PCR amplifications. There are several techniques, but the probe method is used here. In the probe method, an oligonucleotide (probe) modified with a fluorescent material at the 5’ end and a quencher material at the 3’ end is added to the PCR system. The probe is specifically hybridized to the target DNA sequence in the annealing step, and then quencher material is released during in the extension step, thereby emitting fluorescence derived from the fluorescent material. PCR amplifications are monitored in real-time by detecting the fluorescent using a dedicated device and the results are analyzed. Notes are the same as those in the qualitative PCR

(2.2.3.3.).

2.2.4.3.1. Real-time PCR Amplification

Four types of specified ingredients that can be detected by qualitative real-time PCR are wheat, buckwheat, peanut, and walnut. They differ in conditions for real-time PCR amplification. Among the methods described in 2.2.4.3.2. to 2.2.4.3.6., perform inspections using a method appropriate for the type of specified ingredient to be inspected. In addition, for each inspection, DNA for which two replicated DNA extractions are performed in parallel for one prepared sample is prepared to have the specified concentration, and then used as a template DNA to perform real-time PCR. First, perform a PCR by using primer pairs for detecting plant DNA or primer pairs for detecting animal DNA according to 2.2.3.3.1., and then determine the result in light of the judgement cases described in 2.2.3.5., and perform real-time PCR amplification of the specified ingredient to be inspected based on the judgement result.

2.2.4.3.2. Real-time PCR for Detecting Peanut

2.2.4.3.2.1. Real-time PCR Amplification

Prepare the reaction solution in the reaction sample tube for real-time PCR according to the procedures below. Add 2.5 µL of DNA sample solution[*3] (50 ng as DNA) that is prepared so that its concentration becomes 20 ng/µL to the Master Mix containing 12.5 µL of real-time PCR Reagent[*1], 0.2 µL of 5' and 3' primers[*2] respectively (both 50 µM), 0.25 µL of probe[*2] (10 µM), and 9.35 µL of water (see Master Mix Composition (peanut) described below) to make 25 µL of reaction solution in total. Simultaneously prepare solutions for two measurements in parallel, which are solution containing 2.5 µL of the reference plasmid solution[*4] or 2.5 µL of the high-concentration plasmid solution[*4] instead of DNA sample solution. As blank reaction solution for PCR, make sure to prepare the other solutions simultaneously that no DNA sample solution is added. Then, place the reaction sample tube in real-time PCR amplifier[*5]. The reaction conditions are as follows: Keep the temperature at 95 °C for 15 minutes to initiate the reaction. Then, perform real-time PCR amplification for 38 cycles or 42 cycles[*6] depending on the devise type, for which 1 cycle is 95°C for 0.5 minutes and 68 °C for 1 minute. For devices that require input to Reporter or Quencher, set FAM as Reporter and NFQ-MGB (or None) as Quencher.

Master Mix Composition (Peanut)

Reagent	Required volume for each reaction solution (µL)
Real-time PCR Reagent[*1]	12.5
5' Primer (50 µM)[*2]	0.2
3' Primer (50 µM)[*2]	0.2
Probe (10 µM)[*2]	0.25
Water	9.35
Total	22.5

*1 For real-time PCR reagents, use QuantiTect Probe PCR Master Mix (Qiagen), or reagents for which equivalent results are obtained.

*2 Primer pairs and probe for detecting peanut are as follows:
5’ Primer (AI2-F) :5’-TTGGTTCAAAGAGACGGGCTC-3’
3’ Primer (AI2-R) :5’-CACGAGGGTTGTTCTCGACC-3’
Probe (AI2-probe) :5’-FAM-ACCGCGGCAGATGG-MGB-3’

*3 Although DNA sample solution should be prepared at a concentration of 20 ng/μL in principle, depending on the inspection sample, DNA may be poorly extracted and DNA sample solution may be prepared only at concentration of 20 ng/μL or lower. In such cases, prepare the sample solution at maximum concentration closest to the principle. The prepared solution is considered as DNA sample solution.

*4 For plasmid solution, use “Plasmid set for detecting wheat, buckwheat, peanut, and walnut in qualitative real-time PCR” (Fasmac), or plasmid solution for which equivalent results are obtained. The reference plasmid solution is used to determine positive/negative of the sample, and the high-concentration plasmid solution is used to verify whether real-time PCR by this method was performed appropriately.

*5 Real-time PCR amplifier that can achieve the above thermal conditions shall be used.

*6 Similar to ABI 7900HT (Thermo Fisher Scientific Inc.), 38 cycles should be set for models for which any Threshold Line can be set during analysis, and 42 cycles should be set for other models.

2.2.4.3.2.2. Analysis of Real-time PCR Amplification Results

Analyze the fluorescent signal obtained by the real-time PCR device to determine whether the target sequence was amplified or not by real-time PCR for each DNA sample solution. For models for which any Threshold Line can be set during analysis (ABI 7900HT, etc.), calculate Cqvalue (Ct value) by the high-concentration plasmid sample only that was amplified by real-time PCR, after confirming that Threshold Line, and Baseline are set to Auto in Analysis Settings. The obtained Threshold Line that is rounded off to the six decimal place shall be recorded. Next, calculate Cq values using the value of the recorded Threshold Line for all the reaction sample tubes in which amplification was performed by real-time PCR. If the above analysis cannot be performed, or if other models are used, calculate Cq values for all reaction sample tubes using an analysis tool for absolute quantification in the analysis condition with initial set-up of the device. If the calculated Cq value in DNA sample solution is less than the mean Cq value of the reference plasmid solutions measured in 2 parallel replications, the target sequence is determined to be amplified by real-time PCR. If the mean Cq value of the reference plasmid solutions measured in 2 parallel replications minus the mean Cq value of the high-concentration plasmid solutions measured in 2 parallel replications falls outside the range of 4.6 to 6.6, it is judged that real-time PCR amplification by this method was not performed adequately, and retry real-time PCR amplification. In addition, when it is determined that the targeted sequence in the blank reaction solution is amplified by real-time PCR, it is determined that there is a contamination from the experimental environment, and Real-time PCR amplification is performed again.

2.2.4.3.3. Real-time PCR for Detecting Buckwheat

2.2.4.3.3.1. Real-time PCR Amplification

Amplification should be performed according to 2.2.4.3.2.1. It is should be noted that the concentrations of the 5’ and 3’ primers are both 25 μM in this case. Further, the 5′ primer, the 3′ primer, and the probe* are the primer pairs and the probe for detecting buckwheat.

* Primer pairs and probe for detecting buckwheat are as follows:
  5’ primer (Fago-453) :5’-CGCCAAGGACCACGAACAGAAG-3’
  3’ primer (Fago-261) :5’-CGTTGCCGAGAGTCGTTCTGTTT-3’
  Probe (Fago-probe) :5’-FAM-CGGGACGCGCTTC-MGB-3’

2.2.4.3.3.2. Analysis of Real-time PCR Amplification Results

Analyze the results according to 2.2.4.3.2.2.

2.2.4.3.4. Real-time PCR for Detecting Wheat

2.2.4.3.4.1. Real-time PCR Amplification

Amplification should be performed according to 2.2.4.3.2.1. It should be noted that the concentrations of the 5’ and 3’ primers are both 50 μM in this case. Further, the 5’ primer, the 3’ primer, and the probe* are the primer pairs and the probe for detecting wheat.

* Primer pairs and probe for detecting wheat are as follows:
  5’ primer (Tri-F) :5’-CATGGTGGGCGTCCTC-3’
  3’primer (Tri-R) :
  Tri-R1:5’-AAAGGCCATAATGCCAGCTG-3’
  Tri-R2:5’-TGAGGCCGTCATGCCGGCTG-3’
  Tri-R3:5’-TGAGGCCATAATGTCGGCTG -3’
  Mix Tri-R1, Tri-R2 and Tri-R3 in a ratio of 2:1:1 to use.
  Probe (Tri-probe):5’-FAM-CGGATGCACTGCITTGATAAAG-MGB-3’
  “I” in the probe sequence is inosine.

2.2.4.3.4.2. Analysis of Real-time PCR Amplification Results

Analyze the results according to 2.2.4.3.2.2.

2.2.4.3.5. Real-time PCR-H Method for Detecting Walnut

2.2.4.3.5.1. Real-time PCR Amplification

Amplification should be performed according to 2.2.4.3.2.1. It should be noted that the concentrations of the 5’ and 3’ primers are both 25 μM in this case. Further, the 5′ primer, the 3′ primer, and the probe* are the primer pairs and the probe for detecting walnut.

* Primer pairs and probe of H method for detecting walnut are as follows:
  5′ primer (JI2F4): 5′-CCACGACAATCGGTGGTTGAG-3′
  3′ primer (JI2R2): 5′-GTCGAGGAGCACCTTCACA-3′
  Probe (JI2P): 5′-FAM-ACACACGACGGGTCACGAGG-MGB-3′

2.2.4.3.5.2. Analysis of Real-time PCR Amplification Results

Analyze the results according to 2.2.4.3.2.2.

2.2.4.3.6. Real-time PCR-N Method for Detecting Walnut

2.2.4.3.6.1. Real-time PCR Amplification

Prepare the reaction solution in the reaction sample tube for real-time PCR according to the procedures below. Add 2.5 µL of DNA sample solution*3 (50 ng as DNA) that is prepared to 20 ng/µL concentration, to the Master Mix containing 10 µL of real-time PCR Reagent[*1], 0.8 µL of 5' and 3' primers[*2] respectively (both 10 µM), 0.4 µL of probe[*2] (10 µM), and 5.5 µL of water (see the table described below) to make 20 µL of reaction solution in total. As blank reaction solution for PCR, prepare a solution simultaneously to which no DNA sample solution is added. Then, place the reaction sample tube in real-time PCR amplifier[*4]. The reaction conditions are as follows: Keep the temperature at 95 °C for 15 minutes to initiate the reaction. Then, perform real-time PCR amplification for 35 cycles for which 1 cycle is 94 °C for 15 seconds and 60 °C for 1 minute. For devices that require input to Reporter or Quencher, set FAM as Reporter and NFQ-MGB (or None) as Quencher.

Master Mix Composition (Walnut-N Method)

Reagent	Required volume for each reaction solution (µL)
Real-time PCR Reagent[*1]	10
5′ Primer (10 µM)[*2]	0.8
3′ Primer (10 µM)[*2]	0.8
Probe	0.4
Water	5.5
Total	17.5

*1 For real-time PCR reagents, use QuantiTect Probe PCR Master Mix (Qiagen), or reagents for which equivalent results are obtained.

*2 Primer pairs and probe of N method for detecting walnut are as follows:
5′ Primer (JUGr-F) :5′-AAACGGTTGGGAGGGCACGT-3′
3′ Primer (JUGr-R) :5′-CGCCCGTGGTTACTCCTTGTTTA-3′
Probe (JUGr-P) :5′-FAM-TTGGTCAATCTTCTCGTTCC-MGB-3′

*3 Although DNA sample solution should be prepared at a concentration of 20 ng/µL in principle, depending on the inspection sample, DNA may be poorly extracted and DNA sample solution may be prepared only at concentration of 20 ng/µL or lower. In such cases, prepare the sample solution at maximum concentration closest to the principle. The prepared solution is considered as DNA sample solution.

*4 Real-time PCR amplifier that can achieve the above thermal conditions shall be used.

2.2.4.3.6.2. Analysis of Real-time PCR Amplification Results

Analyze the fluorescent signal obtained by the real-time PCR device to determine whether the target sequence was amplified or not by real-time PCR for each DNA sample solution. For models for which any Threshold Line can be used (ABI 7500, etc.), calculate all Cq values after confirming that Threshold Line, and Baseline are set up as Auto in Analysis Settings. If other models are used (Roche LightCycler 96, etc.), calculate Cq values for all reaction sample tubes by using an analysis tool for absolute quantification in the analysis condition with initial set-up of the device. Confirm that no Cq

value was obtained in the blank reaction solution, and then confirm the measured result of DNA sample solution. For the solution for which Cq values are obtained, the target sequence is determined to be amplified by real-time PCR.

2.2.4.4. Judging the Results

2.2.4.4.1. Judging the Inspection Results for Peanut

If it is judged that PCR amplified band of 124 bp or PCR amplified band ranging from 370 to 470 bp is detected in the lane using the primer pair for detecting plant DNA or animal DNA, and moreover the target sequence is amplified by real-time PCR for detecting peanut, the inspection sample is judged to be positive for peanut.

The procedure for judging the results, judgement case, and precautions are the same as those for judging the inspection results for peanut described in 2.2.3.5.1.

2.2.4.4.2. Judging the Inspection Results for Buckwheat

If it is judged that PCR amplified band of 124 bp or PCR amplified band ranging from 370 to 470 bp is detected in the lane using the primer pair for detecting plant DNA or animal DNA, and moreover the target sequence is amplified by real-time PCR for detecting buckwheat, the inspection sample is judged to be positive for buckwheat.

The procedure for judging the results, judgement case, and precautions are the same as those for judging the inspection results for peanut described in 2.2.3.5.1.

2.2.4.4.3. Judging the Inspection Results for Wheat

If it is judged that PCR amplified band of 124 bp or PCR amplified band ranging from 370 to 470 bp is detected in the lane using the primer pair for detecting plant DNA or animal DNA, and moreover the target sequence is amplified by real-time PCR for detecting wheat, the inspection sample is judged to be positive for wheat.

The procedure for judging the results, judgement case, and precautions are the same as those for judging the inspection results for peanut described in 2.2.3.5.1.

2.2.4.4.4. Judging the Inspection Results for Walnut

If it is judged that PCR amplified band of 124 bp or PCR amplified band ranging from 370 to 470 bp is detected in the lane using the primer pair for detecting plant DNA or animal DNA, and moreover the target sequence is amplified by real-time PCR for detecting walnut, the inspection sample is judged to be positive for walnut.

The procedure for judging the results, judgement case, and precautions are the same as those for judging the inspection results for peanut described in 2.2.3.5.1.

2.2.5. Nucleic Acid Chromatography

Extract DNA from foods according to the DNA extraction and purification for PCR (2.2.3.2.) and perform the nucleic acid chromatography described below using the obtained DNA sample solution. Perform two replicated DNA extraction in parallel for one prepared sample, and perform all subsequent steps to PCR amplification confirmation independently in parallel for the two extracted DNA.

2.2.5.1. Sample Preparation

Preparation of samples shall be performed according to the sample preparation for PCR described in 2.2.3.1.

2.2.5.2. DNA Extraction and Purification

DNA extraction and purification shall be performed according to the DNA extraction and purification for PCR described in 2.2.3.2.

2.2.5.3. Nucleic Acid Chromatography

Nucleic acid chromatography is a method in which PCR amplification is performed using tagged primers, and its amplicon is developed on a dedicated strip (nucleic acid chromatography), and visually judged by the presence or absence of target band. Notes are the same as those in the qualitative PCR (2.2.3.3.).

Specified ingredient that can be detected by nucleic acid chromatography is walnut. Perform the inspection using the methods described in 2.2.5.3.1. After preparing each of DNA extracted by two replications in parallel from one prepared sample to the specified concentration, and the prepared DNAs serve as a template NA for PCR amplifications in nucleic acid chromatography. First, perform a PCR by using primer pairs for detecting plant DNA or animal DNA according to 2.2.3.3.1., and then determine the results in light of the judgement cases described in 2.2.3.5., and perform nucleic acid chromatography of the specified ingredient to be inspected based on the determination results.

2.2.5.3.1. Nucleic Acid Chromatography for Detecting Walnut

2.2.5.3.1.1. PCR Amplification

Prepare the reaction solution in the reaction sample tube for PCR according to the procedures below. Add 2.5 μL of DNA sample solution[*3] (50 ng of DNA) that is prepared so that its concentration becomes 20 ng/μL to the solution containing 10 μL of PCR Reagent[*1], 0.6 μL of 5' and 3' primers[*2] respectively (both 10 μM), and 6.3 μL of water to make 20 μL of reaction solution in total. As blank reaction solution for PCR, make sure to prepare the other solutions simultaneously that no DNA sample solution is added. Then, place the reaction sample tube in PCR amplifier[*4]. The reaction conditions are as follows: Keep the temperature at 95 °C for 5 minutes to initiate the reaction. Then, perform real-time PCR amplification for 35 cycles for which 1 cycle is 94 °C for 30 seconds, 67 °C for 30 seconds, and 72 °C for 30 seconds. Next, store at 4 °C, and the obtained reaction solution is considered as a PCR amplification solution.

*1 For PCR reagents, use HotStarTaq Plus Master Mix (Qiagen), or reagents for which equivalent results are obtained.

*2 Primer pairs for detecting walnut are as follows: In this inspection, use the primer pairs for detecting walnut (TBA Co., Ltd.), or primer pairs for which equivalent results are obtained.
5'-primer (JUGc-F) :5'-[F-1]-AAACGGTTGGGAGGGCACGT-3'
3'-primer (JUGc-R) :5'-Biotin-CGCCCGTGGTTACTCCTTGTTTA-3'

*3 Although DNA sample solution should be prepared at a concentration of 20 ng/μL in principle, depending on the inspection sample, DNA may be poorly extracted and DNA sample solution may be prepared only at concentration of 20 ng/μL or lower. In such cases, prepare the sample solution at maximum concentration closest to the principle. The prepared solution is considered as a DNA sample solution.

*4 PCR amplifier shall be used that can achieve the above thermal conditions.

2.2.5.3.1.2. Dipstick Chromatography

Add 10 μL of the developing solution (modified) salinity 0 mM[*1], 1 μL of avidin-coated colored latex solution[*1], and 10 μL of PCR amplification solution to 1.5 mL tube, and mix them well. Hold the test strip (C-PAS(F4))[*1] absorbent pad and insert it into the mixed solution. Then, allow to stand at room temperature to develop it[*2].After 10 to 15 minutes, visually check for the presence of colored lines. If the orange line, which is located above the position marker at the top of the test strip, disappears, the solution is judged to be properly developed. The sample solution with the blue colored line below the bottom position marker is judged that the target sequence is amplified by PCR.

*1 In this inspection, use the reagents and test strips (TBA Co., Ltd.), or items for which equivalent results are obtained.

*2 Use in environments with a humidity of 40% or higher. If the humidity is lower than 40%, care should be taken because non-specific colored lines are more likely to be detected.

2.2.5.4. Judging the Results

2.2.5.4.1. Judging the Inspection Results for Walnut

If it is judged that PCR amplified band of 124 bp or PCR amplified band ranging from 370 to 470 bp is detected in the lane using the primer pair for detecting plant DNA or animal DNA, and the target sequence is amplified by nucleic acid chromatography for detecting walnut, the inspection sample is judged to be positive for walnut.

The procedure for judging the results, judgement case, and precautions are the same as those for judging the inspection results for peanut described in 2.2.3.5.1.

2.3. Quantitative Inspection Method by Improving “2.1. Quantitative Inspection Method”

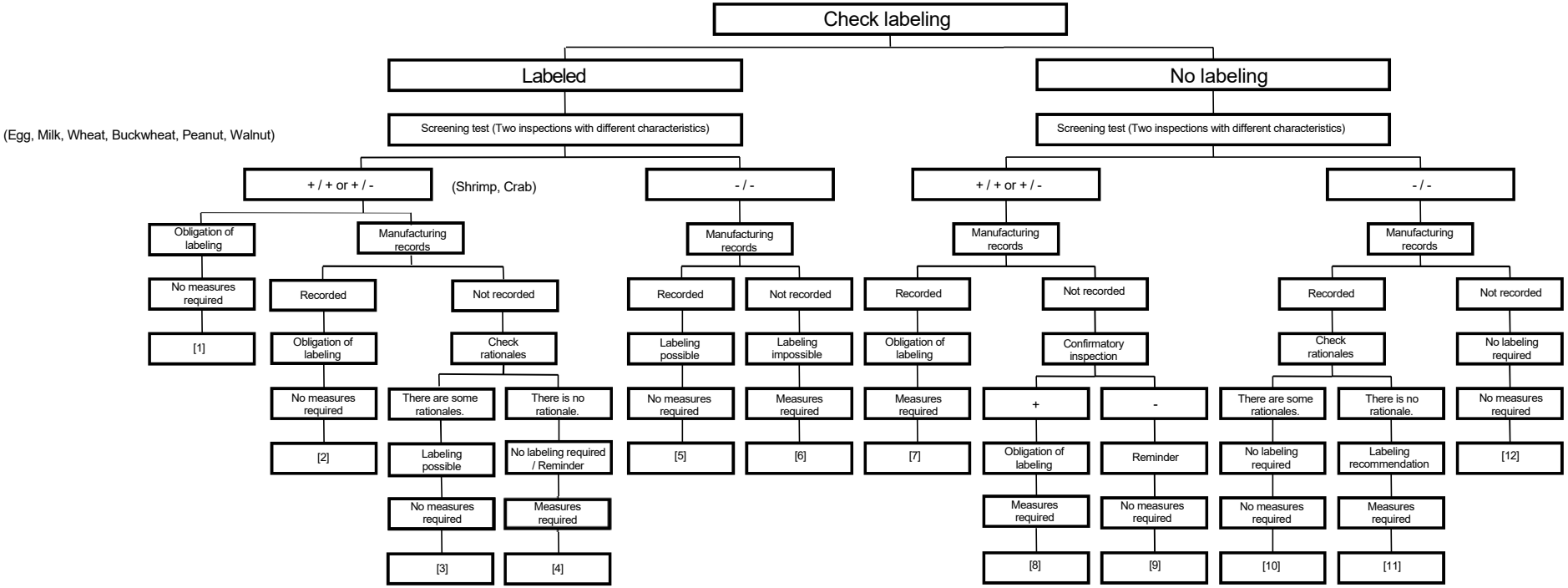
For the quantitative inspection methods for which the inspection method described in the “2.1 Quantitative Inspection Method” (hereinafter referred to as “Conventional Method”) is improved, if it should be evaluated according to the “Guideline for Evaluation of the Improved Inspection Methods for Foods Containing Allergens” (Appendix 5) and have shown having a performance equivalent to or higher than the Conventional Method, the Improved Inspection Method may be used as an inspection method for foods containing allergens in the same way as the Conventional Method.

3. Notes

The inspection of specified ingredients in foods shall be conducted in principle according to the “Decision Tree” in Appendix 1. Also, be sure to see “About Decision Tree” in Appendix 2 reliably.

The standard of reference material used in this inspection method are described in Appendix 3, and should be referred to when inspecting specified ingredients.

(Appendix 1)


Check labeling
Labeled
No labeling
Screening test (Two inspections with different characteristics)
Screening test (Two inspections with different characteristics)
(Egg, Milk, Wheat, Buckwheat, Peanut, Walnut)
+ / + or + / -
(Shrimp, Crab)
- / -
+ / + or + / -
- / -
Obligation of labeling
Manufacturing records
Manufacturing records
Manufacturing records
Manufacturing records
No measures required
Recorded
Not recorded
Recorded
Not recorded
Recorded
Not recorded
Recorded
Not recorded
[1]
Obligation of labeling
Check rationales
Labeling possible
Labeling impossible
Obligation of labeling
Confirmatory inspection
Check rationales
No labeling required
No measures required
There are some rationales.
There is no rationale.
No measures required
Measures required
Measures required
+
-
There are some rationales.
There is no rationale.
No measures required
[2]
Labeling possible
No labeling required / Reminder
[5]
[6]
[7]
Obligation of labeling
Reminder
No labeling required
Labeling recommendation
[12]
No measures required
Measures required
Measures required
No measures required
Measures required
[3]
[4]
[8]
[9]
[10]
[11]

(Appendix 2)

About Decision Tree

1 Basic Precautions

(1) The decision tree is structured with the purpose of avoiding as much as possible the hazard due to mislabeling of foods that may induce allergic symptoms, based on the current scientific knowledge, from the viewpoint of preventing health hazards.
(2) Monitoring of specified ingredients in foods is based on this decision tree in principle.
(3) Foods showing false-positive or false-negative are present in the inspection, and caution should be exercised in determining results. For all inspections, the results for false-positive or false-negative must be checked with reference to false-positive or false-negative information.
(4) In all inspections, the manufacturing records must be checked. (This check may be omitted only for branch [1] of the decision tree).

2 About Screening Test

(1) Screening tests are performed using quantitative inspection methods. Quantitative inspection methods other than ELISA assay may be used, but in such cases, the performance of the inspection methods should be equivalent to or greater than the performance of this assay.
(2) Screening tests should be performed using a combination of two inspections with different characteristics.
(3) A positive in screening test is defined as a case that a protein content from specified ingredient per 1g of the weight of foods collected is 10 μg or more[1].
(4) It should be noted that the screening tests in the monitoring of shrimp and crab do not distinguish between shrimp and crab.

3 Check of Manufacturing Record

(1) The term “Manufacturing Record” as used herein refers to ingredient and manufacturing manuals (including compounding tables), operating procedures, daily work reports, inspection results, Gantt chart (manufacturing schedule for each line), quality (component) warranty letter, product specification charts (including component information), and certificates that specified ingredients are not included.
(2) In the case that the ingredient is described in the manufacturing record but is not indicated in the label, the rationale must be confirmed. In the case that the ingredient is not described in the manufacturing record but is indicated in the label, the rationale must be confirmed.
(3) “Rationale” refers to estimates based on the inspection results or manufacturing records.
(4) If manufacturing record is unknown, it shall be treated in the same manner as “Not recorded”.

4 Confirmatory Inspection

(1) Confirmatory inspection is performed using a qualitative inspection method. Quantitative inspection methods other than Western blot, PCR, real-time PCR, nucleic acid chromatography may be used, but in these cases, the performance of the used methods should be equivalent to or greater than that of the specified methods.
(2) For confirmatory inspection for egg and milk, Western blot is generally commonly used. In this case, the antibody to be used is ovalbumin antibody and ovomucoid

antibody for egg, α-casein antibody and β-lactoglobulin antibody for milk.

(3) For confirmatory inspection for wheat, buckwheat, and peanut, PCR or real-time PCR is generally used. A positive in PCR is defined as a case that a specific gene amplified band is detected. A positive in real-time PCR is defined as a case that the calculated Cq is less than the mean Cq of reference plasmid solutions. For confirmatory inspection for shrimp and crab, PCR is generally used. A positive in PCR is defined as a case that a specific gene amplified band is detected. For confirmatory inspection for walnut, real-time PCR or nucleic acid chromatography is generally used. A positive in real-time PCR-H method is defined as a case that the calculated Cq is less than the mean Cq of reference plasmid solutions. A positive in real-time PCR-N method is defined as a case that the Cq is calculated. A positive in nucleic acid chromatography is defined as a case that a colored line is observed at the predefined position of the test strip.

(4) For confirmatory inspection, samples should be collected from the same prepared sample used in the screening tests. If second obtaining of the sample is not possible, obtain another sample for the same inspection to perform the inspection.

5 Measures to be taken when a violation is found

(1) Until labeling related to foods containing specified ingredients is corrected (or on the decision tree [11], until the rationale for “No labeling” is described in the manufacturing record), guidance should be given to prevent the sale of such foods.

(2) In addition, measures under Article 59 or 60 of the Food Sanitation Act shall be considered as appropriate.

6 Thinking on branches [1] to [12]

(Only monitoring egg, milk, wheat, buckwheat, peanut, and walnut)

[1]	Case that the specified ingredients (egg, milk, wheat, buckwheat, peanut, and walnut) are indicated on the label and at least one of the screening test results with the two inspection types is “+ (positive)”

- Even in this case, it is desirable to check the manufacturing records, and this decision tree does not inhibit this check, but omitting the check is possible.
- No confirmatory inspections are required.
- It is believed that this is adequate labeling, and no administrative measures are required.

(Only monitoring shrimp and crab)

[2]	Case that the specified ingredients (shrimp, crab) are indicated on the label, at least one of the screening test results with the two inspection types is “+ (positive)”, and the specified ingredients are described in manufacturing records

- Confirmation of manufacturing records is required.
- No confirmatory inspections are required.
- It is believed that this is adequate labeling, and no administrative measures are required.
- It should be noted that the screening tests in the monitoring of shrimp and crab do not distinguish between shrimp and crab, and that some crustaceans other than shrimp and crab are also detected.

(Only monitoring shrimp and crab)

[3]	Case that the specified ingredients (shrimp, crab) are indicated on the label, at least one of the screening test results with the two inspection types is “+ (positive)”, the specified ingredients are not described in manufacturing records, and there is a rationale of labeling

- Confirmation of manufacturing records is required.
- Confirmation of rationale that the ingredients are not described in the manufacturing record but are indicated on the label is required.
- No confirmatory inspections are required.
- Labeling is feasible and no administrative measures are required.
- If there is a rationale that the ingredients are not described in the manufacturing record but are indicated on the label, guidance should be given to describe the rationale in the manufacturing record.
- It should be noted that the screening tests in the monitoring of shrimp and crab do not distinguish between shrimp and crab, and that some crustaceans other than shrimp and crab are also detected.

(Only monitoring shrimp and crab)

[4]	Case that the specified ingredients (shrimp, crab) are indicated on the label, at least one of the screening test results with the two inspection types is “+ (positive)”, the specified ingredients are not described in manufacturing records, and there is no rationale of labeling

- Confirmation of manufacturing records is required.
- Describing the precaution in the margin of ingredients section is feasible.
- It should be noted that the screening tests in the monitoring of shrimp and crab do not distinguish between shrimp and crab, and that some crustaceans other than shrimp and crab are also detected.
- If necessary, perform confirmatory inspections.

[5]	Case that the specified ingredients are indicated on the label, both of screening test results with the two inspection types are “- (negative)”, and the specified ingredients are described in manufacturing records

- Confirmation of manufacturing records is required.
- No confirmatory inspections are required.
- Labeling is feasible and no administrative measures are required.
- When the total protein content of specified ingredients, etc. in foods is less than several μg/ml (concentration) or several μg/g (contained amount), labeling is not required, but the decision to indicate or not indicate it is made by the manufacturer or the seller.
- Note that the “- (negative)” of the screening test result does not mean that the total protein content of the specified ingredient is 0 (zero).

[6]	Case that the specified ingredients are indicated on the label, both of screening test results with the two inspection types are “- (negative)”, and the specified ingredients are not described in manufacturing records

- Confirmation of manufacturing records is required.
- No confirmatory inspections are required.
- The specified ingredients should not be indicated on the label and the labels should be corrected.
- If there is a rationale that the ingredients are not described in the manufacturing record but are indicated on the label, guidance should be given to describe the rationale in the manufacturing record.

[7]	Case that the specified ingredients are not indicated on the label, at least one of the screening test results with the two inspection types is “+ (positive)”, and the specified ingredients are described in manufacturing records

- Confirmation of manufacturing records is required.
- No confirmatory inspections are required.
- Labeling is required and the labels should be corrected.
- It should be noted that the screening tests in the monitoring of shrimp and crab do not distinguish between shrimp and crab, and that some crustaceans other than shrimp and crab are also detected.

[8]	Case that the specified ingredients are not indicated on the label, at least one of the screening test results with the two inspection types is “+ (positive)”, the specified ingredients are not described in manufacturing records, and the confirmatory inspection results are “+ (positive)”

- Confirmation of manufacturing records is required.
- Confirmatory inspections are required.
- The confirmatory inspection determined that the screening test results are not false-positive, so labeling is required and the labels should be corrected.
- However, in cases that contamination due to materials that are not usually treated as an ingredient is considered (e.g. “boiled water when wheat noodles are boiled with hot water which is used for boiling buckwheat noodles” or “fried oil used for frying foods such as tempura or cutlet” etc.), precautions notes in the margin area are desirable.
- It should be noted that the screening tests in the monitoring of shrimp and crab do not distinguish between shrimp and crab, and that some crustaceans other than shrimp and crab are also detected.

[9]	Case that the specified ingredients are not indicated on the label, at least one of the screening test results with the two inspection types is “+ (positive)”, the specified ingredients are not described in manufacturing records, and the confirmatory inspection results are “- (negative)”

- Confirmation of manufacturing records is required.
- Confirmatory inspections are required.
- Since the confirmatory inspection did not determine that the screening test results are not false-positive, prevent the labels from being corrected.
- However, it should be noted that the “+ (positive)” as a screening test result is not denied completely by “- (negative)” as a confirmatory inspection result.
- Describing the precaution in the margin of ingredients section is feasible.

- It should be noted that the screening tests in the monitoring of shrimp and crab do not distinguish between shrimp and crab, and that some crustaceans other than shrimp and crab are also detected.

[10]	Case that the specified ingredients are not indicated on the label, both of the screening test results with the two inspection types are "- (negative)", the specified ingredients are described in manufacturing records, and there is a rationale that the specified ingredients are not indicated on the label

- Confirmation of manufacturing records is required.
- No confirmatory inspections are required.
- Confirmation of rationale that the ingredients are described in the manufacturing record but are not indicated on the label is required.
- There is no mandatory label for the ingredients, and the labeling is adequate.

[11]	Case that the specified ingredients are not indicated on the label, both of the screening test results with the two inspection types are "- (negative)", the specified ingredients are described in manufacturing records, and there is no rationale that the specified ingredients are not indicated on the label

- Confirmation of manufacturing records is required.
- No confirmatory inspections are required.
- Confirmation of rationale that the ingredients are described in the manufacturing record but are not indicated on the label is required.
- It is desirable to indicate. Because both screening test results are "- (negative)", correcting the label is not necessary but indicating of the ingredients is recommend.
- However, if there is a rationale that the specified ingredients are described in the manufacturing record but are not indicated on the label, guidance should be given to describe the rationale in the manufacturing record. In addition, even if the results of screening tests are used as a rationale for not labeling, the voluntary inspection results are accepted as a rationale, but the results of administrative inspection are not accepted as a rationale for not labeling.

[12]	Case that the specified ingredients are not indicated on the label, both of the screening test results with the two inspection types are "- (negative)", the specified ingredients are not described in manufacturing records

- Confirmation of manufacturing records is required.
- No confirmatory inspections are required.
- Considering it as an appropriate labeling, there is no problem even if there is no indicating of the ingredients.

7 The contents in this section are based on the conclusion in the interim report of the "Allergy Labeling Review Committee of Study Group on the Social Impact of Food Labeling, Countermeasures for the Impact and Its International Comparison" by using the Health Labour Sciences Research Grant dated October 29, 2001 that "The labeling is considered necessary for foods that contain protein, such as specified ingredients, when its total amount of contained protein is not lower than several μg/ml (concentration) or several μg/g (contained amount)".

(Appendix 3)

Reference Material Standard

1. Standard Reference Solution for Detecting Egg

1.1. Preparation Method

Prepare egg primary reference powder, undiluted egg reference solution, diluted egg primary solution, and high concentration egg reference solution according to the following methods. The process from preparing the undiluted egg reference solution up to preparing high concentration egg reference solution should be performed within one day.

Preparation of Egg Primary Reference Powder

Remove eggshells from 1kg of fresh egg of white Leghorn species (laying hens) and perform homogenization uniformly, then freeze-dry it. Then finely grind freeze-dried eggs to make egg primary reference powder.

Preparation of Undiluted Egg Reference Solution

Collect 0.2 g of egg primary reference powder in 50 mL PP tube, add 20 mL of buffer solution for extraction*, mix by thorough shaking, disperse the solid, and extract with a shaking apparatus (90 to 110 rpm) all night. Centrifuge the extract solution at 10,000 × g for 30 minutes, filter the supernatant through a micro-filter with a 0.8 μm pore size to make undiluted egg reference solution.

For extraction, place the centrifuge tube sideways on the shaking apparatus, and set the shaking amplitude to about 3 cm, and set the number of shakes so that the solution hits both ends by shaking. Sometimes turn over the tube to disperse the sample adhering around the surface of the liquid.

* Buffer Solution for Extraction: PBS (pH 7.4) containing 0.6 % SDS and 0.1 M sodium sulfite.

Preparation of Diluted Egg Primary Solution

Dilute the undiluted egg reference solution by a factor of 10 with PBS (pH 7.4) to make diluted egg primary solution.

Preparation Method of High Concentration Egg Reference Solution

Dilute the diluted egg primary solution by a factor of 2 with PBS (pH 7.4) containing 0.2 % BSA to make high concentration egg reference solution. The procedure of preparing the undiluted egg reference solution to make high concentration egg reference solution should be performed within one day.

1.2. Standard

Standard of Undiluted Egg Reference Solution

Electrophoresis Image

When subjected to electrophoresis with SDS-PAGE, distinct bands appear around 200, 130, 75, 40 kDa.

Protein Content

When the protein is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is between 4.1 and 6.2 mg/mL.

Reference Information The following values are for reference.

When the protein of diluted egg primary solution is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is 0.08 to 0.12 times that of undiluted egg reference solution. When SDS-PAGE is performed on the undiluted egg reference solution, the electrophoretic image as shown in 8. is provided.

2. Reference Solution for Detecting Milk

2.1. Preparation Method

Prepare milk primary reference powder, undiluted milk reference solution, diluted milk primary solution, and high concentration milk reference solution according to the following methods. The process from preparing the undiluted milk reference solution up to preparing high concentration milk reference solution should be performed within one day.

Preparation of Milk Primary Reference Powder

Stir 1L of fresh milk of Holstein (dairy cow) while cooling it with ice, and filter the mass of milk fat produced by the coagulation of milk fat through absorbent cotton. Repeat this procedure three times to remove fat, and then freeze-dry the filtrate, and finely grind the dried product to make milk primary reference powder.

Preparation of Undiluted Milk Reference Solution

Collect 0.2 g of milk primary reference powder in 50 mL PP tube, add 20 mL of buffer solution for extraction*, mix by thorough shaking, disperse the solid, and extract with a shaking apparatus (90 to 110 rpm) all night. Centrifuge the extract solution at 10,000 × g for 30 minutes, filter the supernatant through a micro-filter with a 0.8 μm pore size to make undiluted milk reference solution.

For extraction, place the centrifuge tube sideways on the shaking apparatus. Set the shaking amplitude to about 3 cm, and set the number of shakes so that the solution hits both ends by shaking. Sometimes turn over the tube to disperse the sample adhering around the surface of the liquid.

* Buffer Solution for Extraction: PBS (pH 7.4) containing 0.6 % SDS and 0.1 M sodium sulfite.

Preparation of Diluted Milk Primary Solution

Dilute the undiluted milk reference solution by a factor of 10 with PBS (pH 7.4) to make diluted milk primary solution.

Preparation of High Concentration Milk Reference Solution

Dilute the diluted milk primary solution by a factor of 2 with PBS (pH 7.4) containing 0.2 % BSA to make high concentration milk reference solution. The process from preparing the undiluted milk reference solution up to preparing high concentration milk reference solution should be performed within one day.

2.2. Standard

Standard of Undiluted Milk Reference Solution

Electrophoresis Image

When subjected to electrophoresis with SDS-PAGE, three distinct bands appear in ranging from 40 to 25 kDa and one distinct band appears around 16 kDa.

Protein Content

When the protein is quantified by 2-D Quant kit (Global Life Sciences Technologies

Japan K.K.), the protein concentration is between 2.1 and 3.2 mg/mL.

Reference Information The following values are for reference.

When the protein of diluted milk primary solution is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is 0.08 to 0.12 times that of undiluted milk reference solution. When SDS-PAGE is performed on the undiluted milk reference solution, the electrophoretic image as shown in 8. is provided.

3. Reference Solution for Detecting Wheat

3.1. Preparation Method

Prepare wheat primary reference powder, undiluted wheat reference solution, diluted wheat primary solution, and high concentration wheat reference solution according to the following methods. The process from preparing the undiluted wheat reference solution up to preparing high concentration wheat reference solution should be performed within one day.

Preparation of Wheat Primary Reference Powder

Grind the mixture of 14 brands of wheat described below. The ground wheat mixture that passes through a 14-mesh sieve (aperture = 1.18 mm) is considered as wheat primary reference powder.

Brands Contained in the Mixture

No.1 Canada Western Red Spring	7.14	%	
US No.2 or better (Dark) Northen Spring	7.14	%	
US Hard Red Winter - High Protein	7.14	%	
US Hard Red Winter - Semi Hard	7.14	%	
Canada Western Amber Durum - Triticum durum	7.14	%	
US Western White (White Club + Soft White)	7.14	%	(Club 1.6 %)
Australian Premium White for Japan	7.14	%	
Australian Prime Hard	7.14	%	
Hokushin	7.14	%	
Haruyutaka	7.14	%	
Norin No.61	7.14	%	
Chikugo Izumi	7.14	%	
Bando-wase	7.14	%	
Shirogane	7.14	%	

Preparation of Undiluted Wheat Reference Solution

Collect 1 g of wheat primary reference powder in 50 mL PP tube, add 20 mL of buffer solution for extraction*, mix by thorough shaking, disperse the solid, and extract with a shaking apparatus (90 to 110 rpm) all night. Centrifuge the extract solution at 10,000 × g for 30 minutes, filter the supernatant through a micro-filter with a 0.8 μm pore size to make undiluted wheat reference solution.

For extraction, place the centrifuge tube sideways on the shaking apparatus. Set the shaking amplitude to about 3 cm, and set the number of shakes so that the solution hits both ends by shaking. Sometimes turn over the tube to disperse the sample adhering around the surface of the liquid.

* Buffer Solution for Extraction: 0.1 M Tris-HCl (pH 8.6) containing 0.6 % SDS and 0.1 M

sodium sulfite

Preparation of Diluted Wheat Primary Solution

Dilute the undiluted wheat reference solution by a factor of 10 with PBS (pH 7.4) to make diluted wheat primary solution.

Preparation of High Concentration Wheat Reference Solution

Dilute the diluted wheat primary solution by a factor of 2 with PBS (pH 7.4) containing 0.2 % BSA to make high concentration wheat reference solution. The procedure of preparing the undiluted wheat reference solution and diluted wheat primary solution to make high concentration wheat reference solution should be performed within one day.

3.2. Standard

Standard of Undiluted Wheat Reference Solution

Electrophoresis Image

When subjected to electrophoresis with SDS-PAGE, four or more distinct bands appear in ranging from 32 to 120 kDa.

Protein Content

When the protein is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is between 4.0 and 6.0 mg/mL.

Reference Information The following values are for reference.

When the protein of diluted wheat primary solution is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is 0.08 to 0.12 times that of undiluted wheat reference solution. When SDS-PAGE is performed on the undiluted wheat reference solution, the electrophoretic image as shown in 8. is provided.

4. Reference Solution for Detecting Buckwheat

4.1. Preparation Method

Prepare buckwheat primary reference powder, undiluted buckwheat reference solution, diluted buckwheat primary solution, and high concentration buckwheat reference solution according to the following methods. The process from preparing the undiluted buckwheat reference solution up to preparing high concentration buckwheat reference solution should be performed within one day.

Preparation of Buckwheat Primary Reference Powder

Mix buckwheat produced in Ibaraki Prefecture and China (northern China) in equal amount and grind the mixture. The ground buckwheat mixture that passes through a 14-mesh sieve (aperture = 1.18 mm) is considered as buckwheat primary reference powder.

Preparation of Undiluted Buckwheat Reference Solution

Collect 1 g of buckwheat primary reference powder in 50 mL PP tube, add 20 mL of buffer solution for extraction*, mix by thorough shaking, disperse the solid, and extract with a shaking apparatus (90 to 110 rpm) all night. Centrifuge the extract solution at 10,000 × g for 30 minutes, filter the supernatant through a micro-filter with a 0.8 μm pore size to make undiluted buckwheat reference solution.

For extraction, place the centrifuge tube sideways on the shaking apparatus. Set the shaking amplitude to about 3 cm, and set the number of shakes so that the solution hits

both ends by shaking. Sometimes turn over the tube to disperse the sample adhering around the surface of the liquid.

* Buffer Solution for Extraction: 20 mM Tris-HCl (pH 7.5) containing 0.6 % SDS, 0.1 M sodium sulfite and 0.5 M sodium chloride

Preparation of Diluted Buckwheat Primary Solution

Dilute the undiluted buckwheat reference solution by a factor of 10 with PBS (pH 7.4) to make diluted buckwheat primary solution.

Preparation of High Concentration Buckwheat Reference Solution

Dilute the diluted buckwheat primary solution by a factor of 2 with PBS (pH 7.4) containing 0.2 % BSA to make high concentration buckwheat reference solution. The process from preparing the undiluted buckwheat reference solution up to preparing high concentration buckwheat reference solution should be performed within one day.

4.2. Standard

Standard of Undiluted Buckwheat Reference Solution

Electrophoresis Image

When subjected to electrophoresis with SDS-PAGE, one distinct band appears around 22 kDa and four or more distinct bands appear in ranging from 32 to 83 kDa.

Protein Content

When the protein is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is between 2.7 and 4.0 mg/mL.

Reference Information The following values are for reference.

When the protein of diluted buckwheat primary solution is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is 0.08 to 0.12 times that of undiluted buckwheat reference solution. When SDS-PAGE is performed on the undiluted buckwheat reference solution, the electrophoretic image as shown in 8. is provided.

5. Reference Solution for Detecting Peanut

5.1. Preparation Method

Prepare peanut primary reference powder, undiluted peanut reference solution, diluted peanut primary solution, and high concentration peanut reference solution according to the following methods. The process from preparing the undiluted peanut reference solution up to preparing high concentration peanut reference solution should be performed within one day.

Preparation of Peanut Primary Reference Powder

Pulverize Virginia peanuts produced in Chiba Prefecture in a mortar to make a paste-like peanut. Collect 1 g of paste-like peanut in 50 mL PP tube, add 10 mL of acetone, stir the solution for 1 minute using a vortex mixer, and then centrifuge it at 10,000×g for 30 minutes to remove the supernatant. Repeat this procedure three times. Place the tube on an aluminum bath at 45 °C, dry the tube for about 7 hours to make peanut primary reference powder.

Preparation of Undiluted Peanut Reference Solution

Add 20 mL of buffer solution for extraction* to 0.4 g of peanut primary reference powder, mix by thorough shaking, disperse the solid, and extract with a shaking apparatus (90 to 110 rpm) all night. Centrifuge the extract solution at 10,000 × g for 30 minutes, filter the supernatant through a micro-filter with a 0.8 μm pore size to make undiluted peanut reference solution.

For extraction, place the centrifuge tube sideways on the shaking apparatus. Set the shaking amplitude to about 3 cm, and set the number of shakes so that the solution hits both ends by shaking. Sometimes turn over the tube to disperse the sample adhering around the surface of the liquid.

* Buffer Solution for Extraction: 20 mM Tris-HCl (pH 7.5) containing 0.6 % SDS, 0.1 M sodium sulfite and 0.5 M sodium chloride

Preparation of Diluted Peanut Primary Solution

Dilute the undiluted peanut reference solution by a factor of 10 with PBS (pH 7.4) to make diluted peanut primary solution.

Preparation of High Concentration Peanut Reference Solution

Dilute the diluted peanut primary solution by a factor of 2 with PBS (pH 7.4) containing 0.2 % BSA to make high concentration peanut reference solution. The process from preparing the undiluted peanut reference solution up to preparing high concentration peanut reference solution should be performed within one day.

5.2. Standard

Standard of Undiluted Peanut Reference

Electrophoresis Image

When subjected to electrophoresis with SDS-PAGE, one distinct band appears around 70 kDa and three or four distinct bands appear in ranging from 15 to 30 kDa.

Protein Content

When the protein is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is between 3.2 and 4.8 mg/mL.

Reference Information The following values are for reference.

When the protein of diluted peanut primary solution is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is 0.08 to 0.12 times more than that of undiluted peanut reference solution. When SDS-PAGE is performed on the undiluted peanut reference solution, the electrophoretic image as shown in 8. is provided.

6. Reference Solution for Detecting Crustacean*

* The name of this reference solution is the reference solution for detecting crustacean because shrimp and crab are detected without distinguishing them in screening with ELISA kit.

6.1. Preparation Method

Prepare crustacean primary reference powder, undiluted crustacean reference solution, diluted crustacean primary solution, and high concentration crustacean reference solution according to the following methods. The process from preparing the undiluted crustacean reference solution up to preparing concentration crustacean reference solution should be

performed within one day.

Preparation of Crustacean Primary Reference Powder

Collect tail muscles of giant tiger prawn (black tiger shrimp) (cultured shrimp) and freeze-dry the muscles after homogenization uniformly with ice cooling. Finely grind the dried product to make crustacean primary reference powder.

Preparation of Undiluted Crustacean Reference Solution

Collect 0.1 g of crustacean primary reference powder in 50 mL PP tube, add 20 mL of buffer solution for extraction*, mix by thorough shaking, disperse the solid, and extract with a shaking apparatus (90 to 110 rpm) all night. Centrifuge the extract solution at 10,000 × g for 30 minutes, filter the supernatant through a micro-filter with a 0.8 μm pore size. Heat the filtered solution at 100 °C for 10 minutes to make undiluted crustacean reference solution.

For extraction, place the centrifuge tube sideways on the shaking apparatus. Set the shaking amplitude to about 3 cm, and set the number of shakes so that the solution hits both ends by shaking. Sometimes turn over the tube to disperse the sample adhering around the surface of the liquid.

* Buffer Solution for Extraction: PBS (pH 7.4) containing 0.6 % SDS, 0.1 M sodium sulfite, 1% Inhibitor Cocktail and 5mM EDTA (Halt Protease Inhibitor Cocktail Kit, Thermo Fisher Scientific Inc.)

Preparation of Diluted Crustacean Primary Solution

Dilute the undiluted crustacean reference solution by a factor of 10 with PBS (pH 7.4) to make diluted crustacean primary solution.

Preparation of High Concentration Crustacean Reference Solution

Dilute the diluted crustacean primary solution by a factor of 2 with PBS (pH 7.4) containing 0.2 % BSA to make high concentration crustacean reference solution. The process from preparing the undiluted crustacean reference solution up to preparing high concentration crustacean reference solution should be performed within one day.

6.2. Standard

Standard of Undiluted Crustacean Reference Solution

Electrophoresis Image

When subjected to electrophoresis with SDS-PAGE, one distinct band appears around 160 kDa, 41 kDa and 37 kDa respectively, and four distinct bands appear in ranging from 20 to 16 kDa.

Protein Content

When the protein is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is between 2.7 and 4.1 mg/mL.

Reference Information The following values are for reference.

When the protein of diluted crustacean primary solution is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is 0.08 to 0.12 times more than that of undiluted crustacean reference solution. When SDS-PAGE is performed on the undiluted crustacean reference solution, the electrophoretic image as shown in 8. is provided.

7. Reference Solution for Detecting Walnut

7.1. Preparation Method

Prepare walnut primary reference powder, undiluted walnut reference solution, diluted walnut primary solution, and high concentration walnut reference solution according to the following methods. The process from preparing the undiluted walnut reference solution up to preparing high concentration walnut reference solution should be performed within one day.

Preparation of Walnut Primary Reference Powder

Pulverize Chandler walnuts in a mortar, collect 3 g of the pulverized walnuts in 50 mL PP tube, add 30 mL of acetone, stir the solution for 1 minute using a vortex mixer, and then centrifuge it at 10,000×g for 30 minutes to remove the supernatant. Repeat this procedure three times. Place the tube on an aluminum bath at 45 °C, dry the tube for about 7 hours. The dried walnut that passes through a sieve (aperture = 500 μm) is considered as walnut primary reference powder.

Preparation of Undiluted Walnut Reference Solution

Collect 0.1 g of walnut primary reference powder in 50 mL PP tube, add 20 mL of buffer solution for extraction*, stir the solution for 1 minute using a vortex mixer to disperse the solid, and then extract with a shaking apparatus (90 to 110 rpm) all night. Centrifuge the extract solution at 10,000 × g for 30 minutes, filter the supernatant through a micro-filter with a 0.8 μm pore size to make undiluted walnut reference solution.

For extraction, place the centrifuge tube sideways on the shaking apparatus. Set the shaking amplitude to about3 cm, and set the number of shakes so that the solution hits both ends by shaking. Sometimes turn over the tube to disperse the sample adhering around the surface of the liquid.

* Buffer Solution for Extraction: 120 mM Tris-HCl (pH 7.5) containing 0.6 % SDS, 0.1 M sodium sulfite and 0.05% Tween 20

Preparation of Diluted Walnut Primary Solution

Dilute the undiluted walnut reference solution by a factor of 10 with PBS (pH 7.4) containing 0.6 % SDS, 0.1 M sodium sulfite to make diluted walnut primary solution.

Preparation of High Concentration Walnut Reference Solution

Dilute the diluted walnut primary solution by a factor of 2 with PBS (pH 7.4) containing 0.6 % SDS, 0.1 M sodium sulfite and 0.2 % BSA to make high concentration walnut reference solution. The process from preparing the undiluted walnut reference solution up to preparing high concentration walnut reference solution should be performed within one day.

7.2. Standard

Standard of Undiluted Walnut Reference Solution

Electrophoresis Image

When subjected to electrophoresis with SDS-PAGE, one distinct band appears around 10 Da, and two or more distinct bands appear ranging from 23 to 18 kDa and from 35 to 30 kDa respectively.

Protein Content

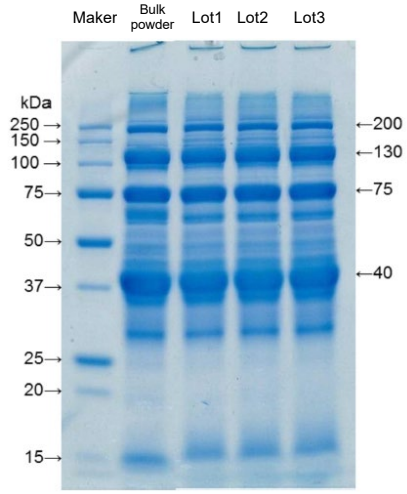
When the protein is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is between 1.9 and 2.9mg/mL.

Reference Information The following values are for reference.

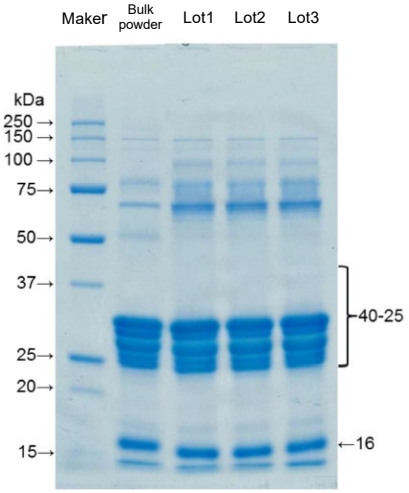
When the protein of diluted walnut primary solution is quantified by 2-D Quant kit (Global Life Sciences Technologies Japan K.K.), the protein concentration is 0.08 to 0.12 times more than that of undiluted walnut reference solution. When SDS-PAGE is performed on the undiluted walnut reference solution, the electrophoretic image as shown in 8. is provided.

8. Electrophoresis Image with SDS-PAGE in Each Undiluted Walnut Reference Solution

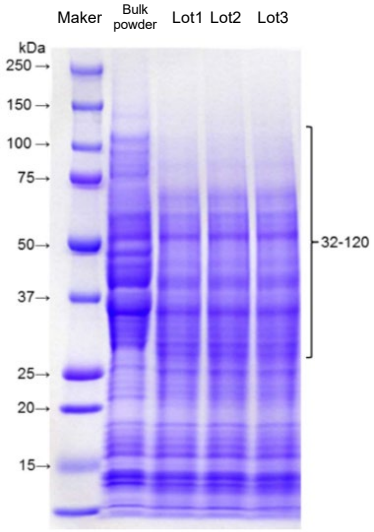
Egg



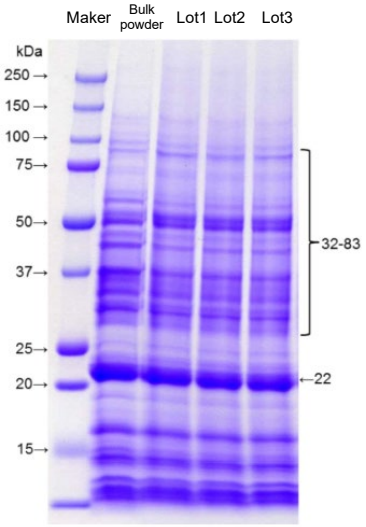
Milk



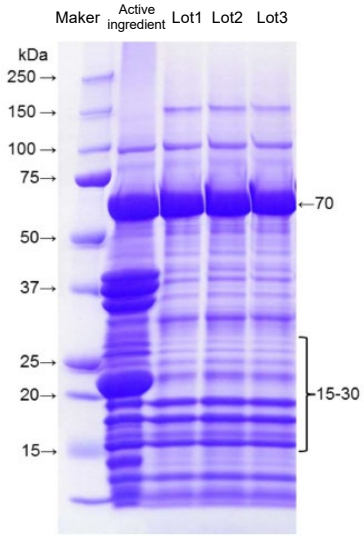
Wheat



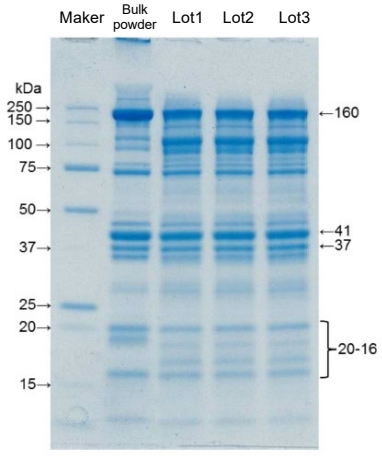
Buckwheat



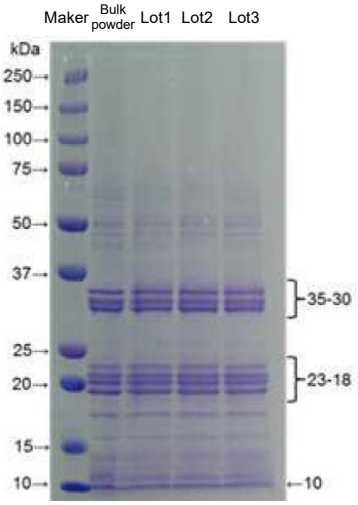
Peanut



Crustacean



Walnut



Bulk powder: Primary Reference Powders of Egg, Milk, Wheat, Buckwheat, Peanut, Crustacean, Walnut
Lot 1-3: Lot Number

(Appendix 4)

Guideline for Evaluating Inspection Methods for Foods Containing Allergens

Introduction

In recent years, there has been an increase in allergies caused by foods and allergies often cause severe symptoms. This led to the creation of labeling system for foods containing allergenic substances (allergens) in April 2001.

To verify that this labelling system is properly practiced, the inspection methods for foods containing specified ingredients are needed. In November 2002, the “Inspection Methods for Foods Containing Allergenic Substances” was notified, and the inspection methods for five specified ingredients were established. Additional inspection methods were notified in November 2005. However, with improvement of technology and discovery of new allergens through subsequent research, it is necessary to always review the inspection methods and strive for appropriate consumer protection. Evaluation of inspection techniques should also be performed to avoid health harm from inappropriate inspection methods. Validation of analytical methods has been established in many fields as an evaluation method for inspection techniques, but the characteristics of inspection for allergen in foods indicate that the evaluation method of conventional analytical methods alone is difficult to evaluate appropriately. Therefore, it has been decided to develop guidelines and determine the evaluation method of appropriate inspection methods for the verification of allergy labeling. In this guideline, the following guidance are provided: Evaluation method of the inspection methods of the allergic foods, characteristics required for the inspection methods for the verification of the labeling system, reliability assurance to be carried out by the inspection method practitioner.

1. Inspection Methods for Specified Ingredients in Foods

1.1. Quantitative Inspection Method (ELISA Assay)

In this method, antigen level in the sample is quantified based on the amount of antigen bound to the antibody which are produced by immunized with antigen in an animal. There are two types of currently developed methods; using antibodies that react with multiple proteins and using antibody that react with particular protein in foods of interest. In addition, in the latter, method using either polyclonal antibody or monoclonal antibody is possible. Selection of such antibodies alters selectivity, cross-reactivity, detection limit, applicability to foods, etc. Antibodies with high affinity for a particular protein may improve specificity, but the protein may become undetectable if the protein is modified by foods processing. In addition, when only a portion of the ingredient is used and the portion contains no protein of interest, false negatives are increased due to undetectability. On the other hand, using antibodies that may bind to multiple proteins can avoid such problems, but they bind more to proteins derived from foods other than the foods of interest, increasing the probability of a false-positive result.

1.2. Qualitative Inspection Method (Western Blot, PCR)

In Western blot, proteins are separated by electrophoresis and then detected by antigen-antibody reaction. This method has higher specificity and is less likely to produce false positives than ELISA assay, because it uses antibodies directed against a particular protein and also provides information about the molecular weight on the basis of the band location. In the current notification, this method is positioned as a confirmatory inspection for egg and milk due to such property. Because the bands are visually confirmed in Western blot, it is not a quantitative inspection method, and validation as a qualitative

inspection method is necessary.

PCR is the method that amplifies DNA regions peculiar to the foods which show the antigenicity by PCR and detects the regions. If the appropriate region is set, the method has high specificity. Therefore, in the current notification, it is considered as a confirmatory inspection method of wheat, buckwheat and peanut. On the other hand, in chicken meat and egg, their DNA is identical and difficult to distinguish by PCR.

Based on the above characteristics, the current inspection method for foods containing allergens uses the quantitative inspection method as a screening test and the qualitative inspection method for confirmation.

2. Evaluation of Inspection Method

2.1. Standard for Evaluating Quantitative Inspection Method

The performance parameters that serve as standard for the evaluation of quantitative inspection method are provided in Codex or the Japanese Pharmacopoeia, etc. While the performance parameters are defined slightly differently in ISO, Codex, and the Japanese Pharmacopoeia, etc., but performance is evaluated using quantities as described in Table 1. The appropriate parameters should be selected and evaluated according to the intended use of the inspection method to be evaluated. In general, the accuracy (recovery rate) and precision (repeatability/intermediate precision) must be checked for any purpose of inspection method. Quantitation limit and detection limit are important in the inspection of the residue level, and when the expected concentration of the object substance changes greatly, the scope in which the inspection can be applied becomes an important parameter.

These parameters are determined by validation. In many cases, the reliability of the obtained parameters varies depending on the number of body participating in the validation and the number of samples used, etc., since these are statistically estimated by repeated test based on the Design of Experiments.

Table1. Performance Parameters

Accuracy	Precision (Repeatability, Intermediate Precision, Reproducibility)
Specificity	Limit of Detection
Linearity	Quantitation Limit
Range	Robustness

2.2. Standard for Evaluating Qualitative Inspection Method

The qualitative inspection method does not give a numerical result, unlike with the quantitative inspection method. Therefore, the parameters of the quantitative inspection method cannot be applied as they are. Concepts that combine accuracy and precision include the correct response rate, false positive rate, and false negative rate. Moreover, because the decision becomes inaccurate if the concentration is low, the limit concentration for which correct judgement is available is also an important performance parameter.

2.3. Inter-laboratory Validation

In inter-laboratory validation, a large number of laboratories analyze common samples and statistically analyze the results to validate accuracy, repeatability and reproducibility. Codex also employs methods that have been validated in inter-laboratory validation and

published. OMA (Official method of analysis) of AOAC INTERNATIONAL is an analytical method evaluated in inter-laboratory validation. In AOAC, inter-laboratory validation is called as collaborative study and its protocols are defined. Similar protocols are provided in ISO5725(JIS Z8402).

Accuracy (recovery rate), repeatability, and reproducibility are validated in the collaborative study. Robustness is also guaranteed as the collaborative study is performed in a number of laboratories. The requirements for the collaborative study in quantitative inspection method are as follows:

Five samples, eight laboratories, number of repeats: one or two

Prior to the collaborative study, the following performances including robustness are evaluated in one laboratory:

- Calibration curve: Determine the concentration range in which the analytical method can be used. It is not necessary to be linear.
- Specificity: Degree of inhibition of a substance which is expected to exist.
- Bias (accuracy): Estimate the systematic error from the spike and recovery test.
- Determine instrument performance and stability of analytical system.
- Precision: Repeatability, Intermediate Precision, Robustness.
- Comparison with existing methods.

Perform the collaborative study only if the in-laboratory performance validation is acceptable.

2.4. Peer Review

A method for confirming the performance by a third party body after performance evaluation by developers is called as peer review. Unlike inter-laboratory validation, reproducibility is not required. For peer review, the following analytical performance should be evaluated in advance:

- Calibration Curve

  Calibration curves in minimum five concentrations are required (not including case that concentration is 0) for the quantitative inspection method. It is not necessary to be linear. Describe both in standard solution and matrix.

  In the qualitative inspection method, confirm the qualitative range with samples containing negative controls. For each concentration, repeat the evaluation from five to ten times. Plot the positive rates for each concentration.
- Applicable Matrices

  Describe applicable matrices explicitly.
- Accuracy

  The recovery rates from samples with an appropriate range of concentrations are used as an indicator of accuracy in the quantitative inspection method. Describe recovery rates at three concentrations for the six samples, respectively.

  Compare the peer review with the existing methods in the qualitative inspection method.
- Precision

  Provide RSD between different days, between analysts, between calibration curves, between reagents, and between matrices in the quantitative inspection method. The precision is expressed as correct response rate, false positive rate, and false negative rate at several concentrations in the qualitative inspection method.
- Comparison with existing methods

  If comparable, comparison with existing method (preferably validated methods) is strongly recommended.

- Cross-reactivity
  Reactivity to similar materials, metabolites and components that may be present in the matrices.
- Stability
  Evaluate the robustness of each part of the kit in terms of time, temperature, freezing and thawing cycles.
- Limit of Detection
  The mean value + 3σ of the matrix blank is converted to the concentration of analyte in the quantitative inspection method.
- Limit of Quantification
  For each matrix, at least six added samples are actually analyzed to determine the limit of quantification.
- False-positive and False-negative Rates
  They are applied in the qualitative inspection method.
- Robustness
  Check of the degree of variability of the test system due to slight changes that may occur in the test environment.

2.5. Validation in a Single Laboratory (Single Laboratory Validation)

Confirm the feasibility of the analytical method prior to inter-laboratory inspection. Provide evidence for the reliability of analytical method when no collaborative data are available or when the implementation of a formal collaborative trial is not realistic. Validation in a single laboratory is performed for the purpose of ensuring that the previously validated method is used correctly. For this validation, IUPAC technical report provides the guideline for harmonizing. The following items are recommended in the guideline:

- Analytical methods that have been evaluated for performance in collaborative trials adapted to international protocols are used, where possible and realistic.
- In the absence of such an analytical method, the analytical method is validated in the laboratory prior to the provision of analytical data to the client.
- In single-laboratory validation, select and evaluate the appropriate performance among: applicability, specificity, accuracy, precision, scope, quantitation limit, detection limit, sensitivity and robustness. The choice of performance is determined by considering the client's demands.
- Evidence for which these performances were evaluated is to be made available if requested by the client.

2.6. Problems in the Evaluation of Detection Method for Specified Ingredients

Antibody-based enzyme-linked immunosorbent assay (ELISA) or Western blot, which are commonly used as a detection method for specified ingredient protein, have some problems that are not belonging to other instrumental analyses. In many physicochemical and microbiological inspections, the physical properties and structures of analyte are clear. Appropriate method is selected based on the information of this physical property and structure and an analytical method is established. In the meantime, the object cannot be fixed in the allergen detection method of the food. For example, when detecting egg, the label indicates whether or not the whole egg is included, but the subjects to be detected include all proteins of egg, a particular protein specific to egg, proteins of egg with antigenicity and genes of egg (hen). When the subjects to be detected are all proteins, the real nature is not clear. For a particular protein, the physical properties are

clear, but the quantitative relationship with whole egg or protein having antigenicity to be described in the labeling must be clarified. At a minimum, the nature of the standard protein used in the calibration curve should be clarified in order to determine the results. Since the label covers the entire protein of specified ingredient, it is desirable that this “standard protein” contains all proteins if possible, not limited to a particular protein or a protein with antigenicity alone.

Denaturation of proteins by processing treatments such as heating is also an important problem. All processed foods are subject to the labeling system and the specified ingredient proteins contained in them are subject to the different degrees of denaturation during processing. This results in a change in binding with the antibody being used. In DNA detection method, cleavage of the amplified site is responsible for variability. For this reason, it is not surprising that different antibodies used in the kit can produce different results in the same sample. It is important to have an acceptable degree of accuracy in a wide range of foods as an inspection method for confirmation of label, rather than aiming for high accuracy. It is imperative that degeneration or inhibition may make accuracy greater than 100% or very small. However, such information should be published whenever possible to increase the reliability of the inspection.

Reference materials are required to evaluate accuracy. Use them that meet the specifications of reference material standard specified in Appendix 3. If other reference materials are to be used, their development, nature and differences from the current references should be identified in order to determine correct interpretation of test results.

3. Reliability Assurance in Laboratory

Even if an inspection method with high performance assurance is adopted, there are some factors that may cause the inspection results to be inaccurate due to the inadequacy of the inspection method performed in the laboratory. As with other food analytics, this should be addressed by the reliability assurance system of each inspection body.

3.1. Validation at the Time of Test Introduction

When inspection for allergens in food is newly initiated in the laboratory, the inspection method whose performance was evaluated and published should be introduced. In addition, a single-laboratory validation should be performed at the time of introduction to ensure that the published performance of inspection method (kits) can be achieved. Precision (repeatability, intermediate precision) and bias should be checked at least. If there are large differences from published data, the procedures should be reviewed with referring to the management of procedure shown in 3.3.

3.2. Internal Quality Control

In attached document of Notice No. 0323003 of the Department of Food Safety (March 23, 2004) about the operating control guidelines for product inspection at registered inspection body, performing of quality control (internal quality control) is specified to routinely evaluate inspection skills. It is desirable to conduct internal quality control using appropriate control samples in order to demonstrate bias at the time of introduction and the evidence that abilities such as intermediate precision are retained.

3.3. Management of Procedure

Sampling

Establishment of standard sampling procedures is necessary because many processed foods are extremely heterogeneous and cause of large variability may exist in the

sampling and sample preparation stages.

Analytical Instruments

In many cases, a cubic curve or four-parameter logistic curve, etc. is fitted to the relation between concentration and measurements to create a calibration curve. Since the four-parameter logistic curve is nonlinear, a correct calibration curve function cannot be obtained if the criteria for determining initial value or convergence are inappropriate. In such cases, large errors in the analytical values may occur.

Since deviation of absorbance due to difference in position in the plate reader and variability of the amount of injection by pipettes greatly affect the repeatability, routine inspection of the instruments used is also important.

Structure of Precision

In the sandwich ELISA used in the allergen inspection, the repeatability (variability between wells) that can be achieved in the absence of inhibition can be calculated from error of injecting fluid by micropipette, the variability of absorbance between the plate wells, etc. by the following equation.

$$\rho_T^2 = \rho_X^2 + \rho_S^2 + \left(\frac{\sigma_W}{f(X)}\right)^2$$

ρ_T : RSD of measured values
ρ_X : RSD of injected volume of analyte (variability of pipette)
ρ_S : Impact of variability in volume of reaction substrate solution on variability in absorbance measurements
ρ_S = (RSD of injected volume by pipetting) x (2/3)
σ_W: SD for absorbance of wells in themselves (SD for absorbance between wells)
f(X):Calibration curve representing absorbance (X is the concentration of the analyte)

Typically, if ρ_X =0.6%, ρ_S = 0.4%, and σ_W =0.004 Abs, RSD is about 1-5% at absorbances ranging from 0.2 to 1.5, subjected to quantification by ELISA kits. When RSD around absorbance 1 widely exceeds 5 % in the case that the reference solution or the same test solution is measured repeatedly in the actual inspection, the pipette injection precision, cleaning operation of the plate, positioning of the plate reader, etc. seem to be abnormal, so the cause should be determined and the accuracy should be improved.

4. Suggestions on the performance and scope of inspection methods to be publicized by developers of specified ingredient detection methods

When specified ingredient inspection methods, such as ELISA assay, Western blot, and PCR, are developed, the developers should perform the inter-laboratory validation and demonstrate that the performance of the methods is in the following ranges.

Inter-laboratory Validation of Quantification Inspection Method

The number of laboratories shall be eight or more, and the number of samples should be 5 or more for the inter-laboratory validation.

As one of the specified ingredient protein concentration levels included in the sample, 10 μg/g is adopted, which is a definition of trace. Samples should include model processed foods that are made by adding specified ingredients to ingredients and then using production methods such as heating.

For immunochemical quantification inspection methods such as ELISA assay, it is expected that the quantitative value will differ depending on the antibody used, i.e. the accuracy will differ. However, the recovery rate should be between 50% and 150%, inclusive in terms of maintaining the health-being of allergic subjects. In addition, the reproducibility should be 25% or less.

Inter-laboratory Validation of Qualification Inspection Method

The number of laboratories shall be six or more, and the number of samples should be five or more for the inter-laboratory validation.

As specified ingredient protein concentration levels that are included in the sample, blank and 10 μg/g, which is a definition of trace, are adopted. Samples should include model processed foods that are made by adding specified ingredients to ingredients and then using production methods such as heating.

Send two or more identical samples with same concentration to each laboratory and evaluate the determination rate using the samples. The positive rate of a sample containing a specified ingredient protein should be 90% or more, and the negative rate of a blank sample should be 90% or more. Meanwhile, in both case, it is desirable that positive rate or negative rate is 95% or more.

Since the inspection methods are applied to many kinds of processed foods, it is desirable to select samples to be evaluated by validation from foods with a certain characteristic such as foods of animal origin, foods of vegetable origin, foods with high degree of processing (long-time heating, high-pressure cooking), and acidic foods.

A single-laboratory validation in the developer's laboratory should be performed prior to inter-laboratory validation. Here, for a representative model processed sample, the accuracy and intermediate precision at a concentration of 10 μg/g after adding substance should be confirmed, and the impacts of a wide range of matrices, and false-positive and false-negative data of many antigens should be obtained using samples with antigen added to extracts of various foods and the information should be disclosed. For qualitative inspection methods such as PCR and Western blot, the rate of false positives should be checked in various matrices with at least twenty different properties and degrees of processing. A low concentration naturally leads to a high rate of false positives. In a case where the rate of false positives is estimated to be 50% or more, it is desirable to consider the concentration as decision limit.

To prepare reference solutions for the calibration curve and confirm the accuracy, it is desirable to use a reference material that conforms to the reference material standard described in Appendix 3. If it cannot be used, the used reference solution, concentration relationship between the used standard solution and the reference should be clarified. And information to allow interpretation of results between detection methods should be provided.

5. Suggestions on the reliability assurance system for inspectors of specified ingredient

Facilities that conduct inspections for specified ingredients, such as ELISA, Western blot, and PCR, should ensure the reliability of the inspection results by implementing “Validation at the Time of Test Introduction”, “Internal Quality Control”, and “Management of Procedure” as shown in “3. Reliability Assurance in Laboratory”.

Description example 1: Inter-laboratory validation of quantitative inspection method

(Forms for publication of validation results for analytical method using fictional data are shown. Please refer to the form in the preparation of the documents attached to the kits, etc.)

Subject to Validation

Kit X for detecting egg

Samples

Sausages, Beef retort pouches, Biscuits, Orange juice, Jam. For each sample, egg primary reference powder was added so that a protein concentration could be 10 μg/g.

Participating bodies : Ten bodies

- A Company, AA Laboratory
- B Laboratory
- C Association, XX Laboratory
- D Company, DD Laboratory
- E Laboratory
- F Company, FF Center
- G Company, GG Department
- H Research Center
- I Analysis Center
- J Company, JJ Laboratory

Procedures

Documents on extraction methods, kit operation methods and report forms, samples (five types), and kits were sent to each participating body. Participating bodies performed two rounds of extraction and measurement for each sample. Measurement of each extract was performed in three wells, and calibration curves at eight concentrations (including blanks) were measured on the same plate, and the obtained results were returned to the coordinator.

The coordinator determined the mean, repeatability and reproducibility of data sent by participating bodies after Cochran and Grubbs tests to exclude outliers (significance levels of 2.5% for both) in accordance with AOAC INTERNATIONAL or JIS Z 8402-2 procedures.

Validation Results

The recovery rates, repeatability (RSD_r) and reproducibility (RSD_R) derived from each kit validation are described in Table A-1. Both the recovery rate and the reproducibility (RSD_R) meet the criteria shown in the “Appendix: Inspection Methods for Foods Containing Allergens”.

Table A-1 Validation Results of Kit X for Detecting Egg

Samples	Number of bodies included in the calculation	Recovery rate	Repeatability (RSD%)	Reproducibility (RSD%)
Sausages	10	67.2	4.1	14.5
Beef retort	10	76.3	2.2	9.6
Biscuits	9	66.1	4.7	10.8
Orange juice	10	97.7	2.4	6.6
Jam	10	95.3	2.7	5.9

Description example 2: Inter-laboratory validation of qualitative inspection method

(Forms for publication of validation results for analytical method using fictional data are shown. Please refer to the form in the preparation of the materials attached to the kits, etc.)

Subject to Validation

Inspection method of peanut by PCR

Samples

Biscuits, Chocolates, Curry paste, Cereal, Meat paste. Defatted peanut powder was added so that a protein concentration could be 0, 2 or 10 μg/g.

Participating Bodies

Six bodies

- A Company, AA Laboratory
- B Laboratory
- C Association, XX Laboratory
- D Company, DD Laboratory
- E Laboratory
- F Company, FF Center

Procedures

Thirty samples (five samples × three concentrations × two times, randomly coded), two primers, and experimental protocols were sent to each participating body. Within two weeks, the participating bodies measured each sample and sent the results.

The validation results are described in the Table A-2. In all samples, the results in the primers for detecting plant DNA were positive. In the blank sample with peanut concentration of 0 μg/g, the results in peanut specific primer were negative in all processing samples, and all results were positive in the sample including peanut with concentration of 10 μg/g. Based on the above, the negative rate in blank samples and the positive rate in added samples with 2 mg/kg and 10 mg/kg were 90% or more, which satisfies the criteria for “Appendix: Inspection Methods for Foods Containing Allergens”.

Table A-2

Peanut Concentration (mg/kg)	Samples	Primers for detecting plant DNA Positive rate	Peanut-specific primers Positive rate
0	Biscuits	12/12	0/12
	Chocolates	12/12	0/12
	Curry paste	12/12	0/12
	Cereal	12/12	0/12
	Meat paste	12/12	0/12
2	Biscuits	12/12	12/12
	Chocolates	12/12	12/12
	Curry paste	12/12	11/12
	Cereal	12/12	12/12
	Meat paste	12/12	12/12
10	Biscuits	12/12	12/12
	Chocolates	12/12	12/12
	Curry paste	12/12	12/12
	Cereal	12/12	12/12
	Meat paste	12/12	12/12

Description example 3: Precision of ELISA kits for quantitative inspection

As described in “Structure of Precision” of “3.3. Management of Procedure”, in the sandwich ELISA assay used in allergen detection, the repeatability (variability between wells) that can be achieved in a non-inhibition situation is calculated from the injection error of fluid with the micropipette, the absorbance variability between the plate wells, and so on. Here, the repeatability of the absorbance obtained by actually dispensing the reference solution into six wells and the precision (Precision profile) obtained from the calculation formula are shown.

Used Kits

A. Manufactured by Morinaga Institute of Biological Science, Inc. FASPEK specified ingredient measuring kit (Ovalbumin) B. Manufactured by NH Foods Ltd. FASTKIT ELIZA Version II Series (Wheat)

Calculation of Precision Profile

Precision of each concentration was calculated based on the following expressions:

$$\rho_T^2 = \rho_X^2 + \rho_S^2 + \left(\frac{\sigma_W}{f(X)}\right)^2$$

ρ_T : RSD of measured values
ρ_X : RSD of injected volume of analyte (variability of pipette)
ρ_S : Impact of variability in volume of reaction substrate solution on variability in absorbance measurements
ρ_S = (RSD of injected volume by pipetting) x (2/3)
σ_W: SD for absorbance of wells in themselves (SD for absorbance between wells)
f(X):Calibration curve representing absorbance (X is the concentration of the analyte)

The precision profile obtained as a ρ_X =0.6%, σ_W = 0.004 and the precision of actual measurement are described in Fig. A-1.

Variations in absorbance measured in the same solutions are generally less than or equal to 5% as RSD%, except for low-concentration range where the absorbance is small.

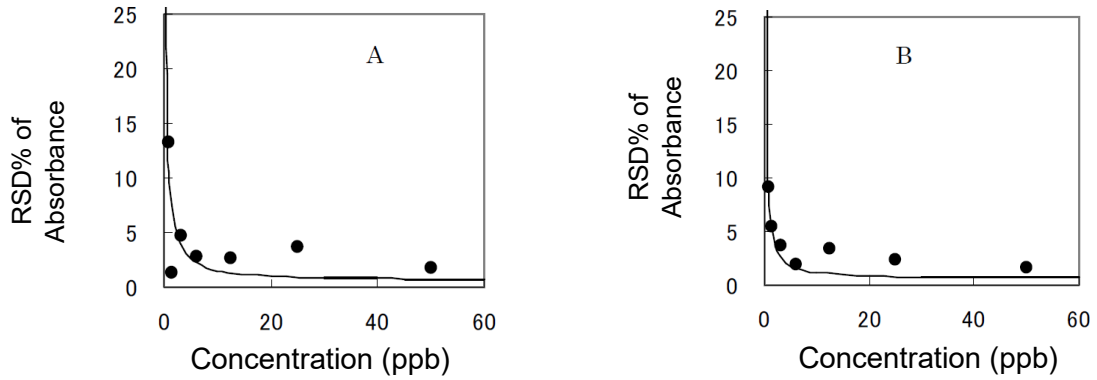


Fig. A-1 Accuracy Profile of Specified Ingredient Detection Kit
A. Manufactured by Morinaga Institute of Biological Science, Inc. FASPEK specified ingredient measuring kit (Ovalbumin)
B. Manufactured by NH Foods Ltd. FASTKIT ELIZA Version II Series (Wheat)

● Repeatability of reference solutions at each concentration (n=6) Solid Line: Precision calculated from the expression A1

(Appendix 5)

Guideline for Evaluation of the Improved Inspection Methods for Foods Containing Allergens

For the improved quantitative inspection methods (hereinafter referred to as “Improved Inspection Method”) established by modifying the quantitative inspection methods that have been evaluated for their performance through inter-laboratory validation and have shown meeting the criteria shown in “Appendix: Inspection Method for Foods Containing Allergens” or the methods that have already been recognized as having a performance equivalent to or higher than the quantitative inspection methods (hereinafter referred to as “Conventional Method”), if the Improved Inspection Method were evaluated for the following performances in a single laboratory and have shown having a performance equivalent to or higher than the Conventional Method, the Improved Inspection Method shall be regarded as inspection methods for foods containing allergens in the same way as the Conventional Method.

1 Calibration Curve

Show that the concentration range and quantitativeness of the calibration curve of the Improved Inspection Method are equivalent to those of the Conventional Method.

2 Correlation with the Conventional Method

Multiple samples are quantified using the Conventional Method and the Improved Inspection Method to show that the Improved Inspection Method is equivalent to the Conventional Method.

Specifically, quantitative values by the Conventional Method are put on the X-axis, and quantitative values by the Improved Inspection Method are put on the Y-axis, and the correlation is plotted. For this plot, calculate an approximate line ($Y = aX$) with the Y-intercept as zero and show that its slope ranges from 0.75 to 1.25 and that the correlation coefficient is 0.9 or more.

For each test method, ten or more kinds of samples whose quantitative values are distributed without bias ranging from several μg/g to about 10,000 μg/g (However, sample with quantitative value exceeding 10,000 μg/g may be included if it is difficult to secure samples whose quantitative value is within the concentration range of interest) shall be examined, and the correlation between the Conventional Method and the Improved Inspection Method shall be plotted.

Further, in addition to the above-mentioned examination, especially for the range from several μg/g to several 10 μg/g, plot the quantitative values of ten or more kinds of samples distributed without bias separately (however, if it is difficult to secure samples whose quantitative value is within the concentration range of interest, the measured value when a high concentration sample is diluted may be used), and confirm that the correlation satisfying the above criteria is observed.

As a sample, use foods like the following.

- Commercial processed foods
- Model processed foods prepared by adding specified ingredient proteins to food materials

- Processed foods for which processed foods that contain specified ingredients and similar processed foods that do not contain specified ingredients are mixed to adjust specified ingredient protein concentrations
- Processed foods for which specified ingredient protein is added to processed foods do not contain specified ingredients. In addition, it is desirable to use foods having various characteristics as a sample, such as foods of animal origin, foods of vegetable origin, foods having a high degree of processing, acidic foods, or the like.

When the slope of the above approximate line is 0.8 or less or 1.2 or more, it is recommended that, in addition to the above examination, the recovery rate should be examined using three or more kinds of samples (one of concentration level of specified ingredient protein contained in the sample should be about 10 μg/g). The recovery rate should be between 50% and 150%, inclusive.

As a sample, use processed foods similar to that described above and having a known concentration of specified ingredient protein.

3 Precision

Samples containing approximately 1-20 μg/g of the specified ingredient protein (approximately 2-3 samples) should be used to examine repeatability (five or more trials) and daily variability (approximately 3-5 days).

The F-test should be performed to show that the accuracy and variability of these methods are equivalent between the Conventional Method and the Improved Inspection Method, and the accuracy of the Improved Inspection Method is higher if they are not equivalent.

In addition, it is desirable to examine the daily variability, variability between analysts, variability between devices, etc.

4 Limit of Detection and Limit of Quantification

For the Improved Inspection Method, these values are equivalent to or smaller than those of the Conventional Method.

5 Specificity

Foods with false positives and false negatives are examined to show concordances and differences with Conventional Method clearly.