FOOD COMPOSITION AND ADDITIVES

Determination of Walnut Protein in Processed Foods by Enzyme-Linked Immunosorbent Assay: Interlaboratory Study

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Because food allergens from tree nuts, including walnuts, are a frequent cause of adverse food reactions for allergic patients, the labeling of foods containing ingredients derived from tree nuts is required in numerous countries. According to Japanese regulations, the labeling of food products containing walnuts is recommended. To ensure proper labeling, a novel sandwich ELISA kit for the determination of walnut protein in processed foods (Walnut Protein [2S-Albumin] Kit; Morinaga Institute of Biological Science, Inc.; "walnut kit") has been developed. We prepared seven types of incurred samples (model processed foods: biscuits, bread, sponge cake, orange juice, jelly, chicken meatballs, and rice gruel) containing 10 μg walnut soluble protein/g of food for use in interlaboratory evaluations of the walnut kit. The walnut kit displayed sufficient reproducibility relative standard deviations (interlaboratory precision: 5.8–9.9% RSD_R) and a high level of recovery (81–119%) for all the incurred samples. All the repeatability relative standard deviation (RSD_r) values for the incurred samples that were examined were less than 6.0%. The results of this interlaboratory evaluation suggested that the walnut kit could be used as a precise and reliable tool for determination of walnut protein in processed foods.

Over recent decades, food allergies have emerged as an important public health concern in industrialized countries. Up to 8% of young children and 2% of adults are estimated to have food allergies, and these percentages appear to be increasing (1–5). The clinical manifestations of food allergies vary from mild symptoms, such as oral allergy syndrome or mild urticaria, to severe anaphylactic reactions with fatal consequences. The most effective means of preventing allergic reactions to food is to avoid foods that contain allergens; therefore, patients with food allergies must be able to obtain accurate information regarding the presence of food allergens in processed foods. Nevertheless, various studies have shown that severe allergenic reactions can be induced by the accidental intake of food products containing allergenic materials (6, 7). Accordingly, information regarding potentially allergenic ingredients in food products is necessary.

The issue of a labeling system for allergenic ingredients in food products has been discussed by international organizations such as the Codex Alimentarius Commission of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to label eight kinds of foods containing known allergens, including tree nuts and nut products (8, 9). Tree nuts are regarded as one of the "big eight" allergenic foods believed to be responsible for 90% of all food allergies (10). Subsequently, various countries or regions have considered the labeling list established by the Codex guidelines and have developed their own lists of the most commonly allergenic foods. In response to the FAO/WHO recommendation, walnut labeling has become mandatory in the United States, European Union, Canada, and Australia/New Zealand.

Since April 2002, the Ministry of Health, Labour, and Welfare (MHLW) of Japan has enforced a labeling system for allergenic food materials to ensure that information on these foods is available to allergic consumers. According to Japanese regulations, the labeling of food products containing eggs, milk, wheat, buckwheat, peanuts, shrimp, and crab is mandatory and is recommended for 18 other food materials, including walnut, in light of the number of allergic patients and the degree of the seriousness of their allergic reactions.

Received August 4, 2009. Accepted by SG September 30, 2009.
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In a notification issued in 2002 (11), the MHLW announced the official Japanese methods for detecting allergens requiring mandatory labeling and the threshold value for labeling [10 μg/g (allergenic ingredient soluble protein weight/food weight)]. The MHLW also described the criteria for interlaboratory validation protocols in their official guidelines, which were issued in 2006 (12). Briefly, the interlaboratory validation protocols must meet the following criteria: number of laboratories ≥8, number of incurred samples ≥5, number of dose levels ≥1 [including 10 μg/g (allergenic ingredient soluble protein weight/food weight)], recovery of 50–150%, and reproducibility relative standard deviation (RSD_R) ≤25%. The reference material, the initial extract solution, and the allergen extraction procedure were also specified and standardized in the guidelines.

Tree nuts are regarded as one of the most potent allergenic ingredients among all known allergenic foods and often cause food anaphylaxis and anaphylaxis death (13–16). Walnut allergy is the most common tree nut allergy and occurs in all age groups (17, 18). In addition, walnut allergy is extremely severe, inducing life-threatening allergic reactions similar to those induced by peanut allergy (19–21). In Japan, epidemiological investigations have revealed that the number of patients with walnut allergy is increasing (5, 22). Furthermore, despite labeling precautions, walnut protein remains quite dangerous, as it is often present in commercial foods as a hidden allergen arising from cross-contamination during food processing. In most factories, many different products are manufactured from various ingredients, and sometimes these products are even manufactured on the same production line. Therefore, a reliable method of detecting walnut protein is needed to monitor and ensure accurate labeling. We have developed a highly sensitive ELISA kit capable of detecting walnut protein [Walnut Protein (2S-Albumin) Kit; hereafter referred to as "walnut kit"] (23). In the present paper, we describe the results of an interlaboratory evaluation of the performance of this ELISA kit.

Experimental

Materials and Methods

(**a**) *Preparation of the defatted walnut powder.*—The walnuts (Chandler) were kindly provided by Tabata, Inc. (Chiba, Japan). They were ground in a mill, and the walnut powder was collected. After removing the fats using acetone, the defatted walnut powder was dried for 16 h. The walnut soluble protein (WP) was extracted from the defatted walnut powder using buffer A [120 mM Tris-HCl (pH 7.4), 0.1% (w/v) bovine serum albumin, and 0.05% (v/v) Tween 20] containing 0.5% (w/v) sodium dodecyl sulfate (SDS) and 2% (v/v) 2-mercaptoethanol (2-ME). The WP content was then calculated using a 2-D Quant Kit (GE Healthcare, Buckinghamshire, UK). One gram of defatted walnut powder was obtained from 3.99 g raw walnuts, and the amount of WP/g of defatted walnut powder was approximately 556 mg (23).

(**b**) *Preparation of incurred samples.*—To obtain a final walnut protein concentration of 10 μg/g (WP weight/sample weight) in the incurred samples, the amount of defatted walnut powder to be spiked in the incurred samples at the ingredient stage was calculated, taking into account the protein content of the defatted walnut powder and the change in weight of the incurred samples during preparation. Because the protein amount/g defatted walnut powder was approximately 556 mg, we spiked 18.0 mg defatted walnut powder to make 1 kg of each incurred sample [final concentration of 10 μg/g (WP weight/sample weight)], as the ratio of the WP weight to the defatted walnut powder weight was estimated to be 55.6%.

All the incurred samples were prepared using the general procedures used by food manufacturers. The detailed procedures are described below.

The biscuits were made from wheat flower, sugar, shortening, salt, bicarbonate, dihydroxysuccinic acid, lecithin, and proteinase. All the raw materials and the defatted walnut powder were kneaded, molded, and baked at 240°C for 8.5 min. The cooked biscuits were stored at –40°C before use.

The bread was made from wheat flower, sugar, shortening, yeast, salt, skim milk, and water. All the raw materials and the defatted walnut powder were kneaded, leavened, and baked at 200°C for 20 min. The cooked bread was stored at –40°C before use.

The sponge cake was made from wheat flower, sugar, whole egg, emulsifiable fat, and water. All the raw materials and the defatted walnut powder were kneaded and baked at 180°C for 30 min. The cooked sponge cake was stored at –40°C before use.

The orange juice was made from orange concentrate, sugar, citric acid, ascorbic acid, and water. Orange concentrate, sugar, water, and the defatted walnut powder were mixed and homogenized. After the homogenate was buffered to pH 4.5 with citric acid, it was divided into cans and heated at 90°C for 10 min. The cooked orange juice was stored at –40°C before use.

The jelly was made from sugar, agar, citric acid, sodium citrate, muscat flavoring, and water. The raw materials and the defatted walnut powder were mixed. The mixture was heated to 90°C and divided into cans. After the canned jelly had been hardened at 15°C for 3 h, it was stored at –40°C before use.

The chicken meatballs were made from white chicken meat, lard, potato starch, and sugar. Lard, potato starch, sugar, and the defatted walnut powder were added to ground white chicken meat and thoroughly mixed. The mixture was ground using a small cutter, and the kneaded mixture was manually inserted into casings. The chicken meatballs were stored at –20°C before use.

The rice gruel was made from rice and water. The rice and the defatted walnut powder were cooked in a rice cooker and stored at –20°C before use.

Each type of incurred sample was also prepared as a blank sample that did not contain the defatted walnut powder to examine potential contamination, false-positive, and matrix effects. All the values for the blank samples of the model processed foods that were determined using the walnut kit were less than the LOD values determined by an in-house study (data not shown).

(**c**) *Homogeneity tests of the incurred samples.*—The homogeneity of the incurred samples was verified by the coordinator prior to distribution according to the procedure described in the International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (24); the only modification to this procedure was that the number of homogeneity test materials was six. Twelve test portions of each incurred sample were analyzed using the walnut kit. The resulting WP concentrations were then analyzed using a one-way analysis of variance (ANOVA).

(**d**) *Test materials for interlaboratory study.*—Seven incurred samples (model processed foods: biscuits, bread, sponge cake, orange juice, jelly, chicken meatballs, and rice gruel) containing WP were prepared and used as test materials. The defatted walnut powder was spiked at the ingredient stage before processing to establish a final level of 10 μg/g (WP weight/sample weight). The prepared incurred samples were homogenized with a food processor (DLC-XG; Cuisinart, Stamford, CT) and sent to the participating laboratories as test materials.

Walnut Protein (2S-Albumin) ELISA Kit (Walnut Kit; Ref. 23)

Polyclonal antibodies to 2S-albumin, the allergenic protein in walnuts, were used in the walnut kit, and a WP solution was utilized for calibration. Diluted standard solutions and sample solutions were added to a polyclonal antibody-coated module and incubated for 1 h at room temperature. After the module was washed, anti-2S-albumin antibody labeled with horseradish peroxidase was added and allowed to stand for 30 min at room temperature. After the final washing, a solution of 3,3′,5,5′-tetramethylbenzidine was added, and the module was allowed to stand at 25°C for exactly 10 min. The reaction was stopped by the addition of 0.5 M sulfuric acid, and the absorbances were measured at 450 nm with 620 nm as the reference wavelength.

Information about the walnut kit including specificity was described earlier (23). The walnut kit has a high specificity for walnut, although it has slight cross-reactivity to pecans and hazelnuts at levels greater than the LOD.

The value of 134 mg WP/g was obtained when raw Chandler walnut was examined using the walnut kit (23). This value is in good agreement with the value described above in the *Preparation of the defatted walnut powder* section (556 mg of WP was obtained from 3.99 g raw Chandler walnuts, i.e., the content of WP in the raw walnuts was approximately 13.9%). This result shows the high reliability of the walnut kit to detect raw, not defatted, walnut. In addition, the activity of five raw or roasted walnut varieties (Chandler and Howard grown in California, Miette grown in France, Chinese grown in China, and Shinano grown in Japan) ranged from 82.1 to 125% (ratio to raw Chandler walnut), indicating that the walnut kit has similar reactivities among these walnut varieties (23).

Extraction

The incurred samples were homogenized using a food processor. A 1.0 g portion of the test material was extracted using 19 mL buffer A containing 0.5% (w/v) SDS and 2% (v/v) 2-ME. The mixture was shaken horizontally overnight (16 h) at room temperature, then centrifuged at 3000 × *g* for 20 min after adjusting the pH to 6.0–8.0. The supernatant was filtered, if necessary, diluted 20 times with buffer A, and subjected to ELISA.

Calibration Standard Solution

The calibration standard solution was prepared according to the official Japanese guidelines and the method used in previous reports in Japan (25–27). The initial extract for the calibration standard solution was prepared from defatted walnut powder as follows: a 0.2 g sample defatted walnut powder was added to 20 mL buffer A containing 0.5% (w/v) SDS and 2% (v/v) 2-ME. The mixture was then shaken for 16 h at room temperature prior to extraction. After extraction, the sample was centrifuged at 10 000 × *g* for 30 min, and the supernatant filtered through a 0.8 μm micro-filter paper (DISMIC 25cs; Advantec, Tokyo, Japan) to obtain the extract. The protein content of the initial extract was assayed using a 2-D Quant Kit. The initial extract was diluted with buffer A to prepare the calibration standard solution (50 ng/mL extracted protein) for the walnut kit.

Interlaboratory Study

Twelve laboratories participated in the interlaboratory evaluation, which was organized by the National Institute of Health Sciences (Tokyo, Japan). The participants included manufacturing companies, public research institutes, local public inspection institutes, and private inspection institutes. The organizer sent each laboratory the seven test materials (3 g each), the ELISA kit, the extraction solution, and the calibration standard solution. The participants took two portions from each test material, extracted the protein using the extraction procedure, and assayed the extract using the ELISA kit. The calibration standard solution was diluted and assayed simultaneously with the incurred sample extracts. Each sample extract was analyzed in triplicate (three wells/sample extraction), and the average absorbance of three wells was used for the calculation. The resulting absorbance data for the calibration solutions and test materials were reported to the coordinator.

The organizer calculated a four-parameter logistic calibration curve based on the absorbance data of the calibration standard solution and calculated the concentrations of WP in the incurred samples using the calibration curve. Each set of samples was extrapolated from the standard curve run on the same plate.

Statistical Analysis

Twenty-four data items (two portions evaluated at 12 laboratories) were used in the calculations. The Cochran and Grubbs tests were used to remove outlying data (P =

Table 1. Homogeneity test results of the incurred samples

Sample	Mean	RSD, %[a]	n	F-ratio	Fcrit[b]
Biscuit	9.1	4.2	6	1.8	4.4
Bread	13.2	2.9	6	3.5	4.4
Sponge cake	10.2	2.6	6	1.2	4.4
Orange juice	10.6	4.7	6	0.6	4.4
Jelly	10.3	1.8	6	1.4	4.4
Chicken meatball	10.5	8.3	6	0.3	4.4
Rice gruel	12.1	3.2	6	0.3	4.4

[a] RSD, %, calculated from s_s (SD of sampling) and s_a (SD of analysis).
[b] Fcrit = Critical F value.

2.5%). The Cochran test was used to remove data from laboratories reporting a significantly large variation between the results of the two portions taken from the test material. The Grubbs test was used to remove data from laboratories reporting a mean of the results for the two portions that was significantly different from those of the other laboratories. The use of these statistical tests to identify outliers was in accordance with the AOAC protocol (28). The recovery, repeatability, and reproducibility were then calculated using data generated by a one-way ANOVA applied to the data after the removal of outliers.

Results and Discussion

Homogeneity of the Test Materials

Table 1 shows the average concentration, the RSD percentages calculated from s_s (SD of sampling), and s_a (SD of analysis), the number of test materials, the F-ratio, and the critical F value. The s_s and s_a values were calculated using data generated by a one-way ANOVA. The resultant F-ratios of the homogeneity test for the biscuit, bread, sponge cake, orange juice, jelly, chicken meatball, and rice gruel samples were <3.5. The F-ratios for all the incurred samples were below the critical F value (4.4). For most of the test materials, the RSD values between the portions were <8.3%, which was smaller than the required RSD_R values (≤25%). We, therefore, concluded that the homogeneity of the test materials was acceptable for the purposes of this study.

Calibration Curve

Figure 1 shows the calibration curve for the determination of WP using the walnut kit. The calibration curve was obtained using the four-parameter logistic model. This curve showed an excellent correlation between the protein concentration and the optical density in the range of 0.78 to 50 ng/mL (correlation coefficient >0.999), and a concentration of 25 ng/mL, which corresponds to a WP dose level of 10 μg/g in the test materials, fell within the dynamic range of the curve. We determined the LOD and LOQ according to the guidelines issued by the International Standards Organization and the International Union of Pure and Applied Chemistry (29) in an in-house study. The LOD was calculated as three times the SD of the mean value of the dilution buffer after eight experiments. The LOQ was calculated as 10 times the SD of the mean value of the dilution buffer after eight experiments. The LOD and LOQ of the

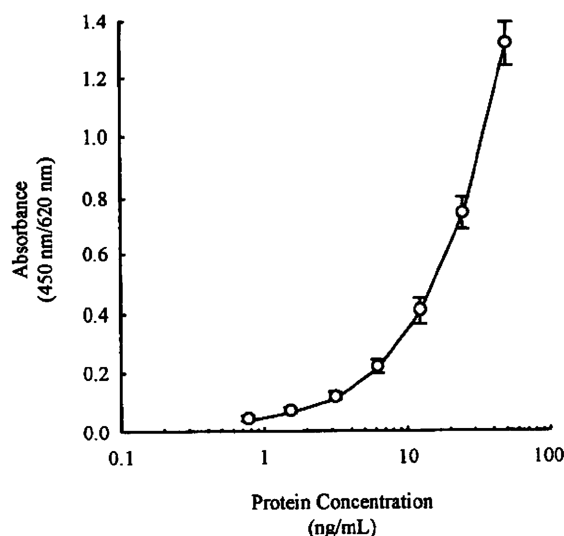

Figure 1. Walnut protein calibration curve for the walnut kit. The calibration curve was obtained using a four-parameter logistic method from the mean value after eight experiments. Concentration of calibration standard solution (calculated WP weight/food weight) = 0.78 ng/mL (0.31 μg/g), 1.56 ng/mL (0.62 μg/g), 3.13 ng/mL (1.25 μg/g), 6.25 ng/mL (2.5 μg/g), 12.5 ng/mL (5 μg/g), 25 ng/mL (10 μg/g), and 50 ng/mL (20.0 μg/g). The equation of the calibration curve is $y = [(A - D)/(1 + (x/C)^B)) + D]$ (x, protein concentration; y, optical density; A = 0.014; B = 1.006; C = 187.327; D = 6.172). The correlation coefficient (r) between the protein concentration and the optical density was found to be >0.999.

Table 2. Results of the interlaboratory study for the walnut kit protein recovery content[a]

	Biscuit		Bread		Sponge cake		Orange juice		Jelly		Chicken meatball		Rice gruel	
Lab	Mean, µg/g[b]	Recovery, %[c]	Mean, µg/g	Recovery, %	Mean, µg/g	Recovery, %	Mean, µg/g	Recovery, %	Mean, µg/g	Recovery, %	Mean, µg/g	Recovery, %	Mean, µg/g	Recovery, %
A	7.8	78	11.1	111	9.5	95	9.0	90	9.2	92	9.0	90	11.3	113
B	8.3[d]	83	10.8	108	9.7	97	9.8	98	10.3	103	9.1	91	11.3	113
C	8.0	80	11.7	117	9.0	90	8.5	85	9.4	94	10.5	105	11.2	112
D	7.8	78	11.1	111	10.2[d]	102	10.0	100	10.2	102	10.1	101	11.2	112
E	7.9	79	12.2	122	9.8	98	9.9	99	10.8	108	11.1	111	12.4	124
F	9.0	90	13.1	131	10.8	108	11.2	112	11.2	112	12.3	123	13.4	134
G	8.1	81	12.3	123	9.8	98	9.5	95	10.2	102	11.1	111	12.0	120
H	9.3	93	10.0	100	9.6	96	10.0	100	10.7	107	10.6	106	12.9	129
I	7.6	76	10.8	108	9.0	90	10.3	103	9.7[d]	97	10.5	105	11.2	112
J	8.1	81	12.0	120	9.9	99	10.6	106	11.0	110	11.6	116	13.0	130
K	8.2	82	11.6	116	9.6	96	10.6	106	10.8	108	10.8	108	12.6	126
L	7.8	78	11.3	113	9.0	90	9.3	93	9.6	96	10.4	104	10.8	108

[a] The incurred samples contained WP of approximately 10 µg/g.
[b] Mean = average concentration of WP (WP weight/food weight).
[c] Recovery = mean/10 (µg/g), %.
[d] Values removed after Cochran test.

walnut kit, as determined using the dilution buffer, were 0.39 ng/mL (equivalent to 0.16 µg/g of food sample) and 0.78 ng/mL (equivalent to 0.31 µg/g of food sample), respectively. Consequently, the practical determination range was between 0.78 and 50 ng/mL.

Recovery, Repeatability, and Reproducibility

The values reported by the participants are summarized in Table 2. As shown in this table, the data for biscuits from "Laboratory B," the data for sponge cake from "Laboratory D," and the data for jelly from "Laboratory I" were detected as outliers using the Cochran test. The number of remaining laboratories after removing the outliers, the average concentration (µg/g), s_r (repeatability SD, µg/g), s_R (reproducibility SD, µg/g), recovery (%), repeatability (RSD_r, %), and reproducibility (RSD_R, %) calculated using an ANOVA are shown in Table 3.

The recoveries of WP from the seven types of test materials using the walnut kit ranged from 81 to 119%. In spite of the high degree of processing, these values indicate a high degree of recovery for all the test materials, especially for the sponge cake, orange juice, and jelly samples (96, 99, and 103%, respectively). The recoveries from bread and rice gruel were slightly high (115 and 119%, respectively), while that of biscuits was slightly low (81%). However, these recoveries tended to follow the mean values of each incurred sample in the homogeneity test (Table 1). Therefore, the recoveries, the average values from each participant, are considered to be reliable. The variations in these recoveries might be affected by the food processing conditions and/or the physical

Table 3. Recovery, repeatability (RSD_r), and reproducibility (RSD_R) values of the walnut kit for WP

Sample[a]	No. of laboratories	Mean, µg/g[b]	s_r, µg/g	s_R, µg/g	Recovery, %[c]	RSD_r, %	RSD_R, %
Biscuit	11	8.1	0.4	0.6	81	4.4	7.1
Bread	12	11.5	0.3	0.9	115	3.0	7.5
Sponge cake	11	9.6	0.3	0.6	96	3.1	5.8
Orange juice	12	9.9	0.6	0.8	99	5.6	8.5
Jelly	11	10.3	0.4	0.7	103	4.3	7.3
Chicken meatball	12	10.6	0.6	1.0	106	6.0	9.9
Rice gruel	12	11.9	0.6	1.0	119	5.0	8.3

[a] The incurred samples contained WP of approximately 10 µg/g.
[b] Mean = average concentration of WP (WP weight/food weight).
[c] Recovery = mean/10 (µg/g), %.

properties of the food matrixes containing the WP. In any event, the recoveries of all incurred samples satisfied the criteria for the interlaboratory validation protocol (50–150%).

Repeatability is a measure of the variation arising from the entire analytical procedure in a particular laboratory. In the intralaboratory evaluations, all the RSD_r values were found to be <6.0%. According to Horwitz's theory, the RSD_r value is generally likely to be less than 2/3 of the RSD_R value (30). In this study, all the RSD_r values for the walnut kit were less than 2/3 of the corresponding RSD_R value. Thus, these satisfactory RSD_r values were considered to guarantee the reliability of this analytical method.

The reproducibilities, expressed by the RSD_R values of the WP content from the seven types of test materials using the walnut kit, ranged between 5.8 and 9.9%. The reproducibilities of all the incurred samples also satisfied the criteria for the interlaboratory validation protocol (≤25%). Thus, the walnut kit displayed a relatively high level of reproducibility in the interlaboratory evaluation results.

The organizer of this study surveyed the instruments (the shaker used for extraction and the microplate reader used for the determination) used by the participants in the interlaboratory evaluation. These instruments were not uniform, but the equipment differences did not affect the data obtained in this study. Therefore, we concluded that this kit would be a very robust tool as a general method for monitoring allergen ingredients to ensure correct labeling.

We previously reported the results of interlaboratory studies examining 13 ELISA kits for the determination of egg, milk, wheat, buckwheat, peanut, crustacean, and soybean proteins in processed foods (25–27). In the case of one buckwheat kit, the results of the recovery, repeatability, and reproducibility for the detection of buckwheat proteins in the five model processed foods were 58–136%, 6–13% RSD_r, and 10–25% RSD_R, respectively. In addition, the recovery of one milk kit was 89–137%, the reproducibility of one crustacean kit was 18–21% RSD_R, and the reproducibility of one soybean kit was 9–13% RSD_R. Thus, the recovery, repeatability, and reproducibility results of the interlaboratory validation of the walnut kit were excellent compared with the results of previous interlaboratory studies examining other ELISA kits.

The Japanese MHLW established an interlaboratory validation protocol in its official guidelines, which were issued in 2006 (12). Briefly, the following criteria for the interlaboratory validation protocol were defined: number of laboratories ≥8, number of incurred samples ≥5, number of dose levels ≥1 [including 10 μg/g (allergenic ingredient soluble protein weight/food weight)], recovery of 50–150%, and RSD_R ≤25%. These criteria were based on the ISO5725 (JIS Z8402) guidelines, which are almost the same as those of the AOAC INTERNATIONAL (28). In the official guidelines, the initial extract solution and the allergen extraction procedure are also specified and standardized.

The present study suggested that the walnut kit is a reliable and precise method of determining the WP content, and that the performance of the walnut kit satisfies the validation criteria described in the official guidelines published by the Japanese government. Because food allergies can induce severe symptoms, the accuracy of the method is crucial. The data obtained in the interlaboratory validation clearly show that the walnut kit can quantify the WP content of the incurred samples very accurately. Moreover, the walnut kit produces good repeatability and reproducibility measures because of the high precision of the assay performance. The present interlaboratory evaluation was performed using seven incurred samples, including highly processed foods, such as biscuits, sponge cake, and rice gruel, as it is necessary to ensure that the kit is able to detect WP in commercial foods to guarantee accurate labeling. The present results demonstrated that the walnut kit is capable of detecting WP in processed foods and could be applicable to monitoring of the food labeling system, in accordance with Japanese regulations. Furthermore, we have already developed a method for detecting walnuts using conventional PCR and electrophoresis (31). By combining these methods, the risk of false-negative and/or false-positive results in inspections for walnut contamination could be minimized.

Conclusions

In conclusion, the present ELISA kit is a rapid, precise, and reliable tool for the analysis of WP in processed foods. The proposed system is capable of accurately monitoring labeling systems in a reliable manner and may be useful for inspections performed in accordance with Japanese regulations.

Acknowledgments

We thank Rieko Matsuda (National Institute of Health Sciences) for her statistical input into this investigation. We thank the following collaborators for participating in the interlaboratory evaluation:

Hiroyuki Haraguchi, FASMAC Co., Ltd, Atsugi, Japan

Takashi Mishima, Japan Food Research Laboratories, Chitose, Japan

Tatsuo Seki and Masamitsu Hirota, Japan Frozen Foods Inspection Corp., Yokohama, Japan

Tayoshi Iidzuka and Yutaka Hirose, Japan Inspection Association of Food and Food Industry Environment, Tokyo, Japan

Yasutaka Minegishi, Nippon Gene Co., Ltd, Toyama, Japan

Hirohito Yamakawa and Satoko Maeda, Nisshin Seifun Group, Inc., Fujimino, Japan

Takeo Yano, Oriental Yeast Co., Ltd, Nagahama, Japan

Koichiro Kojima, Rohto Pharmaceutical Co., Ltd, Kyoto, Japan

Fumihiro Arakawa, San-Ei Gen F.F.I., Inc., Osaka, Japan

Kana Ikeno, Showa Sangyo Co., Ltd, Funabashi, Japan

Kazuo Yasuda, Tokyo Metropolitan Institute of Public Health, Tokyo, Japan

Keiko Oguchi, Wakodo Co., Ltd, Tokyo, Japan

This study was supported by Health and Labour Science Research Grants for Research on Food Safety from the Ministry of Health, Labour and Welfare of Japan.

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 8145–8151 8145
DOI:10.1021/jf100675c

Specific Detection of Banana Residue in Processed Foods Using Polymerase Chain Reaction

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Specific polymerase chain reaction (PCR) methods were developed for the detection of banana residue in processed foods. For high banana specificity, the primer set BAN-F/BAN-R was designed on the basis of the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*) genes of chloroplasts and used to obtain amplified products specific to banana by both conventional and real-time PCR. To confirm the specificity of these methods, genomic DNA samples from 31 other species were examined; no amplification products were detected. Subsequently, eight kinds of processed foods containing banana were investigated using these methods to confirm the presence of banana DNA. Conventional PCR had a detection limit of 1 ppm (w/w) banana DNA spiked in 50 ng of salmon testis DNA, whereas SYBR Green I real-time semiquantitative PCR had a detection limit as low as 10 ppm banana DNA. Thus, both methods show high sensitivity and may be applicable as specific tools for the detection of trace amounts of banana in commercial food products.

KEYWORDS: Allergen; banana; polymerase chain reaction; real-time polymerase chain reaction; ribulose-1,5-bisphosphate carboxylase gene

INTRODUCTION

Banana is a major worldwide fruit that is consumed and produced in Asia, Central and South America, and Africa. The origin of modern bananas derives from two native species of Southeast Asia, *Musa acuminate* and *Musa balbisiana* (*1*). Modern bananas as foods are mostly diploid or triploid cultivars obtained by crossing two wild cultivars. To classify banana genotypes, primers have been developed (*2*); however, studies have yet to determine their cross-reactivity with other biological species and detection sensitivity. Such detection of banana residue in foods needs to be achieved due to its potential allergenicity.

Since April 2002, the Japanese government has enforced a labeling system for allergenic food materials (*3*). In this system, labeling is now mandatory for seven food products (egg, milk, wheat, buckwheat, peanuts, shrimp/prawn, and crab); labeling for shrimp/prawn and crab became mandatory in 2008. In addition to these seven products, 18 additional food materials, including banana, have been recommended for labeling. Banana became recommended for labeling in 2004, based on a 2002/2003 investigation that reported an increase in the number of banana allergy patients in Japan.

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Banana contains food allergens that are common to those in latex or pollens (*4*, *5*). Many clinical studies have reported cross-reactivity of banana and latex, referred to as the latex−fruit syndrome (*6*−*8*). These studies monitored the number of patients with a food allergy in Japan and found that patients with banana allergy comprised the second largest population among those with fruit allergies; kiwifruit allergy was the most prevalent (*9*). The general symptom of the banana allergy is the oral allergy syndrome involving an allergic reaction induced in the mouth and pharynx. The latex−fruit syndrome can also develop as an immediate-type allergic reaction, including generalized urticaria, asthma, and severe anaphylactic reactions, which may have fatal consequences (*6*). Class I chitinases containing the hevein-like domain are major allergens in banana and could be the pan-allergens responsible for the latex−fruit syndrome (*10*, *11*).

For better consumer protection, specific and sensitive methods are required for the detection of trace amounts of allergens in commercial food products. PCR-based methods can be applied to the detection of allergenic food material contamination. Conventional and real-time PCR methods have already been established for the detection of wheat, buckwheat, peanuts, soybeans, walnuts, pork, and kiwifruit (*12*−*20*).

In the present study, we developed novel conventional and real-time PCR methods with high specificity and sensitivity for the detection of banana in processed foods. The primer set was

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Published on Web 07/06/2010 pubs.acs.org/JAFC

Table 1. Designed Primers Used for Detection of Banana[a]

	name	sequence	specificity	amplicon	
A	CP 03−5′	5′-CGG ACG AGA ATA AAG ATA GAG T-3′	chloroplast DNA/sense	plants	123 bp
	CP 03−3′	5′-TTT TGG GGA TAG AGG GAC TTG A-3′	chloroplast DNA/antisense		
B	BAN-F	5′-TCG TCA CCT ATT GGG ATG C-3′	chloroplast DNA/sense	banana	186 bp
	BAN-R	5′-GCT TT A ATA AGT GCT TCG GTG-3′	chloroplast DNA/antisense		

[a] A, primer set used to confirm the validity of the DNA extracted from plants for PCR; B, primer set used to specifically detect banana DNA.

designed to detect the gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbcL*). The present PCR methods were shown to be applicable for the detection of trace amounts of banana contained in commercially processed food products.

MATERIALS AND METHODS

Samples Used in DNA Isolation. Food products including banana, yam, monkey banana, and eight commercial products containing banana (banana puree, fruit juice, banana juice, soy milk, banana chip, chocolate, soft cookie, and a cereal product) were purchased from a local market in Japan. Twenty-three kinds of fruits (apple, pear, Japanese pear, Japanese persimmon, peach, prune, apricot, Japanese apricot, cherry, orange, Satsuma orange, fig, kiwifruit, grape, blueberry, raspberry, juneberry, strawberry, melon, avocado, papaya, pineapple, and mango) were kindly provided by House Foods, Corp. (Tokyo, Japan). For the examination of sensitivity, we prepared a chocolate sample spiked with 1% (w/w) banana chip and a soy milk sample spiked with 0.01% (w/w) banana puree. We used chocolate and soy milk without the addition of banana as negative controls.

Extraction and Purification of Genomic DNA. Genomic DNA was extracted from plant materials including banana using a silica gel membrane-type kit (DNeasy Plant Mini, Qiagen, Hilden, Germany), according to the procedure described in previous studies (*15, 19, 20*). Genomic DNA was extracted from each banana-derived food material and commercial food product using an anion exchange-type kit (Genomic-tip 20/G, Qiagen) as described previously (*14, 18*). Extracted DNA was diluted with an appropriate volume of distilled water to a final concentration of 20 ng/μL and stored at −20 °C until use in the PCR analyses.

PCR Conditions. The reaction mixture for PCR was prepared in a PCR tube. The reaction volume of 25 μL contained 50 ng of template genomic DNA, 0.2 mM each of dNTP, 1× Ex Taq buffer (Mg^{2+} free), 2.0 mM $MgCl_2$, 0.625 unit of Ex Taq (Takara, Shiga, Japan), and 0.2 μM each primer (**Table 1B**), topped up with distilled water. When the concentration of extracted DNA was <20 ng/μL, 2.5 μL of undiluted DNA extract was added to the reaction tube for normalizing volume of template DNA. Amplification was performed in a GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA) as follows: preincubation at 95 °C for 10 min; 40 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 64 °C (60 °C for CP 03−5′/CP 03−3′ primer set (*14*)) for 0.5 min, and extension at 72 °C for 0.5 min; and final extension at 72 °C for 7 min. After PCR amplification, agarose gel electrophoresis of the PCR products (5 μL) was carried out according to previous studies (*14, 18*). The amplified fragments generated with the BAN-F/BAN-R primer set were cloned into pCR II-TOPO vector (TOPO TA cloning kit, Invitrogen, Tokyo, Japan), and the sequences were analyzed using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

SYBR Green I Real-Time PCR. For real-time PCR amplification, the reaction mixture (25 μL) contained 50 ng of template genomic DNA, 0.2 μM each of the primer set, and 12.5 μL of *Power* SYBR Green PCR Master mix (Applied Biosystems). Reactions were performed using the Applied Biosystems 7500 Real-Time PCR System, with the following PCR conditions: 50 °C for 2 min, 90 °C for 10 min, followed by 90 °C for 15 s and 64 °C for 1 min, repeated for 45 cycles. For dissociation after PCR amplification, the protocol included a slow heating from 60 to 95 °C at increments of 0.2 °C. T_m curve analysis was performed using SDS software 1.2 (Applied Biosystems).

RESULTS

Primer Design for Banana DNA Detection. To specifically detect banana by PCR, banana-specific genes have been investigated. For various banana-specific gene sequences, a primer set was designed for PCR amplification of DNA extracted from banana and other food samples including fruits. The banana *rbcL* gene was predicted to have the highest specificity for detection of banana DNA in foods on the basis of previous investigations (*21, 22*). Therefore, in this study we used the gene encoding banana *rbcL* as the banana-specific gene and designed the primer set BAN-F/BAN-R on the basis of its reported sequence (GenBank accession no. AF378770). The primer set CP 03−5′/CP 03−3′ was used for the universal detection of DNA derived from plants to verify DNA extraction (*14*); this primer set generated a 123 bp amplified fragment from genomic DNA extracted from banana. We amplified DNA from banana and other food samples using these primer sets. The PCR results confirmed that products of the target size could be predicted from *Musa* spp. The sequences of the designed primers used in this study are in **Table 1**.

Specificity and Sensitivity of Conventional and Semiquantitative Real-Time PCR. The specificity of the detection method using the BAN-F/BAN-R primer set was confirmed by conventional PCR. The amplification products obtained using the CP 03−5′/CP 03−3′ primer set were detected in all DNA samples (**Figure 1A**), confirming the quality of the template DNA. The conventional PCR fragment (186 bp) amplified using the BAN-F/BAN-R primer set was specifically detected in genomic DNA of banana and monkey banana; no cross-reactivity was detected for DNA extracted from 23 other fruits (**Figure 1B**) and eight crops (wheat, buckwheat, peanut, rice, corn, soybean, walnut, and yam) (data not shown). The sensitivity of conventional PCR was evaluated using from 5000 to 0.005 pg of banana DNA spiked in 50 ng of salmon testis DNA; the detection limit was 0.05 pg of banana genomic DNA, which corresponded to ca. 1 ppm (w/w) banana DNA (**Figure 3A**). The target products were also detected in the chocolate sample spiked with 1% (w/w) banana chip and in the soy milk sample spiked with 0.01% (w/w) banana puree, which corresponded to ca. 380 and 1.1 ppm of banana protein, respectively (**Table 4**, no. 1 and 2).

As the sequences of banana and yam from the GenBank database have similar homology (**Figure 2**), real-time PCR using SYBR Green I was examined for identifying these plants. Nonspecific bands seen in **Figure 1C,D** were detected at low levels under the extension temperature of 64 °C compared to 60 °C. As these PCR products have specific melting temperatures (T_m), melting curve analysis could discriminate between the PCR products of banana (T_m = 76.9 °C) and yam (78.3 °C) genomic DNA. There was no cross-reactivity to 30 other fruits and crops except yam using either real-time or conventional PCR. The amplification curve could be consistently obtained from 50000 to 0.5 pg (10 ppm equivalency) of banana DNA (**Figure 3B**); the calculated R^2 value of the standard curve was 0.995 in the range from 50000 to 50 pg (**Figure 3C**). These results show that banana genomic DNA can be specifically detected using the BAN-F/BAN-R primer set.

The allelic variation of the *rbcL* gene among different banana cultivars from Philippines (four cultivars), Taiwan (two),

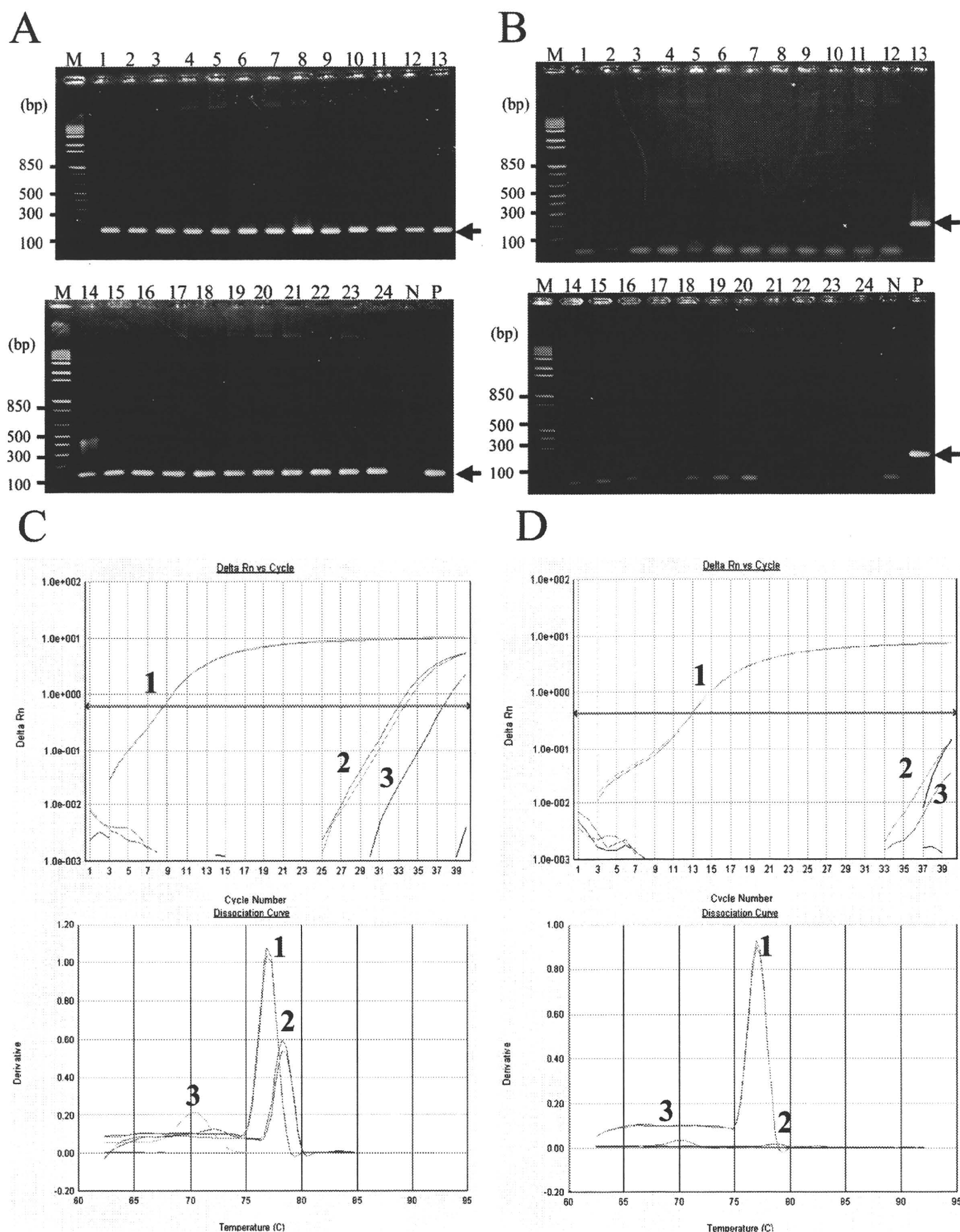

Figure 1. Specificity of PCR using the BAN-F/BAN-R primer set. Amplification of DNA from various plant species was performed with primer sets for plants (**A**) or banana (**B**). The arrowhead indicates the expected PCR product. Lanes: P, amplification of 50 ng of genomic DNA of banana; M, DNA marker (100 bp ladder size standard, Invitrogen); N, nontemplate control; 1−24, amplification of 50 ng of genomic DNA of apple (1), pear (2), Japanese pear (3), Japanese persimmon (4), peach (5), prune (6), apricot (7), Japanese apricot (8), cherry (9), orange (10), Satsuma orange (11), fig (12), monkey banana (13), kiwifruit (14), grape (15), blueberry (16), raspberry (17), juneberry (18), strawberry (19), melon (20), avocado (21), papaya (22), pineapple (23), and mango (24). Amplicons were electrophoresed on a 1.6% agarose gel. Amplification curve and melting curve of banana and yam PCR products were obtained under the extension temperatures of 60 (**C**) and 64 °C (**D**). Curves: 1 and 2, amplifications of 50 ng of banana and yam DNA, respectively; 3, nontemplate control. All reactions were performed in duplicate.

Ecuador (one), Mexico (one), and Colombia (one) was tested using both conventional and real-time PCR. Only the expected PCR products (123 bp in size) with identical size and relative intensity were obtained from these nine cultivars by conventional PCR (**Figure 4**). The melting curve analysis of real-time PCR obtained similar T_m values ranging from 76.2 to 76.7 °C in these nine cultivars using 50 ng of DNA per reaction (**Table 2**). The slight variation in the T_m values might be from the quality of template genomic DNA used. These results suggest that the *rbcL* gene has no allelic variation among the different banana cultivars,

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Banana: TCGTC CCCTA TTGGG ATGCA CTATT AAACC AAAAT TGGGA TTATC TGCAA AAAAC
Yam   : CCGTC CCCTA TTGGG ATGCA CTATT AAACC AAAAT TGGGG TTATC CGCAA AGAAC

Banana: TACGG TAGAG CGGTT TATGA ATGTC TACGT GGTGG ACTTG ATTTT ACCAA AGATG
Yam   : TACGG CAGAG CCGTT TATGA ATGTC TACGT GGTGG ACTTG ATTTT ACCAA GGATG

Banana: ATGAA AACGT AAACT CACAA CCATT TATGC GTTGG AGAGA TCGTT TCTTA TTTTG
Yam   : ATGAA AATGT GAACT CACAA CCATT TATGC GTTGG AGAGA CCGTT TCTTA TTTTG

Banana: CACCG AAGCA CTTTT TAAAG C
Yam   : TGCCG AAGCA CTTTT TAAAG C
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Figure 2. Comparison of gene sequences of banana and yam: partial DNA sequence of the target region on the ribulose-1,5-bisphosphate carboxylase (*rbcL*) gene. The right and left arrows indicate the forward (BAN-F) and reverse (BAN-R) primers, respectively. GenBank accession no.: banana (*Musa acuminata*), AF378770; yam (*Dioscorea japonica*), AF307457.

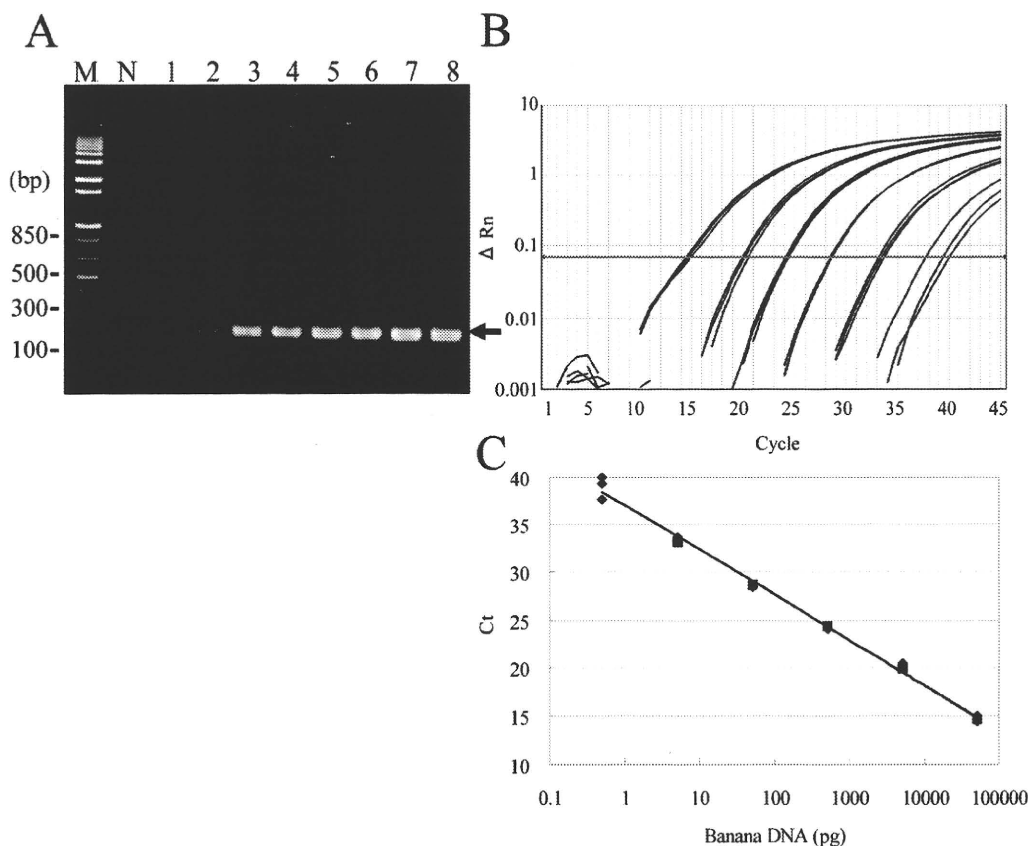

Figure 3. Sensitivity of specific detection method for banana. (**A**) Samples of salmon testis DNA containing various amounts of genomic DNA extracted from banana were used as template DNA. The arrowhead indicates the expected PCR product. Lanes: M, DNA marker (100 bp ladder size standard); N, nontemplate control; 1−8, amplification of genomic DNA samples with banana concentrations of 5 fg (1), 50 fg (2), 500 fg (3), 5 pg (4), 50 pg (5), 500 pg (6), 5 ng (7), and 50 ng (8). Amplicons were electrophoresed on a 1.6% agarose gel. (**B**) Amplification curve generated by serial dilution of banana DNA ranging from 50000 to 0.5 pg. All reactions were performed in triplicate. (**C**) Calibration curve generated from the amplification data in **A**. The relationship between the threshold cycle Ct and log DNA amount was determined to be $y = -4.7273 \log_{10}x + 37.06$ ($R^2 = 0.9945$).

and the copy number of the *rbcL* gene in the tested banana cultivars appears to be consistent.

Repeatability and Reproducibility for Real-Time PCR. Repeatability and reproducibility were evaluated using a dilution of banana genomic DNA (i.e., 50000, 5000, 500, 50, 5, and 0.5 pg). The mean and standard deviation (SD), coefficient of variation (CV %) of the Ct values of repeatability were calculated according to the mean value from three replications performed by one researcher on the same day and on three different days. As seen in **Table 3**, the CV of the Ct values ranged from 0.48 to 3.08% for testing repeatability on one day. For reproducibility, the CV of the Ct values ranged from 2.04 to 5.18% for three different days. All of the results of the repeatability and reproducibility tests indicate that this banana *rbcL* gene quantitative PCR method is reliable.

Application of the Banana DNA Detection Methods in Commercial Products. To investigate the applicability of the banana DNA detection methods for commercial food products, eight food products were purchased from a local market and examined for the presence of banana (**Table 4**, no. 3−10). Labeling indicated that all of the food products contained banana, except for chocolate and soy milk. For PCR, a sufficient amount (20 ng/μL) of extracted DNA was obtained from five processed food products (banana chip, chocolate, soy milk, soft cookie, and cereal product),

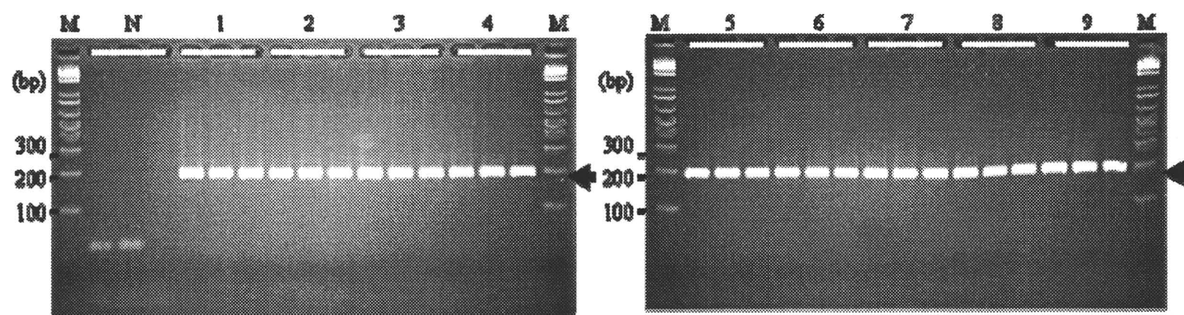

Figure 4. Conventional PCR results. The arrowhead indicates the expected PCR product. Lanes: M, DNA marker (100 bp ladder size standard, Invitrogen); N, nontemplate control; 1−9, amplification of 50 ng of genomic DNA of Philippines-1 (1), Taiwan-1 (2), Taiwan-2 (3), Philippines-2 (4), Philippines-3 (5), Colombia (6), Mexico (7), Ecuador (8), and Philippines-4 (9). Amplicons were electrophoresed on a 2.0% agarose gel. All reactions were performed in triplicate.

Table 2. Melting Curve Analysis Using Real-Time PCR[a]

	real-time PCR	
cultivar	mean T_m	SD
Philippines-1	76.4	0.2
Philippines-2	76.3	0.0
Philippines-3	76.6	0.2
Philippines-4	76.7	0.0
Taiwan-1	76.2	0.2
Taiwan-2	76.7	0.0
Colombia	76.4	0.2
Mexico	76.7	0.0
Ecuador	76.3	0.0

[a] All reactions were performed in triplicate. SD, standard deviation of T_m value

Table 3. Repeatability and Reproducibility of PCR[a]

banana genomic DNA dilution (pg)		same day (n = 3)			
		1	2	3	3 days (n = 9)
50000	mean Ct	15.04	13.81	14.65	14.73
	SD	0.13	0.38	0.33	0.30
	CV	0.86	2.75	2.25	2.04
5000	mean Ct	21.69	20.10	21.09	21.04
	SD	0.57	0.62	0.29	0.80
	CV	2.63	3.08	1.38	3.80
500	mean Ct	26.35	23.89	24.72	25.47
	SD	0.27	0.50	0.53	1.32
	CV	1.02	2.09	2.14	5.18
50	mean Ct	31.07	27.43	27.52	29.76
	SD	0.71	0.68	0.72	1.16
	CV	2.29	2.48	2.62	3.90
5	mean Ct	36.31	32.13	31.96	34.85
	SD	0.31	0.41	0.34	1.30
	CV	0.85	1.28	1.06	3.73
0.5	mean Ct	40.63	36.19	35.29	39.81
	SD	0.34	0.19	0.17	0.95
	CV	0.84	0.53	0.48	2.39

[a] SD, standard deviation of Ct value; CV, coefficient of variation of Ct value (%).

whereas < 20 ng/μL could be obtained from the other products. The universal primer set (CP03−5′/CP03−3′) could generate a specific amplified fragment from all samples (**Table 4**). Banana DNA from all products containing banana was clearly detected by both conventional and real-time PCR using the BAN-F/BAN-R primer set; amplification was not detected for soy milk and chocolate samples lacking banana. The amplified sequence from the processed foods was confirmed to be that for banana reported in the GenBank database (accession no. AF378770). These results suggest that these methods are applicable for the detection of residual banana in a wide variety of processed food products.

DISCUSSION

Bananas are valuable fruits consumed worldwide, but a serious source of fruit allergy. In 1991, cross-reactivity was reported between banana and latex with different kinds of plants (*6*). Half of the patients with latex allergy had simultaneously induced fruit allergies, including those for banana, chestnut, and avocado, a phenomenon termed the latex−fruit syndrome (*7, 8*). Sanchez-Monge et al. (*4*) reported class I chitinases with an N-terminal hevein-like domain to be one of the major allergens in banana, suggesting their pan-allergenic role in the latex−fruit syndrome. Currently, the only effective way to prevent the life-threatening allergic reactions to banana is to strictly avoid consuming banana, directly or through processed foods. Sufficient information about potentially allergenic ingredients in food products is thus necessary, and a specific and sensitive method of detecting banana residue is required, especially because processed foods contain a combination of many kinds of plant and animal ingredients.

In this study, a primer set was designed that specifically detects banana residue in commercial food products. For specific detection of banana DNA with high sensitivity, the gene encoding banana *rbcL* from chloroplast DNA was used. Improvement in the sensitivity of our detection system for trace amounts of banana residue that contaminate processed foods could be successfully achieved by targeting this multicopy chloroplast gene found in the plant cells. These PCR detection methods were examined using genomic DNA from various fruits including banana and yam and found to be specific for detection of banana genomic DNA. Conventional PCR showed a sensitivity of 1 ppm for banana genomic DNA, thus providing a reliable, specific, and sensitive detection method of banana in processed food products. In addition, real-time PCR using SYBR Green I showed a sensitivity of 10 ppm using the fluorescent amplification signal. This method also proved to have sufficient accuracy, with good repeatability (SD of Ct values = 0.5−3.1%) and reproducibility (2.0−5.2%).

To determine the applicability of our banana DNA detection methods, we tested several commercial food products that are known to contain banana. The BAN-F/BAN-R primer set successfully generated banana-specific fragments from all of the examined food products containing banana. The sensitivity of these

Table 4. PCR Analysis of Commercial Products

no.	sample	concentration of template DNA (ng/μL)	conventional PCR[a] CP 03−5′/CP 03−3′	conventional PCR[a] BAN-F/BAN-R	SYBR Green assay BAN-F/BAN-R[a]
1	chocolate spiked with 1% banana chip	20	+	+	+
2	soy milk spiked with 0.01% banana puree	20	+	+	+
3	chocolate	20	+	−	−
4	soy milk	20	+	−	−
5	banana chip	20	+	+	+
6	banana puree	<10	+	+	+
7	fruit juice	<10	+	+	+
8	banana juice	<10	+	+	+
9	soft cookie with dried banana	20	+	+	+
10	cereal product	20	+	+	+

[a] +, DNA amplification; −, no DNA amplification.

methods was also verified; the specific PCR products could detect approximately 1.1 ppm of banana protein in a spiked soy milk sample. Therefore, trace amounts of banana in processed foods can be detected using these PCR methods, although their applicability may be limited to highly processed foods. Notably, the *rbcL* gene was confirmed to have neither allelic variation among nine different banana cultivars originating from five countries and processed foods containing banana nor variation in the copy number of the *rbcL* gene in these tested samples on the market.

The allergens in banana residue are proteins. The developed methods detect banana DNA. Depending on processing conditions, banana protein and banana genomic DNA could be differentially degraded. We consider that it also would be very important to clarify the correlation between both amounts of banana allergen and banana DNA in a food product. Further studies are underway to develop an ELISA method to detecting banana allergen and investigate the correlation of both amounts of banana allergen and banana DNA in a food product.

In conclusion, we successfully designed a primer set (BAN-F/BAN-R) for the detection of banana DNA using both conventional and real-time PCR. These PCR methods are both specific and useful in detecting trace amounts of banana in processed foods. Real-time PCR, in particular, allows many samples to be analyzed in a short time, with little risk of carry-over contamination from PCR-amplified products, as agarose gel electrophoresis is unnecessary. Thus, these methods can be applied to reliable monitoring and inspections of processed food labeling.

ACKNOWLEDGMENT

We thank Drs. S. Watanabe and T. Hirao of House Foods Corp. for kindly providing the fruit DNAs.

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Received for review February 18, 2010. Revised manuscript received June 16, 2010. Accepted June 21, 2010. This study was supported by Health and Labor Science Research Grants for Research from the Ministry of Health, Labor, and Welfare of Japan.