extraction efficiency between target species and other matrices. Therefore, we confirmed the sensitivity of each detection method using various incurred foods containing 10 μg/g (shrimp or crab soluble protein weight/sample weight). As shown in Figure 5, the target size PCR products were detected from the DNA of all positive model foods and were not detected from all negative model foods, although there is a difference in the signal strength of these PCR products. Therefore, we concluded that the two PCR methods would be sensitive enough to detect trace amounts of shrimp and crab species in processed commercial foods and as a confirmation method for positive ELISA screening tests.

Analysis of Commercial Food Products. To compare the sensitivity of PCR and ELISA and to validate the specificity of shrimp- and crab-PCR, we tested 27 commercial products for the presence of shrimp and crab using two ELISA kits and two PCR methods as shown in Table 3. In 15 of 27 samples, the results of PCR amplification were consistent with the declaration in the list of ingredients and the content of crustacean protein measured using ELISA. Sample 14 tested positive with the shrimp-PCR, although shrimp was not declared in the list of ingredients. As the sequence of the amplification product from sample 14 matched with that of Western Australian rock lobster (Panulirus cygnus) in GenBank, sample 14 was thought to be contaminated with a trace amount of it. In Japanese regulation, shrimp and crab must be differentially declared when $\geq$10 ppm (total protein) of an allergenic ingredient is present. Four samples (no. 3, 8, 10, and 18) with declaration contained protein levels of less than 10 ppm with ELISA, but were positive with either of the PCR methods. The other seven samples (no. 4, 6, 7, 9, and 11−13) with declaration contained levels of less than 10 ppm with ELISA, and were also negative with the PCR methods. During processing of foods, proteins and DNA are subject to denaturation and fragmentation, which may render them undetectable by ELISA or PCR methods. It should be kept in mind that, since DNAs are generally less susceptible to degradation than proteins are to denaturation, and PCR methods are highly sensitive, they may detect very low levels of a contaminant that may not be clinically relevant. Therefore, PCR methods are to be used in conjunction with ELISA tests to determine the levels of the contaminating proteins. The shrimp- and crab-PCR methods detected shrimp in samples 1, 2, and 5, and crab in samples 15, 16, and 17, correctly as declared in the respective list of ingredients. Hence, they are particularly useful as confirmatory tests for differential detection of shrimp and crab species after positive ELISA results.

Conclusion. We newly developed the shrimp- and crab-PCR methods for final and differential detection of shrimp and crab species. The PCR methods were sensitive enough to detect 5 pg of DNA extracted from target species and 50 ng of genomic DNA extracted from incurred foods containing 10 ppm (μg/g) total protein of either Black tiger shrimp or King crab, and were considered to be specific enough to detect shrimp and crab separately, although some false-positive and false-negative results occurred. PCR technique targets a specific DNA sequence, not allergenic protein, to detect the presence of an allergenic food, and is particularly suitable for confirmation of positive results from ELISA tests that determine the levels of the contaminating proteins. Both PCR methods developed here met the specifications for, and were included in the notification by Japanese regulatory agency in 2009 as the methods for final identification of the presence of shrimp and crab species after two ELISA assays. They are useful for confirming the validity of food labeling and giving allergic consumers a wider range of food options. The akiami paste shrimp-PCR and mantis shrimp-PCR, developed in this study, complement the shrimp- and crab-PCR in case false-negative or false-positive results are suspected.

Table 3. Analysis of 27 Commercial Food Products for the Presence of Shrimp and Crab

sample		D[a]		ELISA (ppm)		PCR	
no.	description	shrimp	crab	N kit[b]	M kit[c]	shrimp[d]	crab
1	candy I	+	−	>50	23.3	pos	neg
2	instant noodle I	+	−	>50	23.0	pos	neg
3	clam chowder	+	−	<0.78	<0.78	pos	neg
4	curry sauce I	+	−	3.5	3.1	neg	neg
5	bouillabaisse sauce	+	−	25.4	22.3	pos	neg
6	pasta sauce I	+	−	<0.78	<0.78	neg	neg
7	pasta sauce II	+	−	<0.78	<0.78	neg	neg
8	pasta sauce III	+	−	<0.78	<0.78	pos	neg
9	curry sauce II	+	−	<0.78	<0.78	neg	neg
10	stew roux block	+	−	<0.78	0.8	pos	neg
11	chowder roux block	+	−	<0.78	<0.78	neg	neg
12	instant noodle II	−	+	<0.78	<0.78	neg	neg
13	dehydrated soup I	−	+	<0.78	<0.78	neg	neg
14	seasoning	−	+	8.7	7.5	pos	pos
15	soup	−	+	>50	24.8	neg	pos
16	pasta sauce IV	−	+	>50	24.5	neg	pos
17	rice gruel I	−	+	>50	25.7	neg	pos
18	seasoning paste	+	+	<0.78	<0.78	neg	pos
19	rice gruel II	+	+	54.8	27.9	pos	pos
20	candy II	−	−	<0.78	<0.78	neg	neg
21	dehydrated soup II	−	−	<0.78	<0.78	neg	neg
22	curry sauce II	−	−	<0.78	<0.78	neg	neg
23	pasta sauce V	−	−	<0.78	<0.78	neg	neg
24	pasta sauce VI	−	−	<0.78	<0.78	neg	neg
25	pasta sauce VII	−	−	<0.78	<0.78	neg	neg
26	curry sauce III	−	−	<0.78	<0.78	neg	neg
27	curry roux block	−	−	<0.78	<0.78	neg	neg

[a] Labeling of shrimp and crab components: +, declaration of shrimp or crab; −, without declaration. [b] N kit is Food Allergen Test EIA Crustacean "Nissui" (the Nissui Pharmaceutical Co., Ltd.). [c] M kit is crustacean kit "Maruha" (the Maruha Nichiro Holdings, Inc.). Range of quantitation of both ELISA kits is 0.78−50 ppm. [d] The results of shrimp- and akiami paste shrimp-PCR; pos indicates that PCR product with target size was detected with at least shrimp- or akiami paste shrimp-PCR; neg indicates that no PCR product with target size was detected with both shrimp- and akiami paste shrimp-PCR.

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REFERENCES

(1) Lehrer, S. B.; Ayuso, R.; Reese, G. Seafood allergy and allergen: A review. *Marine Biotechnol.* **2003**, *5*, 339–348.

(2) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission relating to the evaluation of allergenic foods for labelling purposes, Request N° EFSA-Q-2003-016. *EFSA J.* **2004**, *32*, 52-58.

(3) Tomikawa, M.; Suzuki, N.; Urisu, A.; Tsuburai, T.; Ito, S.; Shibata, R.; Ito, K.; Ebisawa, M. Characteristics of shrimp allergy from childhood to adulthood in Japan. *Jpn. J. Allergol.* **2006**, *55*, 1536–1542.

(4) Department of Food Safety, the Ministry of Health, Labour and Welfare of Japan, Notification No. 1106001. 2002.

(5) Department of Food Safety, the Ministry of Health, Labour and Welfare of Japan, Notification No. 0603001. 2008.

(6) Department of Food Safety, the Ministry of Health, Labour and Welfare of Japan, Notification No. 0622003. 2006.

(7) Watanabe, T.; Akiyama, H.; Yamakawa, H.; Iijima, K.; Yamazaki, F.; Matsumoto, T.; Futo, S.; Arakawa, F.; Watai, M.; Maitani, T. A specific qualitative detection method for peanut (*Arachis hypogaea*) in foods using polymerase chain reaction. *J. Food Biochem.* **2006**, *30*, 215–233.

(8) Yamakawa, H.; Akiyama, H.; Endo, Y.; Miyatake, K.; Sakata, K.; Sakai, S.; Toyoda, M.; Urisu, A. Specific detection of wheat residues in processed foods by polymerase chain reaction. *Biosci., Biotechnol., Biochem.* **2007**, *71*, 2561–2564.

(9) Yamakawa, H.; Akiyama, H.; Endo, Y.; Miyatake, K.; Sakata, K.; Sakai, S.; Toyoda, M.; Urisu, A.; Teshima, R. Specific detection of buckwheat residues in processed foods by polymerase chain reaction. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 2228–2231.

(10) Seiki, K.; Oda, H.; Yoshioka, H.; Sakai, S.; Urisu, A.; Akiyama, H.; Ohno, Y. A reliable and sensitive immunoassay for the determination of crustacean protein in processed foods. *J. Agric. Food Chem.* **2007**, *55*, 9345–9350.

(11) Shibahara, Y.; Oka, M.; Tominaga, K.; Ii, T.; Umeda, M.; Uneo, N.; Abe, A.; Ohashi, E.; Ushio, H.; Shiomi, K. Determination of crustacean allergen in food products by sandwich ELISA. *Jpn. Food Sci. Technol. Res.* **2007**, *54*, 280–286.

(12) Sakai, S.; Matsuda, R.; Adachi, R.; Akiyama, H.; Maitani, T.; Ohno, Y.; Oka, M.; Abe, A.; Seiki, K.; Oda, H.; Shiomi, K.; Urisu, A. Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the determination of crustacean protein in processed foods. *J. AOAC Int.* **2008**, *91*, 123–129.

(13) Food Labeling Division, the Consumer Affairs Agency of Japan, Notification No. 286. 2010.

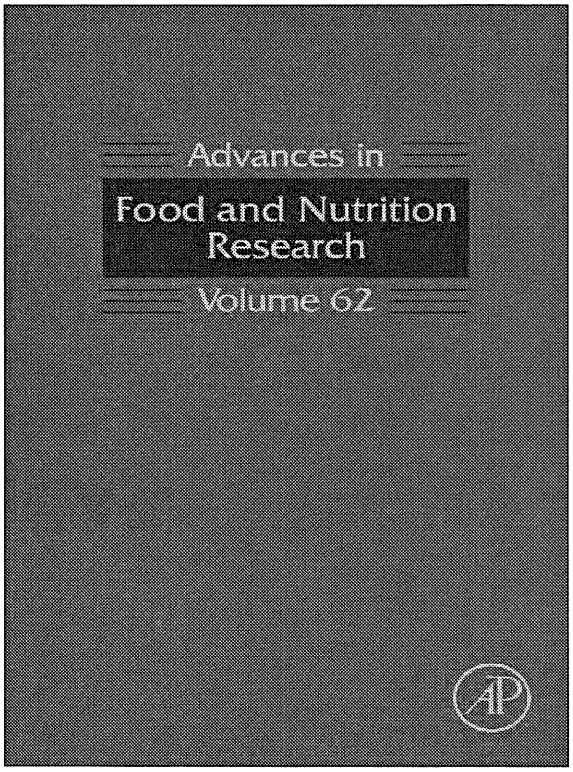
(14) Hirao, T.; Imai, S.; Sawada, H.; Shiomi, N.; Hachimura, S.; Kato, H. PCR method for detecting trace amounts of buckwheat (*Fagopyrum* spp.) in food. *Biosci., Biotechnol., Biochem.* **2005**, *69*, 724–731.

(15) Hirao, T.; Watanabe, S.; Temmei, Y.; Hiramoto, M.; Kato, H. Qualitative polymerase chain reaction methods for detecting major food allergens (peanut, soybean, and wheat) by using internal transcribed spacer region. *J. AOAC Int.* **2009**, *92*, 1464–1471.

(16) Taguchi, H.; Watanabe, S.; Hirao, T.; Akiyama, H.; Sakai, S.; Watanabe, T.; Matsuda, R.; Urisu, A.; Maitani, T. Specific detection of potentially allergenic kiwifruit in foods using polymerase chain reaction. *J. Agric. Food Chem.* **2007**, *55*, 1649–1655.

(17) Pettersson, M.; Bylund, M.; Alderborn, A. Molecular haplotype determination using allele-specific PCR and pyrosequencing technology. *Genomics* **2003**, *82*, 390–396.

(18) Sakai, S.; Adachi, R.; Shibahara, Y.; Oka, M.; Abe, A.; Seiki, K.; Oda, H.; Yoshioka, H.; Shiomi, K.; Urisu, A.; Akiyama, H.; Teshima, R. A survey of crustacean soluble proteins such as "shrimp" and "crab" content in food ingredients. *Jpn. J. Food Chem.* **2008**, *15*, 1–6.



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CHAPTER 4

Japan Food Allergen Labeling Regulation—History and Evaluation

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Abstract According to a national survey of food allergy cases, the food-labeling system for specific allergenic ingredients (i.e., egg, milk, wheat, buckwheat, and peanut) in Japan was mandated under law on April 1, 2002. By Japanese law, labeling of allergens is designated as mandatory or recommended based on the number of cases of actual illness and the degree of seriousness. Mandatory labeling is enforced by the ministerial ordinance, and the ministerial notification recommends that foods containing walnut and soybean be labeled with subspecific allergenic ingredients. Additional labeling of shrimp/prawn and crab has also become mandatory since 2008. To monitor the validity of the labeling system, the Japanese government announced the official methods for detection of allergens in a November 2002 ministry notification. These official methods, including two kinds of enzyme-linked immunosorbent assay kits for screening, Western blotting analyses for egg and milk, and polymerase chain reaction analyses for wheat, buckwheat, peanut, shrimp/prawn and crab as confirmation tests, have provided a means to monitor the labeling system. To standardize the official methods, the Japanese government described the validation protocol criteria in the 2006 official guidelines. The guidelines stipulate that any food containing allergen proteins at greater than 10 mg/kg must be labeled under the Law. This review covers the selection of the specific allergenic ingredients by the Japanese government, the implementation of regulatory action levels and the detection methods to support them, and the assessment of the effectiveness of this approach.

I. ASSESSMENT OF IMMEDIATE-TYPE FOOD ALLERGIES IN JAPAN

Food allergies that cause immediate reactions had already been under investigation prior to any discussion of "allergy food labeling" under the food sanitary law for prepackaged processed foods and food additives. Before implementation of the allergy food-labeling system in Japan, a research group supported by the Ministry of Health and Welfare of Japan had collected epidemiological data on immediate-type food allergies during both childhood and adulthood in Japan in 1998 and 1999. This retrospective study asked hospitals with more than 200 beds to report all immediate-type food allergy cases treated by the emergency department. The questionnaire included information on age, sex, cause of the food allergy, symptoms, IgE CAP RAST, and type of treatment. To focus on the

immediate-type, only cases in which symptoms occurred within 60 min after ingestion of the suspected food were included. Of the 2623 hospitals surveyed, 1623 hospitals responded and 1420 cases were analyzed. As shown in Table 4.1, hen's eggs were the most common allergen, followed by cow's milk, wheat, buckwheat, fishes, fruits, and shrimp. The top three major food allergens were most prevalent among the pediatric population, whereas fishes, buckwheat, and shrimp were mainly reported in adults. Based on these data, the Ministry of Health and Welfare selected 24 candidates that caused more than four cases of adverse reaction for the allergy food-labeling system. Following roundtable discussions among specialists and regulatory officers of the Ministry of Health and Welfare, hen's eggs, cow's milk, wheat, buckwheat, and peanuts were selected as items for mandatory labeling by the 2000 ministerial ordinance; the remaining 19 allergens were designated as items for recommended labeling by a ministerial notification.

To further understand the real-time condition of food allergies in Japan, we investigated prospectively the immediate-type food allergy cases in collaboration with more than 2000 doctors between 2001 and 2002 to account for recall bias in the previous study. The contributing doctors included those working in hospitals with more than 200 beds as well as allergy specialists working in clinics. Contributing doctors were asked to respond to a questionnaire every 3 months for 2 years from 2001 to 2002 and report immediate-type food allergy cases by mail. The same questionnaire as that in the previous studies was used, and only immediate-type food allergies as defined in the previous study were included. A total of 3882 cases were reported within the 2 years (Table 4.2). The cases ranged from 0 to 80 years of age, with 50% (1969) of them below 2 years of age. The most common cause of food allergy was hen's eggs (38.3%), followed by cow's milk (15.9%), wheat (8%), shellfish (6.2%), fruits (6%), buckwheat (4.6%), fishes (4.4%), and peanuts (2.8%). Notably, the cause of food allergy differed greatly among age groups. Food-induced anaphylaxis was seen in 10.9% of the reported cases. As shown in Table 4.3, hen's eggs, cow's milk plus its products, wheat, buckwheat, and peanuts were the major causes of food-induced anaphylaxis in Japan. Compared to our previous investigation, fruit allergies against kiwi and banana seemed to be an increasing trend. Thus, the present Ministry of Health, Labor, and Welfare of Japan (MHLW) has been implementing countermeasures against food allergies to improve the quality of life of afflicted patients. This prospective investigation on immediate-type food allergies has been repeated every 3 years as a means to monitor the condition of food allergies in Japan. The results of these investigations have improved the allergy food-labeling system by including banana as a recommended item by a ministerial notification and shrimp and crab as mandatory items for labeling by a ministerial ordinance.

TABLE 4.1 Immediate type of food allergy cases reported from 1998 to 1999

Offending food, *n* (%)	Total	>1 year	1 year	2–3 years	4–6 years	7–19 years	20+ years
Egg	420 (29.6)	197 (47.4)	72 (30.4)	89 (30.8)	35 (25.0)	19 (9.2)	8 (6.1)
Milk product	324 (22.8)	128 (30.8)	66 (27.8)	70 (24.2)	34 (24.3)	21 (10.1)	5 (3.8)
Wheat	147 (10.4)	40 (9.6)	20 (8.4)	35 (12.1)	12 (8.6)	27 (13.0)	13 (9.9)
Buckwheat	82 (5.8)	1 (0.2)	10 (4.2)	16 (5.5)	10 (7.1)	29 (14.0)	16 (12.2)
Fish	73 (5.1)	15 (3.6)	9 (3.8)	10 (3.5)	5 (3.6)	13 (6.3)	21 (16.0)
Fruits	66 (4.6)	6 (1.4)	13 (5.5)	13 (4.5)	8 (5.7)	19 (9.2)	7 (5.3)
Shrimp	51 (3.6)	0 (0.0)	2 (0.8)	4 (1.4)	4 (2.9)	22 (10.6)	19 (14.5)
Meat	44 (3.1)	9 (2.2)	2 (0.8)	4 (1.4)	4 (2.9)	14 (6.8)	11 (8.4)
Peanut	34 (2.4)	3 (0.7)	12 (5.1)	5 (1.7)	6 (4.3)	5 (2.4)	3 (2.3)
Soybean	27 (1.9)	5 (1.2)	8 (3.4)	4 (1.4)	3 (2.1)	4 (1.9)	3 (2.3)
Other	152 (10.7)	12 (2.9)	23 (9.7)	39 (13.5)	19 (13.6)	34 (16.4)	25 (19.1)
Total	1420	416	237	289	140	207	131

TABLE 4.2 Immediate type of food allergy cases reported from 2001 to 2002

Offending food, *n* (%)	Total	>1 year	1 year	2–3 years	4–6 years	7–19 years	+20 years
Egg	1486 (38.3)	789 (62.1)	312 (44.6)	179 (30.1)	106 (23.3)	76 (15.2)	24 (6.6)
Milk product	616 (15.9)	255 (20.1)	111 (15.9)	117 (19.7)	84 (18.5)	41 (8.2)	8 (2.2)
Wheat	311 (8.0)	90 (7.1)	49 (7.0)	46 (7.7)	24 (5.3)	48 (9.6)	54 (14.8)
Fruits	232 (6.0)	40 (3.1)	30 (4.3)	30 (5.1)	40 (8.8)	45 (9.0)	47 (12.8)
Buckwheat	179 (4.6)	4 (0.3)	23 (3.3)	45 (7.6)	27 (5.9)	54 (10.8)	26 (7.1)
Fish	171 (4.4)	21 (1.7)	32 (4.6)	22 (3.7)	18 (4.0)	37 (7.4)	41 (11.2)
Shrimp	161 (4.1)	4 (0.3)	10 (1.4)	20 (3.4)	29 (6.4)	59 (11.8)	39 (10.7)
Peanut	110 (2.8)	4 (0.3)	22 (3.1)	31 (5.2)	28 (6.2)	22 (4.4)	3 (0.8)
Soybean	76 (2.0)	22 (1.7)	16 (2.3)	9 (1.5)	8 (1.8)	9 (1.8)	12 (3.3)
Meat	71 (1.8)	13 (1.0)	6 (0.9)	7 (1.2)	7 (1.5)	19 (3.8)	19 (5.2)
Other	469 (12.1)	28 (2.2)	88 (12.6)	88 (14.8)	83 (18.3)	89 (17.8)	93 (25.4)
Total	3882	1270	699	594	454	499	366

TABLE 4.3 Anaphylaxis cases reported from 2001 to 2002

No.	Offending food	*n* (%)
1	Egg	109 (27.6)
2	Milk product	93 (23.5)
3	wheat	70 (17.7)
4	Buckwheat	28 (7.1)
5	Peanuts	18 (4.6)
6	Shrimp	14 (3.5)
7	Salmon roe	8 (2.0)
	Peach	8 (2.0)
9	Soybean	7 (1.8)
	Kiwi	7 (1.8)
11	Banana	4 (1.0)
	Yam	4 (1.0)
–	Other	25 (6.3)
	Total	395

II. JAPANESE FOOD ALLERGY-LABELING SYSTEM

Food allergies represent an important health problem in industrialized countries. In Japan, the number of people with food allergies is increasing, especially among young children, due to major changes in dietary habits with the introduction of western foods after World War II.

In 1999, the Joint FAO/WHO Codex Alimentary Commission Session agreed to recommend labeling of eight kinds of food which contain ingredients known to be allergens. This movement has led the Japanese government to take new measures to tackle food allergies in Japan.

A. Japanese regulations for labeling of food allergenic ingredients

The special subcommittee of MHLW held a meeting on the labeling of the Food Sanitation Investigation Council and stated that, "From the viewpoint of preventing the occurrence of health hazards, mandatory labeling of foods containing specific allergenic ingredients should be required." Accordingly, the MHLW decided that the Food Sanitation Law should provide for the mandatory labeling of foods containing allergenic ingredients designated in the 2000 ministerial ordinance.

Since the only therapy for a food allergy is avoidance of the responsible food, it is essential for food allergy patients to eliminate food allergens from their diet. Therefore, the Japanese MHLW decided to improve the

allergen-labeling system by amending the Food Sanitation Law in 2001 (Ebisawa *et al.*, 2003). They organized a labeling study group consisting of clinical experts, patients, researchers, retailers, and food industrialists. The group discussed different labeling system methods. The results were announced as a report. In the report outline, labeling was divided into two stages, mandatory and recommended, based on the number of cases of actual illnesses and the degree of seriousness (Table 4.4). Consequently, eggs, milk, wheat, buckwheat and peanuts, and most recently shrimp and crab require mandatory labeling by the ministerial ordinance; hereinafter, we refer to these seven ingredients as "specific allergenic ingredients." In addition, the ministerial notification recommends labeling of any food that contains the following 18 ingredients: abalone, squid, salmon roe, orange, kiwifruit, beef, walnut, salmon, mackerel, soybean, chicken, banana, pork, Matsutake mushroom, peach, yam, apple, and gelatin. Hereinafter, we refer to these ingredients as "subspecific allergenic ingredients." To the best of our knowledge, Japan is the first country to set up mandatory food allergy labeling and to regulate it under national law in 2002. The additional labeling requirement for shrimp/prawn and crab was introduced by the amendment of the food Sanitation Law under the MHLW in June 2008 due to the almost unlimited use of crustaceans in processed foods in Japan and the frequency of adverse food reactions in allergic patients.

Among shrimp allergy cases, 64.7% of patients showed positive reaction to crabs. The clinical evidence suggests that many shrimp allergy patients react to crabs. On the contrary, as the remaining 35.3% of patients showed no reaction to crabs, some patients with shrimp allergy can eat crabs. Thus, it would be important to label "shrimp" and "crab" separately, rather than as "crustacean" to give consumers more information. Accordingly, the MHLW has revised the mandatory labeling for shrimp

TABLE 4.4 Allergenic ingredients designated by the MHLW of Japan*

Specific allergenic ingredients
Mandatory by ministerial ordinance (seven ingredients)
Egg, milk, wheat, buckwheat, peanut, shrimp/prawn, and crab
Subspecific allergenic ingredients
Recommended by ministerial notification (18 ingredients)
Abalone, squid, salmon roe, orange, kiwifruit, beef, walnut, salmon, mackerel, soybean, chicken, banana, pork, Matsutake mushroom, peach, yam, apple, and gelatin

*Based on the Notification of March 15, 2001 and the newest Notification of June 3, 2008 from the Department of Food Safety, Ministry of Health, Labor, and Welfare (MHLW) of Japan.

and crab to be labeled separately. Since the management of the food-labeling policy was transferred from the MHLW to the Consumer Affairs Agency (CAA) in 2010, CAA announces the Japanese food-labeling system through ministry notifications.

The content scope of allergens for labeling was established based on the Japan Standard Commodity Classification. Japan is the first country to set up mandatory food allergy labeling and to regulate it under national law.

The characteristics of the Japanese labeling system are as follows.

1. Small quantity labeling

The specific allergenic ingredients must be labeled even in cases of carry-over conditions or when used as processing aids. Labeling of the 18 subspecific allergenic ingredients in Table 4.1 is recommended as much as possible.

2. "May contain" labeling

"May contain (name of allergenic ingredients)" type labeling is prohibited.

3. Combination of specified ingredients

With a few exceptions, the use of major item classifications (declaration of meats, cereals, etc.) is prohibited.

4. Declaration of high-grade food ingredients

In cases with high-grade food ingredients such as abalone, salmon roe, and mushroom mixed in very small quantities, a declaration such as "contains xxx extract" is required so as not to mislead consumers.

5. Method of declaring additives

For food additives, labeling shall, in principle, declare the "name of the substance (derived from)."

6. Declaration of flavorings

Aromatic ingredients have not yet been subjected to labeling, but should be labeled as much as possible.

7. Alcoholic beverages and related products

Alcoholic beverages are not currently subject to regulated labeling.

8. Alternative declaration

Alternative vocabulary usage in declaration is allowed for certain items, if the declaration can be considered allergen labeling in that the general (practical) expression used suggests that an allergenic ingredient is being used.

9. Specified processed foods

Specified processed foods generally known to be made from allergenic ingredients do not require declaration of such ingredients. For example, a sandwich using mayonnaise may mention "mayonnaise" instead of "egg."

III. REGULATION OF DETECTION METHODS FOR FOOD ALLERGENIC INGREDIENTS

A. Consideration of Japanese allergen-labeling thresholds

A system of labeling for food allergies is necessary for people with allergies. However, in general, proteins and nucleotides from allergens are not necessarily toxins. The threshold dose for an allergic reaction is often considered to be zero. However, a zero tolerance for the offending food would create enormous practical problems for the food industry. Therefore, the MHLW established a threshold of food allergy labeling and developed the official detection methods for specific allergenic ingredients. To do this, they organized a detection method study group consisting of manufacturing companies, retailers, public research institutes, universities, and private inspection institutes. Thereinafter, we have been developing detection methods for specific allergenic ingredients in foods.

The detection method study group considered how to set the threshold for labeling (Fig. 4.1). They presumed that the limits of detection (LOD) for enzyme-linked immunosorbent assay (ELISA) are generally in the range of 0.1–1.0 μg protein/g food. However, setting up the threshold for labeling in the range of LOD for ELISA would be difficult due to the large deviation in repeatability and reproducibility. In addition, LODs of lateral flow and polymerase chain reaction (PCR) methods would be approximately 5 μg protein/g food.

The labeling study group determined the threshold for the labeling system, that is, the definition of a trace amount. The group stated that, "If more than a few micrograms of protein weight per milliliter of food or a few micrograms of protein per gram of food are contained in a food, labeling of that allergen is necessary."

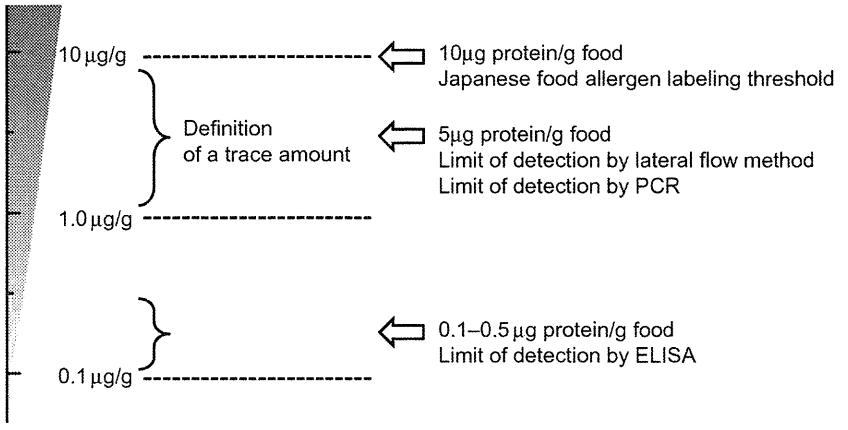

FIGURE 4.1 Consideration of Japanese food allergen-labeling threshold.

Considering these factors, we designated 10 μg protein/g food (the corresponding allergen soluble protein weight/food weight) as a threshold to monitor the labeling using ELISA. We believe that this level is the minimum for controlling the contamination of allergic ingredients using the detection method on an industrial scale.

Therefore, we developed detection methods for determining the presence of proteins on the level of a few micrograms per milliliter or gram of food based on the definition of a trace amount.

Accurate determination of the allergen proteins is difficult, however, as they undergo denaturation and degradation. Further, the standard allergen protein reference could change, as identical allergen proteins cannot always be obtained for every test. In Japan, the labeling of egg, milk, wheat, buckwheat, and peanut ingredients in any processed foods became mandatory in April 2002, while shrimp and crab became mandatory in June 2008. The Japanese official methods consisted of screenings of two different ELISA kits, the Western blot method for egg or milk and the PCR method for wheat, buckwheat, peanut, shrimp/prawn, and crab as the confirmation tests under the ministerial notification (Notification No. 1106001, 2002). The MHLW added the specification and standardization of the extraction buffer, reference material, and the standard solution for the testing of these five allergenic ingredients in 2004 (described in Section III.B). Further, the validation protocol criteria were included in the official guidelines in 2006 to standardize the Japanese official method for allergen detection (Notification No. 1106001, 2002), followed by addition of the ELISA, PCR methods and reference material, and the standard solution for the testing of crustaceans for detection of shrimp/prawn and crab in 2008.

B. Reference material and calibrator

To assess compliance to the mandatory labeling system of allergenic ingredients (eggs, milk, wheat, buckwheat, peanuts, and shrimp/prawn (crustaceans)) in processed foods in Japan established in April 2002, followed by shrimp and crab in June 2008, we have established two types of ELISA. However, some discrepancies exist between the results from the two kits, partly due to the use of different antibodies. Another possibility for the discrepancies could be the differences between the standard solutions provided in the kits. Since the test kits are used for regulatory purposes, we considered that the extraction buffer and reference standard for measurement should be unified and standardized between the test kits. Therefore, the MHLW set the specifications and standardization of the extraction buffer, reference material, and the standard solution for testing the five allergenic ingredients (Notification No. 1106001, 2002).

The specifications and standardization include raw materials, preparation method of the standard solution, concentration of proteins, and the main band on SDS-PAGE. The outline of the procedure for preparation of the calibrators is shown in Fig. 4.2. Table 4.5 shows the raw materials and the preparation method of the initial extract. To prepare the calibrators, the raw materials are extracted by the standard solution containing SDS and mercaptoethanol. The initial extract is prepared by centrifugation and filtration of the extract. The diluted extract is then prepared by 10-fold dilution of the initial extract with phosphate-buffered saline (PBS; pH 7.4). The protein concentration of the diluted extract is assayed using the 2-D Quant kit (Amersham Bio Sciences). The standard solution is then

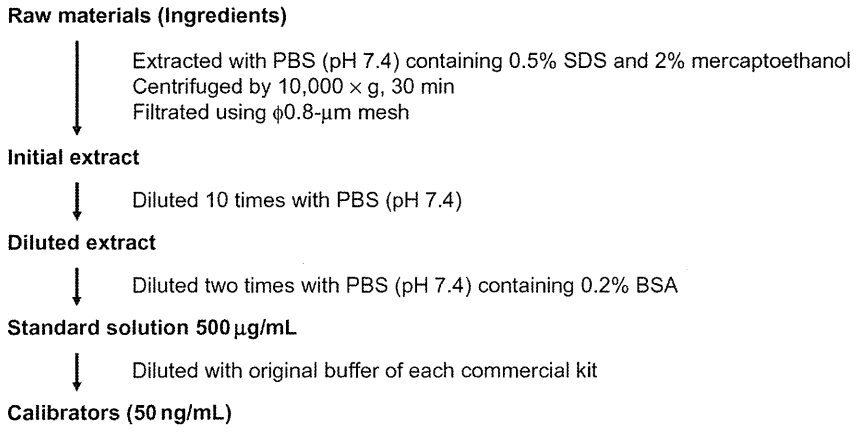

FIGURE 4.2 Procedure for preparing the calibrators.

TABLE 4.5 Raw materials and initial extraction methods

Allergenic food	Raw material (ingredients)	Extraction method (preparation)[a]
Egg	Fresh eggs of white leghorn hen, homogenized, and freeze-dried	0.2 g in 20 mL extraction solution[b] shaken overnight
Milk	Fresh milk of cows, freeze-dried after defatting by churning	0.2 g in 20 mL extraction solution shaken overnight
Wheat	Mixture of 14 species of wheat, pulverized	1.0 g in 20 mL extraction solution shaken overnight
Buckwheat	Mixture of buckwheat produced in Ibaraki Prefecture and China, pulverized	1.0 g in 20 mL extraction solution shaken overnight
Peanut	Virginia species produced in Chiba Prefecture, ground in a mortar	0.4 g in 20 mL extraction solution defatted by acetone and shaken overnight
Shrimp/prawn (Crustacean)	Fresh muscle of black tiger, homogenized, and freeze-dried	0.1 g in 20 mL extraction solution shaken overnight

[a] The protein content of the initial extract was determined using the 2-D Quant kit (Amersham Bio Sciences). The initial extract was diluted 20 times to make up the calibration standard solution.
[b] Extraction solution: buffer containing 0.5% SDS and 2% mercaptoethanol.

prepared by a twofold dilution with PBS (pH 7.4) containing 0.2% BSA. The calibrator included in each commercial kit is prepared by dilution of the standards (concentrated standard solution) to 50 ng/mL with each company kit's original buffer containing the carrier protein.

Three lots of initial extracts for each allergic ingredient were prepared following this procedure to assess the conformity to the specifications. The reproducibility of the protein concentration and the SDS-PAGE pattern of the initial extract solution were also checked (Table 4.6, Fig. 4.3). The initial extract solutions were stored at −80 °C for 6 months to evaluate their stability. The protein concentration and the SDS-PAGE pattern of the 3 lots were equivalent, and no significant variability occurred during the storage period. The calibration standard solution was stored at 4 and 37 °C. The calibration standard solutions were tested by the relevant ELISA kits once a month during storage, and the stability was checked by the obtained absorbance.

TABLE 4.6 Reproducibility of protein concentration determination

	Lot				
	1	2	3	Average	RSD%
Egg	4.55	4.69	4.88	4.71	3.52
Milk	2.57	2.63	2.52	2.57	2.14
Wheat	4.95	4.96	5.10	5.00	1.68
Buckwheat	3.37	3.47	3.59	3.48	3.17
Peanut	3.99	4.47	4.86	4.44	9.81
Shrimp/prawn	3.42	3.46	3.37	3.42	2.00

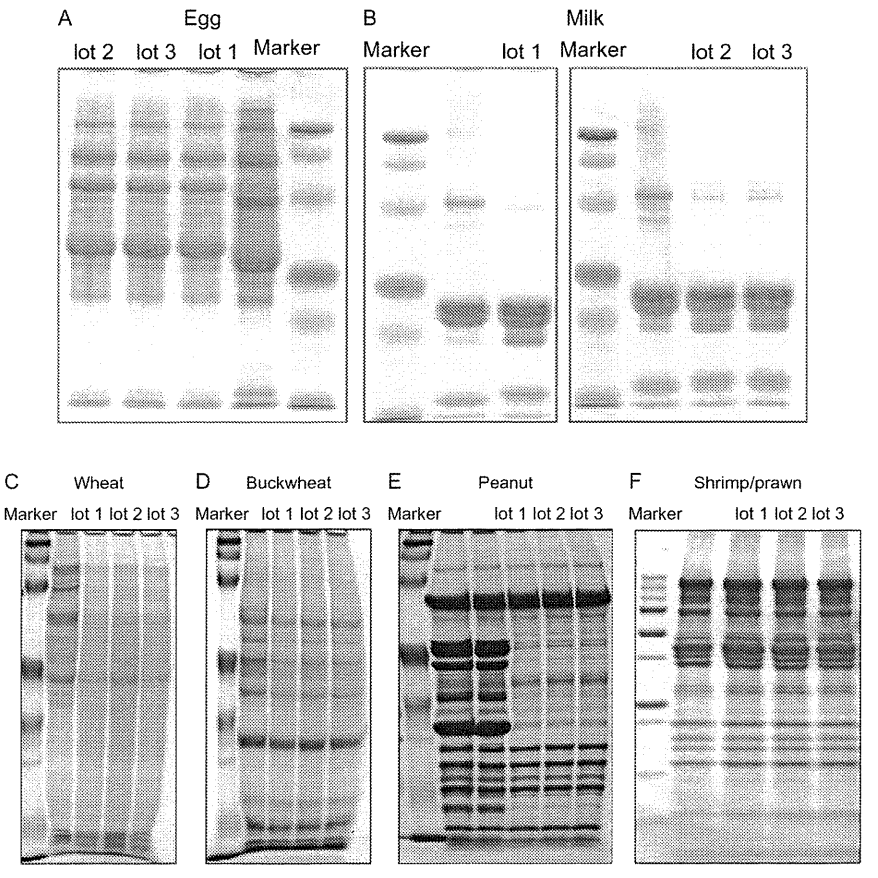

FIGURE 4.3 Reproducibility of SDS-PAGE results.

C. Japanese guideline criteria for validation protocol of specific allergenic ingredient detection method

The MHLW described the validation protocol criteria in the 2006 official guidelines to standardize the Japanese official method for specific allergenic ingredient detection. The outlines of the validation protocol criteria for the food allergenic ingredient quantitative and qualitative detection methods are shown in Tables 4.7 and 4.8, respectively.

The validation protocol criteria for the food allergenic ingredient quantitative detection method are as follows: (1) Eight or more laboratories (independent from the ELISA developer). (2) Five or more food samples (matrices). (3) A concentration of 10 μg/g food specific allergenic ingredient in the food sample (the corresponding allergenic ingredient soluble protein weight/food weight), the concentration defined as the "trace amount of contamination" (Any food containing the specific allergenic ingredient protein greater than 10 μg/g must be labeled for the relevant food specific allergenic ingredients under the Food Sanitation Law; if the specific allergenic ingredient protein level is less than 10 μg/g, labeling is not required). The food sample should be prepared by common processing methods, such as heating, baking, frying, acidifying, and

TABLE 4.7 Japanese guideline criteria for validation protocol of quantitative detection methods for food allergenic ingredients[a]

Number of laboratories	≥8
Number of incurred samples	≥5
Number of dose levels	≥1 including 10 μg/g[b]
Recovery	50–150%
RSDr	≤25%

[a] Based on Notification Nos. 1106001 of November 6, 2002, and 0622003 of June 22, 2006, from the Department of Food Safety of the MHLW of Japan.
[b] The corresponding allergenic ingredient soluble protein weight/food weight.

TABLE 4.8 Japanese guideline criteria for validation protocol of qualitative detection methods for food allergenic ingredients[a]

Number of laboratories	≥6
Number of incurred samples	≥5
Number of dose levels	≥2 including negative control (blank) and positive control (10 μg/g[b])
Precision	≥90%

[a] Based on Notification Nos. 1106001 of November 6, 2002, and 0622003 of June 22, 2006, from the Department of Food Safety of the MHLW of Japan.
[b] The corresponding allergenic ingredient soluble protein weight/food weight.

pressurizing processes, hereinafter termed "model processed (incurred) food." It is recommended that food samples comprising animal product, plant product, highly processed food (long heating, high-pressure preparation), or acidic foods be evaluated during the validation to ensure that the ELISA method is applicable to various types of processed foods. (4) The recovery rate from the model processed food should be in the range of 50% and 150%, and the interlaboratory precision (RSD_r) should be less than 25%. (5) The matrix effect data by adding the target specific allergenic ingredient protein to the matrix extract, that of foods showing a false-positive (cross-reactivity) or false-negative result and that of matrices for which the ELISA method hardly applies, should be fully examined and disclosed. (6) "Reference Material for Monitoring Foods Containing Specific Allergenic Substances" should be applied for preparing kit standards as well as model processed food samples (Notification No. 1106001, 2002).

In the guidelines and reference materials, the initial extract solution and the extraction procedure from specific allergenic ingredients are also specified and standardized. For developing a food specific allergenic ingredient ELISA, the ELISA performance should fulfill the following interlaboratory validation criteria of the "Collaborative Study" protocol based on ISO5725 (JIS Z8402), which is basically the same as that of AOAC, and the obtained performance data must be available to the public.

D. Detection methods for specific allergenic ingredients (Notification No. 1106001, 2002)

1. ELISA

ELISA is the most commonly used method in the food industry and official food control agency laboratories for detecting and quantifying trace specific allergenic ingredients in foods. We introduced two assays using ELISA as the Japanese official method (Matsuda *et al.*, 2006). The best antibody for detecting specific allergenic ingredients in foods was previously determined. Antibodies can be classified into two groups: monoclonal and polyclonal. A polyclonal antibody was chosen for detecting a variety of allergen proteins.

One of the kits for the five allergenic ingredients (eggs, milk, wheat, buckwheat, and peanuts) is the FASTKIT ELISA Ver. II® (Food Allergen Screening Test Kit). This kit uses polyclonal antibodies against multiplex antigens and is produced and commercialized by Nippon Meat Packers, Inc. The concept of this kit is to use polyclonal antibodies to detect whole allergen proteins. Basically, many allergenic ingredients contain multiple allergenic proteins, for example, eggs contain ovalbumin, ovomucoid, and lysozyme, and these proteins can be denatured, degraded, and

combined with other proteins by food processing. To solve this problem, this kit uses multiple antibodies for the native protein, in addition to antibodies for the denatured proteins. The series of FASTKIT ELISA Ver. II® for each allergenic ingredient has been commercialized.

The other ELISA kit for these five allergenic ingredients is the FASPEK KIT®. The concept of this kit uses polyclonal antibodies to detect purified specific proteins or single specific proteins of specific allergenic ingredients. This kit is produced and commercialized by Morinaga Institute of Biological Sciences Co., Ltd. For ELISA, target proteins can be divided into whole proteins and proteins specific to the allergenic ingredient. For the FASPEK KIT®, these specific proteins are used as the target proteins. The target proteins are ovalbumin and ovomucoid for egg, casein, and β-lactoglobulin for milk, gliadin for wheat, the main protein complex for buckwheat, and the protein complex including Ara h2 for peanut. The series of FASPEK KIT® for ovalbumin, ovomucoid, casein, β-lactoglobulin, gliadin, buckwheat main protein complex, and peanut protein complex including Ara h2 has been commercialized. The ovalbumin kit for egg and the casein kit for milk are used as the Japanese official methods because the proportion of these proteins in egg and milk are significant.

In September 2010, CAA announced the addition of ALLERGENEYE® ELISA series of kits for egg, milk, wheat, buckwheat, and peanut as Japanese official methods based on their validation determined by the Japanese validation protocol.

Detection of every kind of protein with consistent sensitivity within a foodstuff is impossible using one kind of ELISA system, as the contents and denaturation of proteins vary greatly. Determination by ELISA is affected by denaturation and extraction efficiency of the target protein. Conventional methods cannot be easily applied to heat- and pressure-processed foods such as retorted and canned foods. Therefore, we developed a unique buffer for extracting insoluble antigens produced during heat and pressure processing (Watanabe *et al.*, 2005) as well as new polyclonal antibodies of the extracted allergen proteins using the new extraction buffer for the Japanese official method kits.

Since the MHLW designated shrimp/prawn and crab for mandatory labeling in June 2008 due to the almost unlimited use of crustacean in the processed foods in Japan and the status as a frequent cause of adverse food reactions in allergic patients, two ELISA methods for the determination of crustacean protein in processed foods have been developed (Seiki *et al.*, 2007; Shibahara *et al.*, 2007): FA test EIA–Crustacean [Nissui]® produced by Nissui Pharmaceutical Co., Ltd. and Crustacean Kit [Maruha®] produced by Maruha Nichiro Foods, Inc. Both kits have been validated according to the Japanese validation protocol (Sakai *et al.*, 2008) and are commercially available. All the commercial ELISA kits are shown in Table 4.9.

TABLE 4.9 Commercial ELISA kits for specific allergenic ingredients

Specific allergenic ingredient	ELISA kits	Target protein
Egg	FASTKIT ELISA Ver.II® for egg	Egg soluble protein
	FASPEK KIT® for egg	Ovalbumin
	ALLERGENEYE® ELISA for egg	Ovalbumin
Milk	FASTKIT ELISA Ver.II® for milk	Milk soluble protein
	FASPEK KIT® for milk	β-lactoglobulin
	ALLERGENEYE® ELISA for milk	Casein
Wheat	FASTKIT ELISA Ver.II® for wheat	Wheat soluble protein
	FASPEK KIT® for wheat	Gliadin
	ALLERGENEYE® ELISA for wheat	Gliadin
Buckwheat	FASTKIT ELISA Ver.II® for buckwheat	Buckwheat soluble protein
	FASPEK KIT® for buckwheat	Soluble peanut protein mixture
	ALLERGENEYE® ELISA for buckwheat	24-kDa protein
Peanut	FASTKIT ELISA Ver.II® for peanut	Peanut soluble protein
	FASPEK KIT® for peanut	Soluble peanut protein mixture
	ALLERGENEYE® ELISA for peanut	Ara h1 protein
Crustacean	Crustacean Kit [Maruha®]	Tropomyosin
	FA test EIA–Crustacean [Nissui]®	Tropomyosin

2. Western blotting method for egg and milk

Western blotting is another protein-based qualitative method. This method has high specificity, because specific proteins are separated according to their molecular mass, irrespective of their original electrochemical charge. Figure 4.4 shows a flowchart of the procedures for Western blotting. First, samples are prepared for polyacrylamide gel electrophoresis (PAGE) and then subjected to blotting and blocking. Next, it is reacted with the primary antibody, followed by the secondary antibody, and then reacted with the avidin-labeled alkaline phosphatase-biotin conjugate, followed by the substrate. The final step is detection of the protein-derived allergens. Western blotting method is prescribed as the confirmation test for egg and milk in the Japanese official methods. The Western blotting kits for egg and milk, FASPEK Western Blot KIT® for egg and milk, are produced and commercialized by Morinaga Institute of Biological Sciences Co.