Interlaboratory Evaluation of Two Enzyme-Linked Immunosorbent Assay Kits for the Determination of Crustacean Protein in Processed Foods

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The labeling of foods containing material derived from crustaceans such as shrimp and crab is to become mandatory in Japan because of increases in the number of allergy patients. To ensure proper labeling, 2 novel sandwich enzyme-linked immunosorbent assay (ELISA) kits for the determination of crustacean protein in processed foods, the N kit (Nissui Pharmaceutical Co., Ltd, Ibaraki, Japan) and the M kit (Maruha Nichiro Holdings, Inc., Ibaraki, Japan), have been developed. Five types of model processed foods containing 10 and/or 11.9 μg/g crustacean soluble protein were prepared for interlaboratory evaluation of the performance of these kits. The N kit displayed a relatively high level of reproducibility relative standard deviation (interlaboratory precision; 4.0–8.4% RSDR) and sufficient recovery (65–86%) for all the model processed foods. The M kit displayed sufficient reproducibility (17.6–20.5% RSDR) and a reasonably high level of recovery (82–103%). The repeatability relative standard deviation (RSDr) values regarding the detection of crustacean proteins in the 5 model foods were mostly <5.1% RSD, for the N kit and 9.9% RSDr, for the M kit. In conclusion, the results of this interlaboratory evaluation suggest that both these ELISA kits would be very useful for detecting crustacean protein in processed foods.

In industrialized countries, food allergies have represented an important health problem in recent years, and it is estimated that approximately 8% of children and 2% of adults in these countries have some type of food allergy (1, 2). Burks et al. (3) estimated that approximately 120 deaths related to food allergies occur yearly in the United States. In Japan as well, the number of patients with food allergies, especially among young children, is increasing (4). To prevent possible life-threatening reactions, the only effective measure is to strictly avoid the consumption of allergenic foods because of the lack of effective medical treatment for food allergies. However, various studies have shown that severe allergenic reactions can be induced by the accidental intake of food products containing allergenic materials (5, 6). Therefore, sufficient 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 8 kinds of foods that contain ingredients known to be allergens, including Crustacea (7). In Japan, the Ministry of Health, Labor and Welfare (MHLW) has enforced a labeling system for allergenic food materials since April 2002 in order to provide information about these foods to the allergic
consumer. According to the Japanese regulations, labeling for food products, including eggs, milk, wheat, buckwheat, and peanuts, is mandatory, and is recommended for 20 other food materials such as shrimp and crab in light of the number of patients and the degree of seriousness. In a ministry notification in 2002 (8), the MHLW announced the official Japanese methods for the detection of allergens for mandatory labeling and the threshold value for labeling [10 μg/g (the corresponding allergenic ingredient soluble protein weight/food weight)]. The MHLW also described the intervalidation protocol criteria in the official guidelines in 2006 (9). The outline of the intervalidation protocol criteria is as follows: number of laboratories, ≥8; number of incurred samples, ≥5; number of dose level, ≥1, including 10 μg/g (the corresponding allergen protein weight/food weight); recovery, 50–150%; reproducibility relative standard deviation (RSDR), ≤25%. In the guideline, reference material, the initial extract solution, and the extraction procedure from allergen were also specified and standardized.

Epidemiological investigations in Japan have shown that the number of patients with a crustacean allergy such as to shrimp and crab has increased (10). In recommendations regarding labeling, Crustacea labeling would be particularly important because of the almost unlimited uses of Crustacea as an ingredient and because the number of patients with allergies to Crustacea has been increasing, although crustacean allergy is still less prevalent than, for example, milk allergy in the food-allergic population (11, 12). Crustacean allergic reactions can be elicited by the intake of trace amounts of crustacean proteins, and anaphylaxis caused by Crustacea has been reported (13, 14). Considering current conditions, the MHLW will have a plan for crustacean labeling to be mandatory in 2008. Therefore, a reliable detection method for crustacean protein monitoring is necessary to ensure accurate labeling. We have developed 2 enzyme-linked immunosorbent assay (ELISA) kits capable of highly sensitive crustacean protein detection. This paper describes the results of an interlaboratory evaluation of the performance of these ELISA kits.

Experimental

Materials and Methods

Preparation of crustacean powder.—Crustacean powder was prepared by homogenization followed by freeze-drying of the tail muscle of the shrimp (black tiger prawn, *Penaeus monodon*). The crustacean soluble protein (CP) from crustacean powder was extracted using the extraction buffer phosphate buffered saline (PBS), pH 7.4, containing 0.5% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol. The CP content was then calculated using a 2-D Quant Kit (GE Healthcare, Buckinghamshire, UK). The amount of CP/1 g crustacean powder was approximately 684 mg.

Test Materials

Five model processed foods (fish sausage, freeze-dried egg soup, tomato sauce, creamy croquette, and chicken balls) containing CP were prepared and used as test materials. Crustacean powder was spiked at the ingredient stage before processing to establish a final level of 10 μg/g (CP weight/sample weight). In the case of freeze-dried egg soup, the CP content was calculated to be 11.9 μg/g (CP weight/sample weight). The prepared model processed foods were homogenized with a food processor (DLC-XG; Cuisinart, Stamford, CT) and sent to the participants as test materials.

Preparation of Model Processed Foods

All the model processed foods were prepared using procedures described by the manufacturers. To make a final protein concentration of 10 μg/g (CP weight/sample weight) in these foods, the amount of crustacean powder spiked in the foods at the ingredient stage was calculated, taking into account the protein content of the crustacean powder and the change in weight of the foods during preparation. Because the protein amount per 1 g crustacean powder was ca 684 mg, we spiked 14.6 mg crustacean powder to make 1 kg of each model processed food [the make-up of the final concentration is calculated for 10 μg/g (CP weight/sample weight)] because we estimated the ratio of CP weight to crustacean powder weight to be 68.4%.

The fish sausages were composed of minced fish flesh (yellow goatfish, atka mackerel, and walleye pollack), lard, sugar, salt, ice water, and crustacean powder. Lard, salt, sugar, ice water, and the crustacean powder were added to minced fish flesh and thoroughly mixed. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into fish sausage casings. These were then heated at 121°C for 15 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5°C overnight.

The freeze-dried egg soup was composed of egg, potato starch, milk sugar, salt, and crustacean powder. Eggs, potato starch, milk sugar, salt, and the crustacean powder were thoroughly mixed. The mixture was dissolved in a plastic tray, frozen in a deep-freezer at −80°C, and then lyophilized for 20 h.

Tomato sauce was composed of tomato, onion, olive oil, garlic, bay leaf, sugar, salt, black pepper, and crustacean powder. The measured raw materials were thoroughly mixed and placed in an aluminum pouch. The sauce was heated at 121°C for 1 min, cooled in flowing water for 5 min, and then placed in a refrigerator at 5°C overnight.

The creamy croquettes were composed of milk, butter, wheat, potato starch, salt, sugar, black pepper, flour, and crustacean powder. The raw materials were homogeneously mixed and kneaded for 20 min. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into casings, which were then preserved in a deep-freezer at −20°C until interlaboratory evaluation.
The chicken balls were composed of white meat of chicken, lard, potato starch, sugar, and crustacean powder. Lard, potato starch, sugar, and the crustacean powder were added to ground white meat and thoroughly mixed. The mixture was ground up using a small cutter, and the kneaded mixture was manually placed into casings, which were then preserved in a deep-freezer at -20°C until interlaboratory evaluation.

Each type of model processed food was also prepared as a blank sample which did not include CP for the purpose of confirming potential contamination, determination of the limit of detection (LOD), the limit of quantification (LOQ), false positive, interference, and matrix effects. All the values determined in the blank samples of the model processed foods using both kits were less than respective LOD in in-house validation (data not shown).

**Homogeneity Tests of the Test Materials**

The homogeneity of the test materials was verified by the coordinator before distribution following the procedure described in the International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (15), except that the number of test materials was 6. Twelve test portions of each test material were analyzed using the N kit. The obtained concentrations of CP were submitted to one-way analysis of variance (ANOVA). Table 1 shows the average concentration, the relative standard deviation (RSD) percentages calculated from \( s_x \) (SD of sampling) and \( s_a \) (SD of analysis) as well as the \( F \)-ratios. The \( F \)-ratios for all test materials were below the critical \( F \)-value.

### Table 1. Homogeneity test\(^a\) results of processed food samples

<table>
<thead>
<tr>
<th>Food</th>
<th>Mean, ( \mu g/g )</th>
<th>RSD, %(^b)</th>
<th>( n )</th>
<th>( F )-ratio</th>
<th>( F_{crit} )^(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish sausage</td>
<td>6.5</td>
<td>4.5</td>
<td>6</td>
<td>1.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Freeze-dried egg soup(^d)</td>
<td>8.5</td>
<td>2.4</td>
<td>6</td>
<td>0.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Tomato sauce</td>
<td>9.0</td>
<td>4.9</td>
<td>6</td>
<td>1.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Creamy croquette</td>
<td>8.5</td>
<td>4.3</td>
<td>6</td>
<td>1.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Chicken ball</td>
<td>7.1</td>
<td>5.5</td>
<td>6</td>
<td>3.8</td>
<td>4.4</td>
</tr>
</tbody>
</table>

\(^a\) Homogeneity test was carried out with N kit.

\(^b\) RSD% calculated from \( s_x \) (SD of sampling) and \( s_a \) (SD of analysis).

\(^c\) \( F_{crit} \) = Critical \( F \)-value.

\(^d\) CP was spiked at 11.9 \( \mu g/g \).

ELISA Kits

**Crustacean kit "Maruha" (M kit; 16).**—The polyclonal and monoclonal antibodies to tropomyosin in the black tiger prawn (Penaeus monodon) are used in the M kit with the CP solution used as a calibrator. The detection procedure was performed according to the manufacturer’s instructions. Diluted standard solutions and sample solutions were added to a monoclonal antibody-coated module and incubated for 1 h at 25°C. After the module was washed, a solution of the polyclonal antibody, labeled with horseradish peroxidase, was added and allowed to stand for 1 h. The reaction was stopped by addition of 1 M sulfuric acid, and the absorbances were measured at 450 nm with 650 nm as the reference wavelength. The M kit was highly specific for the Decapoda group, apart from minor cross-reactivities to other Crustacea and Mollusks.

We determined the LOD and LOQ according to International Standards Organization (ISO) and International Union of Pure and Applied Chemistry (IUPAC) guidelines (17). The LOD was calculated as 3 times the SD of the dilution buffer mean value after 25 experiments. The LOQ was calculated as 10 times the SD of the dilution buffer mean values after 25 experiments. The LOD of the M kit determined using the dilution buffer was 0.71 ng/mL, equivalent to 0.29 mg/kg samples, and the LOQ was 0.78 ng/mL, equivalent to a 0.31 mg/kg sample. Consequently, the practical determination range is between 0.78 and 50 ng/mL.

**Food Allergen Test EIA Crustacean "Nissui" (N kit; 18).**—The polyclonal and monoclonal antibodies to tropomyosin in the black tiger prawn (Penaeus monodon) are used in the N kit, with the CP solution used as a calibrator. Diluted standard solutions and sample solutions were added to a monoclonal antibody-coated module and incubated for 1 h at 25°C. After the module was washed, a solution of the polyclonal antibody, labeled with horseradish peroxidase, was added and allowed to stand for 1 h at 25°C. After the second washing, a solution of 3,3',5,5'-tetramethylbenzidine was added and the module was allowed to stand at 25°C for exactly 20 min. The reaction was stopped by addition of 1 M sulfuric acid, and the absorbances were measured at 450 nm with 650 nm as the reference wavelength. The M kit was highly specific for the Decapoda group, apart from minor cross-reactivities to other Crustacea and Mollusks. We determined the LOD and LOQ according to ISO and IUPAC guidelines (17). The LOD was calculated as 3 times the SD of the dilution buffer mean value after 36 experiments. The LOQ was calculated as 10 times the SD of the dilution buffer mean values after 36 experiments. The LOD of the N kit determined using the dilution buffer was 0.4 ng/mL, equivalent to 0.16 mg/kg samples, and the LOQ...
was 0.78 ng/mL, equivalent to a 0.31 mg/kg sample. Consequently, the practical determination range is between 0.78 and 50 ng/mL.

**Extraction**

The extraction procedure was common to both ELISA series. A 1.0 g portion of the test material was extracted using 19 mL of the extraction buffer. This extraction buffer was also common to both ELISA kits. The mixture was shaken horizontally overnight (16 h) at room temperature, and then centrifuged at 3000 \( g \) for 20 min after adjustment of the pH to 6.0–8.0. The supernatant was filtered if necessary, diluted 20 times using each kit dilution buffer, and subjected to ELISA.

**Calibration Standard Solutions**

The calibration standard solutions of CP were provided by Nippon Gene Co., Ltd (Toyama, Japan).

**Interlaboratory Study**

Ten laboratories participated in the interlaboratory evaluation, 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 5 test materials (3 g each) and 2 ELISA kits, plus the extraction solution and the calibration standard solutions. The participants took 2 portions from each test material, extracted the protein using the extraction procedure, and assayed each extract using the ELISA kits. The calibration standard solution was diluted and assayed simultaneously with the test material extracts. Each sample extract was analyzed in triplicate (3 wells/sample extraction), and the average absorbance of 3 wells was used for the calculation. The obtained absorbance data of calibration solutions and test materials were reported to the coordinator.

The organizer calculated a 4-parameter logistic calibration graph based on the absorbance data of the calibration standard solution and calculated the concentrations of CP in the test material using the calibration graph. Each set of samples was extrapolated from the standard curve run in the same plate.

The study was performed from July to August 2006.

**Statistical Analysis**

The values reported by the participants are summarized in Tables 2 and 3. Twenty data items, as 2 portions from 10 laboratories, were fed into the calculation. Cochran's test and Grubbs' test were used to remove outlying data (\( P = 2.5\% \)). Cochran's test was used to remove the laboratory that reported a significantly large variability between the results of

| Table 2. Results of the interlaboratory study for M kit: protein recovery content |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| Fish sausage | Freeze-dried egg soup | Tomato sauce | Creamy croquette | Chicken ball |        |
| Lab | µg/g | % | µg/g | % | µg/g | % | µg/g | % | µg/g | % |
| A | 11.4 | 114 | 12.2 | 103 | 11.6 | 116 | 10.0 | 100 | 13.2 | 132 |
| B | 11.6 | 116 | 11.8 | 100 | 10.8 | 108 | 10.2 | 102 | 10.8 | 108 |
| C | 10.1 | 101 | 8.6 | 72 | 11.6 | 116 | 8.7 | 87 | 8.0 | 80 |
| D | 8.6 | 86 | 11.0 | 93 | 8.7 | 87 | 7.5 | 75 | 8.8 | 88 |
| E | 13.3 | 133 | 14.0 | 117 | 9.9 | 99 | 9.0 | 90 | 9.6 | 96 |
| F | 10.0 | 100 | 9.5 | 80 | 7.7 | 77 | 6.4 | 64 | 10.4 | 104 |
| G | 6.8 | 68 | 9.7 | 81 | 7.5 | 75 | 6.0 | 60 | 8.9 | 89 |
| H | 11.8 | 118 | 13.4 | 113 | 10.6 | 106 | 9.0 | 90 | 9.1 | 91 |
| I | 7.6 | 76 | 14.5 | 122 | 9.0 | 90 | 7.8 | 78 | 8.2 | 82 |
| J | 11.6 | 116 | 12.3 | 103 | 8.2 | 82 | 7.5 | 75 | 13.1 | 131 |

*CP was spiked at 11.9 µg/g.*

*Value removed after Cochran's test.
the 2 portions taken from the test material. Grubbs’ test was used to remove the laboratory that reported the mean of the results of 2 portions significantly different from other laboratories. The use of statistical tests for outliers follows the AOAC protocol (19). The removed values are also shown in Tables 2 and 3. Recovery, repeatability, and reproducibility were calculated by one-way ANOVA using the remaining data after the removal of outliers.

Results and Discussion

Homogeneity of the Test Materials

The resultant $F$-ratios of the homogeneity test regarding fish sausage, freeze-dried egg soup, tomato sauce, and creamy croquettes were <1.6. The critical value of $F$ was 4.4, and the homogeneity of the test materials was sufficient for the interlaboratory evaluation. The $F$-ratio from chicken balls was slightly higher than the others, but lower than the critical $F$.

For most test materials, the RSD values among portions were <5.5% and smaller than the required RSDR values (≤25%).

Calibration Graph

Figure 1 shows the calibration graphs of the 2 ELISA kits. Both graphs have good correlation between 0.78 and 50 ng/mL, and give sufficiently high absorbance at 25 ng/mL, corresponding to 10 μg/g CP in the test materials.

Recovery

The recovery, repeatability ($RSD_r$), and reproducibility ($RSD_R$) calculated using ANOVA are shown in Table 4 with the number of remaining laboratories after removing the outliers. As shown in Table 4, the recoveries of CP from 5 types of test materials using the M kit were found to be 82–103%, whereas those using the N kit were 65–86%. The recoveries of CP determined by the M kit displayed a relatively high degree of recovery compared with those of the
N kit for all test materials in the interlaboratory evaluation, being especially significant for fish sausage and chicken balls. We considered that the discrepancies in the recoveries of CP between the 2 kits could be due to some differences of the property of the antibody in each kit because the sample extract solutions from the test material were identical.

A 2-way ANOVA of the results of each kit and laboratory was performed for each test material. Significant differences were seen between the values determined by 2 kits. In addition, there appear to be differences among the results of the laboratories, although the differences are not statistically significant. Table 4 shows that all the values of the test materials determined by the M kit are higher than those determined by the N kits. These results suggest that the values determined by the M kit have a tendency to be higher than those of the N kits in identical materials.

**Repeatability**

Repeatability is a measure of the variance arising from the extraction and determination procedure in a particular laboratory. In intralaboratory evaluations, the RSDr values of most cases were found to be <9.9%. The RSDr values for the M kit materials in regard to tomato sauce and creamy croquette were approximately twice as large as those of the N kit. According to Horwitz theory, the RSDr value generally is likely to be less than 2/3 of the RSDR value (20). In this study, most RSDr values for both kits were less than 2/3 of the corresponding RSDR value, although some RSDr values for the N kit were similar level to the corresponding RSDR values due to the low RSDR values.

**Reproducibility**

The reproducibilities, expressed by RSDR values of CP from 5 types of test materials using the M kit, ranged between 17.6 and 20.5%, whereas those using the N kit ranged between 4.0 and 8.4%. The RSDR values of the M kit were more than double those of the N kit. The N kit displayed a relatively high level of reproducibility compared with the M kit in the interlaboratory evaluation results.

With regard to repeatability and reproducibility, we considered that the differences between the 2 kits could be due to differences of the ruggedness of the assay performance in each kit, because the sample extract solution from the test material and calibration standard were almost identical except for the dilution buffer. Accordingly, the precision of the N kit performance is higher than that of the M kit in terms of the deviation effects of incubation times, reagent volumes, and reaction time with substrate.

**Conclusions**

The Japanese government MHLW established the interlaboratory validation protocol in the official guidelines published in 2006 (9). The outline of the interlaboratory validation protocol is as follows: number of laboratories, ≥8, number of incurred samples, ≥5; number of dose level, ≥1, including 10 μg/g (the corresponding allergen protein

<table>
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<th>Sample</th>
<th>N</th>
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* CP was spiked at 11.9 μg/g.
weight/food weight); recovery, 50–150%; RSDR, ≤25%. These criteria are based on ISO 5725 (JIS Z8402), which is mostly the same as that of AOAC (19). In the guidelines, the initial extract solution and the extraction procedure from allergen were specified and standardized.

The present study suggests that both test kits are reliable and precise methods to determine CP content, and the performance of both kits satisfies the validation criteria described in the official guidelines published by the Japanese government. Because food allergies can induce severe disease, the accuracy of the method is crucial. It is apparent from the data of interlaboratory evaluation that the M kit is more accurate than the N kit. On the other hand, the N kit produces much better repeatability and reproducibility measures than does the M kit because of the high precision of the assay performance of the N kit. The present interlaboratory evaluation was performed using 5 processed model foods, including highly processed foods such as fish sausage, freeze-dried egg soup, and tomato sauce, to ensure that these kits are able to detect CP from commercial foods and thereby guarantee accurate labeling. The creamy croquettes and chicken balls used in this study are not highly processed because they are generally marketed as frozen foods without undergoing heat processing in Japan. The present results demonstrate that both kits would be able to detect the CP contained in processed foods and thus support the food labeling system according to Japanese regulations.

Among the remarkable features of these kits are their unified extraction solutions and identical calibration standard solutions. These standardized features can provide a comparison of the assay performance of the 2 kits by comparing their results determined with an identical calibrator as a common ruler without considering the deviation effects of the extraction and calibration standard.

In conclusion, the 2 ELISA kits provide rapid, precise, and reliable tools for the quantitative analysis of CP in processed foods. This proposed system is able to accurately monitor the labeling system in a reliable manner and can be useful for the inspection mandatory in the Japanese regulation.

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