Table 2. Results of the interlaboratory study for M kit: protein recovery content

	Fish sausage		Freeze-dried egg soup[a]		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[b]	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

[a] CP was spiked at 11.9 μg/g.
[b] Value removed after Cochran's test.

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 were identical in both ELISA kits except for the dilution buffer from the initial extract used for the calibration standard solution. The initial extract for calibration standard solution was prepared from crustacean powder as follows: A 0.1 g sample of the crustacean powder was added to 20 mL PBS (10 mM Na-phosphate, 154 mM NaCl, pH 7.4) containing 5 g/L SDS, 20 mL/L β-mercaptoethanol, 10 μL/mL protease inhibitor cocktail, and 10 μL/mL 0.5 M EDTA (Halt protease inhibitor cocktail kit; Pierce, Rockford, IL). The mixture was then shaken for 15 h at room temperature for extraction. After the extraction, the sample was centrifuged at 10 000 × *g* for 30 min and the supernatant was filtered through a 0.8 μm micro-filter paper (DISMIC 25 cs; Advantec, Tokyo, Japan) to obtain the extract. The extract was then heated at 100°C for 10 min. The protein content of the initial extract was assayed using a 2-D Quant Kit (GE Healthcare). The initial extract was diluted with each kit dilution buffer to compose the calibration standard solution (50 ng/mL of extracted protein) for each kit. 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 3. Results of the interlaboratory study for N kit: protein recovery content

	Fish sausage		Freeze-dried egg soup[a]		Tomato sauce		Creamy croquette		Chicken ball	
Lab	μg/g	%	μg/g	%	μg/g	%	μg/g	%	μg/g	%
A	5.5[b]	55	7.7	65	7.7	77	7.0	70	6.4	64
B	6.4	64	9.0	76	8.7	87	8.2	82	7.3	73
C	6.4	64	9.8	82	9.7	97	7.9	79	7.6	76
D	5.1[b]	51	8.2	69	8.3	83	7.2	72	6.7	67
E	6.4	64	9.2	77	8.4	84	8.1	81	6.9	69
F	6.4	64	8.2	69	8.6	86	8.0	80	7.1	71
G	6.4	64	8.4	70	8.6	86	7.6	76	6.9	69
H	6.4	64	8.6	72	8.6	86	7.7	77	7.8	78
I	6.8	68	9.8	83	9.0	90	7.9	79	8.3	83
J	6.7	67	8.6	72	8.4	84	7.9	79	7.2	72

[a] CP was spiked at 11.9 μg/g.
[b] Value removed after Grubbs' 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 RSD_R 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

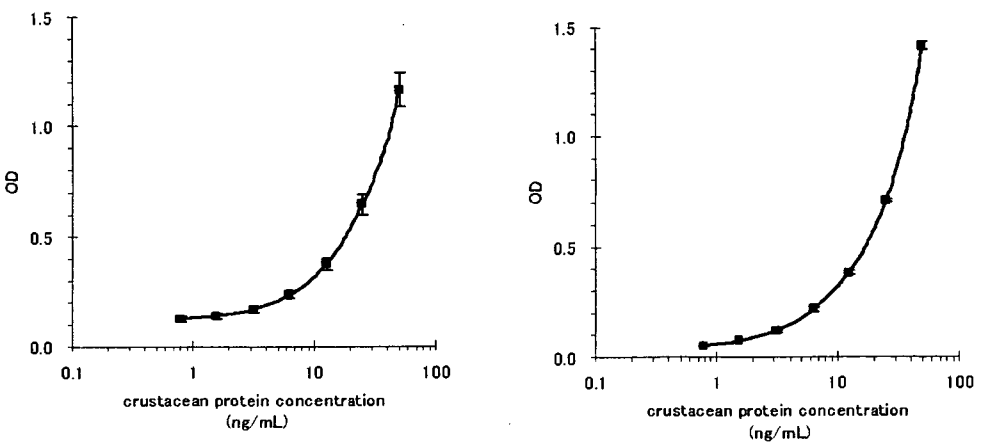

Figure 1. Crustacean protein calibration graphs of M kit (left) and N kit (right).

Table 4. Recovery, repeatability (RSD_r), and reproducibility (RSD_R) of the M kit and N kit for CP

Sample	No. of laboratories		Mean, μg/g		s_r, μg/g		s_R, μg/g		Recovery, %		RSD_r, %		RSD_R, %	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N
Fish sausage	10	8	10.3	6.5	0.5	0.3	2.1	0.3	103	65	4.9	4.0	20.5	4.0
Freeze-dried egg soup[a]	9	10	11.5	8.8	0.7	0.4	2.0	0.7	97	74	3.6	4.1	17.6	8.4
Tomato sauce	10	10	9.6	8.6	0.9	0.4	1.7	0.6	96	86	9.3	4.7	17.6	6.8
Creamy croquette	10	10	8.2	7.8	0.8	0.4	1.5	0.5	82	78	9.9	4.6	18.8	5.9
Chicken ball	10	10	10.0	7.2	0.6	0.4	1.9	0.6	100	72	6.1	5.1	19.2	8.4

[a] CP was spiked at 11.9 μg/g.

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 RSD_r values of most cases were found to be <9.9%. The RSD_r 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 RSD_r value generally is likely to be less than 2/3 of the RSD_R value (20). In this study, most RSD_r values for both kits were less than 2/3 of the corresponding RSD_R value, although some RSD_r values for the N kit were similar level to the corresponding RSD_R values due to the low RSD_R values.

Reproducibility

The reproducibilities, expressed by RSD_R 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 RSD_R 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

weight/food weight); recovery, 50–150%; RSD_R, ≤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 wih 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.

Acknowledgments

We thank the following collaborators for participating in the interlaboratory evaluation:

S. Futo and H. Haraguchi, FASMAC Co., Ltd, Atsugi, Japan

H. Sato and A. Yamaguchi, Japan Food Research Laboratories, Chitose, Japan

Y. Mori and M. Hirota, Japan Frozen Foods Inspection Corp., Yokohama, Japan

T. Iidzuka and Y. Hirose, Japan Inspection Association of Food and Food Industry Environment, Tokyo, Japan

S. Kanayama and Y. Minegishi, Nippon Gene Co., Ltd, Toyama, Japan

H. Yamakawa, Nisshin Seifun Group, Inc., Fujimino, Japan

T. Koike and K. Kojima, Rohto Pharmaceutical Co., Ltd, Kyoto, Japan

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

R. Nishihara, Showa Sangyo Co., Ltd, Funabashi, Japan

K. Yasuda and K. Kan, Tokyo Metropolitan Institute of Public Health, Tokyo, Japan

This study was supported by Health and Labor Science Research Grants for Research on Food Safety from the MHLW of Japan.

References

(1) Jansen, J.J., Kardinaal, A.F., Huijbers, G., Vlieg-Boerstra, B.J., Martens, B.P., & Ockhuizen, T. (1994) *J. Allergy Clin. Immunol.* **93**, 446–456

(2) Bousquest, J., Bjorksten, B., Bruijnzeel-Koomen, C., Hugget, A., Ortolani, C., Warner, J.O., & Smith, M. (1998) *Allergy* **53**, 3–21

(3) Burks, W., Bannon, G.A., Sicherer, S., & Sampson, H.A. (1999) *Int. Arch. Allergy Immunol.* **119**, 165–172

(4) Ebisawa, M., Ikematsu, K., Imai, T., & Tachimoto, H. (2003) *J. World Allergy Org.* **15**, 214–217

(5) Bock, S.A., & Atkins, F.M. (1989) *J. Allergy Clin. Immunol.* **83**, 900–904

(6) Furlong, T.J., DeSimone, J., & Sicherer, S.H. (2001) *Allergy Clin. Immunol.* **108**, 867–870

(7) Hefle, S.L., Nordlee, J.A., & Taylor, S.L. (1996) *Crit. Rev. Food Sci. Nutr.* **36**, S69–S89

(8) Department of Food Safety, the Ministry of Health, Labor and Welfare of Japan (2002) *Notification No. 1106001 of November 6, 2002*, Tokyo, Japan

(9) Department of Food Safety, the Ministry of Health, Labor and Welfare of Japan (2006) *Notification No. 0622003 of June 22, 2006*, Tokyo, Japan

(10) Ebisawa, M. (2007) *Arerugi* **56**, 10–18

(11) Bock, S.A., & Atkins, F.M. (1990) *J. Pediatr.* **117**, 561–567

(12) Sicherer, S.H., Furlong, T.J., Maes, H.H., Desnick, R.J., Sampson, H.A., & Gelb, B.D. (2000) *J. Allergy Clin. Immunol.* **106**, 53–56

(13) Sampson, H.A., & Metcalfe, D.D. (1992) *J. Am. Med. Assoc.* **268**, 2840–2844

(14) Foucard, T., & Malmheden Yman, I. (1999) *Allergy* **54**, 261–265

(15) Thompson, M., & Wood, R. (1993) *J. AOAC Int.* **76**, 926–940

(16) Seiki, K., Oda, H., Yoshioka, H., Sakai, S., Urisu, A., Akiyama, H., & Ohno, Y. (2007) *J. Agric. Food Chem.* **55**, 9345–9350

(17) International Conference on Harmonization (1996) *ICH Harmonized Tripartite Guideline, Validation of Analytical Procedures: Methodology*, Brussels, Belgium

(18) Shibahara, Y., Oka, M., Tominaga, K., Ii, T., Umeda, M., Uneo, N., Abe, A., Ohashi, E., Ushio, H., & Shiomi, K. (2007) *Nippon Shokuhin Kagaku Kogaku Kaishi* **54**, 280–286

(19) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Appendix D

(20) Pocklington, W.D. (1990) *Pure Appl. Chem.* **62**, 149–162