

ELISA Kit for Determination of Egg White Proteins: Interlaboratory Study

KVĚTA TOMKOVÁ

SEDIUM RD, Ltd, Železničního pluku 1361, 530 02 Pardubice, Czech Republic

PETR CUHRA

Czech Agriculture and Food Inspection Authority, Za Opravnou 300/6, 150 06 Praha 5, Czech Republic

JANA RYSOVÁ, PETR HANÁK, and DANA GABROVSKÁ¹

Food Research Institute Prague, Radiová 7, 102 31 Praha 10, Czech Republic

Collaborators: A. Baranda; S. Baršová; J. Brychta; H. Bulawová; A. Čenčíč; J.A.G. Del Barco; P. Dvorská; S. Iametti; E. Jimenez; L. Karšulínová; M. Kubík; T. Langerholc; M.-L. Laukkanen; I. Martínez de Marañon; E. Suñen Pardo; M. Angel Pardo; L. Píknová; J. Plicka; J. Martínez Quesada; I. Postigo Resa; F. Šturm; K. Takkinen

An interlaboratory study was conducted in 11 laboratories to validate an ELISA method developed for the quantitative determination of egg white proteins (EWPs) in foods. The ELISA kit used for this study is based on sheep polyclonal antibody. It does not produce any false-positive results or cross-reactivity in a broad food matrix range with zero EWP content. All participants obtained the Egg ELISA Kit-native with standard operational procedure and the list of samples, as well as the samples and a protocol for recording test results. The study included 10 food samples. Four samples of food matrix with zero EWP content showed EWP content lower than the first standard (EWP content 0.5 mg/kg). One sample of food matrix with zero EWP content revealed EWP content higher than standard 3 (1.5 mg EWP/kg). Five food samples containing EWP as an ingredient tested positive and one negative. The statistical tests (Cochran, Dixon, and Mandel) and analysis of variance were used to evaluate the interlaboratory study results. Repeatability and reproducibility limits, as well as LOQ (1.4 mg EWP/kg) and LOD (0.43 mg EWP/kg), were calculated for the kit.

Egg allergy represents one of the most frequent human allergies at levels in affected individuals similar to that for milk and peanut allergies. Sensitization to egg with subsequent allergic symptoms in childhood often occurs without known oral exposure. Egg sensitization rates at 12 months of age in infants with an atopic history are about 7%, and are predictive of asthma in later life. The frequency in

childhood is 2–3%, similar to that reported in adults (1). Clinical features are comparable to those seen in other food allergic diseases, from mild oral and skin reactions to severe anaphylaxis and death. Egg allergy prevalence estimates based on parental perception vary between 3 and 7% at the age of 2 years (1, 2). On the basis of positive skin tests, the prevalence was estimated to be 3.2% (3). In an opinion poll-like questionnaire-based study of 33 110 individuals in France, representing a sample of the normal population, 3.5% reported symptoms of a food allergy, of which 4% thought they were reacting to eggs (4).

Major allergens of the eggs of the species *Gallus domesticus* are known, characterized, and classified as *Gal d 1-5* by the International Union of Immunological Societies, although major allergen sources are still unassigned (5–13), and other not yet well-characterized proteins. Ovomucoid, one of the major egg allergens for clinical reactions (14), is relatively heat-stable; individuals reacting to this allergen will react to raw and cooked eggs. Egg lysozyme is used in the preparation of medications and in the food industry as a bactericide to prevent the growth of anaerobic bacteria. Egg lecithin is also used as an emulsifier. There is the possibility of residual allergenicity of these preparations in severely allergic patients. Egg albumin is used as a clarifying or fining agent in the production of red wine. Reports of allergies to the protein content after clarification appear to be rare, although well-conducted studies are lacking (15, 16).

The effects of heat and food processing are not likely to abolish allergenicity, and most egg allergic individuals react to cooked and raw eggs (17). There are a number of reports investigating the effects of heat and chemical denaturation procedures on major egg allergens in vitro (5, 7, 11, 12, 18–21). As shown in the comparison of four commercial ELISA kits (22), heat treatment influences the extractability of egg proteins, mainly from baked products, and results in the decrease in egg protein content in the products.

According to European Union (EU) and Czech national legislation, all selected substances with allergenic effect are

Received February 24, 2010. Accepted by SG June 23, 2010.

¹ Corresponding author's e-mail: d.gabrovskaa@vupp.cz

Table 1. Food samples used in this study

1	Pancake mixture
2	Pizza mixture
3	Potato dumpling mixture
4	Rice
5	Bread roll mixture
6	Farmer soup
7	Egg pasta
8	Red wine
9	Red wine
10	Yogurt cake mixture
11	Flour mixture 1
12	Flour mixture 2
13	Flour mixture 3
14	Flour mixture 4
15	Flour mixture 5
16	Flour mixture 6
CS 1	Negative sample from the kit
CS 2	Positive sample from the kit

subject to labeling rules in common foods and raw materials. This means that all food producers have to provide appropriate information to consumers who suffer from food allergies (23–26). This EU food legislation would result in better food quality and labeling control by state inspection authorities. New validated analytical methods are needed in this respect (23–26). Several ELISA kits are already available; nevertheless, some are suitable only for certain food commodities (22). A newly developed ELISA method may broaden the selection of kits available on the market.

Experimental

Samples

The selected foods with and without reported egg content were purchased in the supermarket. The samples were homogenized by an IKA® rotor-speed mill. The food samples were distributed, together with the ELISA kit for interlaboratory study. The list of the samples is presented in Table 1.

ELISA Kit Chemicals

Egg ELISA Kit-native.—Cat. No. FA 00408 (SEDIUM RD, Ltd, Pardubice, Czech Republic). The format of the newly developed ELISA kit for egg white protein (EWP) determination in food and raw materials is a sandwich assay based on a developed sheep polyclonal antibody (specificity against ovomucoid, lysozyme, ovalbumin, and conalbumin) used both for solid-phase coating and signal conjugate with horseradish peroxidase from Boehringer Ingelheim, am Rhein, Germany (Cat. No. 121606). EWPs (ovalbumin,

conalbumin, ovomucoid, lysozyme; Sigma Aldrich, St. Louis, MO) were used for preparation of standard solutions (a combination of proteins naturally present in egg white: ovalbumin 67.5%; conalbumin 15.1%; ovomucoid 13.3%; and lysozyme 4.2%). TMB (3,3',5,5'-tetramethylbenzidine) liquid substrate for ELISA (Sigma-Aldrich; Cat. No. T5513) was used as a chromogenic substrate.

ELISA Kit Composition

(a) *Microtiter plate with lid.*—96 wells (arranged in 12 strips, each containing eight wells), inner walls of which are coated with the specific antibody (ready for use).

(b) *Assay buffer.*—Buffer solution for the first incubation (phosphate-based buffer solution colored blue); one bottle containing 18 mL of solution (ready for use).

(c) *Standards.*—Five vials, each 1.0 mL volume, containing egg white proteins of concentrations 0, 0.5, 1.5, 5.0, and 15 mg/kg, colored yellow (ready for use).

(d) *Control samples.*—Two vials; CS1, negative control; CS2, positive control, each containing 1.0 mL, colored yellow (ready for use).

(e) *Concentrate of extraction buffer, yellow-colored (5x).*—Tris-glycin sodium dodecyl sulfate buffer; two bottles, each containing 45 mL concentrate. The concentrated solution must be diluted five times prior to its use with distilled water.

(f) *Concentrate of conjugate (80x).*—One vial, containing 0.5 mL (concentrated conjugate of the specific antibody with peroxidase in a phosphate buffer solution containing stabilizers).

(g) *Buffer solution for dilution of conjugate.*—Phosphate buffer solution; pink-colored; one bottle containing 24 mL (ready for use).

(h) *Concentrate of wash solution (20x; borate buffer).*—One bottle, containing 50 mL concentrate. The concentrated solution must be diluted prior to use.

(i) *TMB substrate.*—One bottle containing 22 mL (ready for use).

(j) *Stop solution.*—One vial containing 6.0 mL (ready for use) 2 M solution of hydrochloric acid.

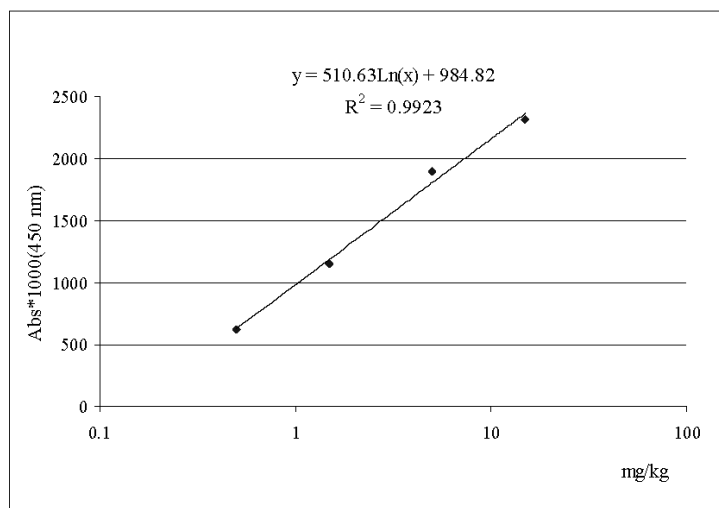
(k) *Vial for conjugate dilution.*—One piece.

Assay Parameters

In-house validation of the kit was performed for determination of assay parameters. Three analysts carried out the in-house validation. The target CV value was calculated for intra-assay, interassay, and extraction effect (27).

Analytical sensitivity.—The analytical sensitivity is defined as the lowest determinable concentration that is still distinguishable from zero. First standard (0.50 mg/kg) was diluted with zero standard twice and four times to obtain solutions with concentrations of 0, 0.125, 0.25, and 0.50 mg/kg (mg/kg). These solutions were measured 10x (zero standard 20x) and analytical sensitivity, as LOD from in-house validation (LOD_{IIH}) was calculated. The value determined by this procedure is at the level of 0.06 mg/kg.

Functional sensitivity.—Seven samples contaminated by EWP were analyzed 10 times in duplicate. The average and



EWP mg/kg	A450*1000	SD
0.0	231	5.0
0.5	623	24.0
1.5	1158	16.0
5.0	1902	67.0
15.0	2314	77.5

Figure 1. Calibration curve.

CV was determined for each sample. Dependence between CV and concentration of EWP was not found; however, CV was always 20%. This result indicates that functional sensitivity (LOQ from in-house validation – LOQ_{IH}) is not worse than the value of the first standard, i.e., 0.5 mg/kg.

Repeatability

Extraction.—Four samples (ketchup, muesli, potato starch, and juice) were contaminated by EWP. Each sample

was extracted four times. Extracts were analyzed in one run, and maximum CV was 9.02%. Extraction repeatability is influenced by the kind of matrix, especially by its homogeneity.

Intra-assay.—Intra-assay variance of the kit was tested for an extract of three samples contaminated by EWP in 25 multiplicates; maximum CV was 9.96%.

Interassay.—Interassay variance of the kit was determined for three samples contaminated by EWP in 10 runs and

Table 2. Content of EWP in the samples (average of two replicates)

Lab No.	Sample (mg EWP protein/kg)								
	CS1	CS2	1	2	3	4	5	6	7
1	<st	2.45	2.29	>st	<st	<st	35.2	<st	84.3
2	<st	3.70	2.30	>st	<st	<st	48.1	5.11	65.3
3	<st	4.05	2.10	>st	<st	<st	39.3	1.62	73.4
4	<st	3.94	2.07	>st	<st	<st	31.6	<st	23.8
5	<st	3.29	2.92	>st	<st	<st	45.7	4.85	59.0
6	<st	4.17	2.05	>st	<st	<st	53.0	3.29	87.4
7	<st	3.76	1.97	>st	<st	<st	43.4	5.41	63.8
8	<st	7.36	0.12	1.0	<st	<st	<st	0.75	<st
9	<st	3.83	2.27	>st	<st	<st	10.4	0.88	<st
10	<st	3.37	4.37	1.40	<st	<st	4.4	1.68	<st
11	<st	4.24	1.66	>st	<st	<st	14.4	5.65	0.58

Table 3. Content of EWP in the samples (average of two replicates)

Lab No.	Sample (mg EWP protein/kg)								
	8	9	10	11	12	13	14	15	16
1	<st	<st	<st	6.95	1.89	8.26	0.7	0.55	<st
2	<st	<st	<st	8.54	1.56	7.89	0.6	<st	<st
3	<st	<st	<st	6.48	1.16	8.11	0.8	<st	<st
4	<st	<st	<st	7.27	2.79	7.10	1.1	<st	<st
5	<st	<st	<st	7.39	1.61	9.87	1.0	<st	<st
6	<st	<st	<st	48.80	2.70	9.72	0.9	0.50	<st
7	<st	<st	<st	5.66	1.32	7.63	0.8	<st	<st
8	<st	<st	<st	<st	<st	<st	<st	<st	<st
9	<st	<st	<st	6.64	1.41	6.42	0.9	0.51	<st
10	<st	<st	<st	1.20	<st	0.76	<st	<st	<st
11	<st	<st	<st	3.59	1.87	6.93	0.7	0.60	<st

maximum CV was 11.62%. The CV value was always <20% for intra-assay, interassay, and extraction effect. These results are sufficient for the purpose of the kit (27).

Accuracy

Recovery.—Addition of the defined EWP was added to four naturally EWP-free matrixes, increasing the content to levels of 5.0 and 10.0 mg/kg, respectively. Recovery was in the range of 71–105%, which was a very sufficient result.

Dilution effect.—Sets of diluted samples using dilution buffer from extracts of three real samples with natural content of EWP were prepared. All samples were then analyzed in one run. Recovery was in the range of 93.4–124.9%, which was a very satisfactory result (28).

Zero matrixes.—Analyses included raw materials for foodstuffs, the nature of which should justify the expectation of their zero content of egg proteins (wheat flour, rye flour, maize flour, fish meat, beef and pork meat, rice, sugar, ketchup, cocoa, juice, vegetable oil, milk, beer, oat flakes, soy, fish, hazelnut, walnut, lentils, pine nut, peanut, barley, sesame, coconut, oats, rye, sunflower seeds, almond, chickpea, soybean, cashew nut, maize, apricot, celery, mustard, barley, and wheat). The analyses detected no EWP in any of these matrixes. Regarding samples taken from chicken meat that have not been heat-processed during the previous steps of production, it is recommended that heating be applied (up to 70 °C, 30 min) while using a water bath. Then the steps of extraction and downstream centrifuging (*see above*) should follow.

Influence of Temperature on Amount of EWP in Samples

Two real matrixes that naturally contained EWP were chosen. Samples were heated at 30, 40, 50, 60, 70, and 80 °C for 5 min before extraction. The prepared samples were

analyzed according to working protocol. Results of this experiment showed that EWPs are unstable at temperature higher than 70 °C. Based on the results obtained, it was decided to produce this kit in the future as Egg ELISA Kit-native. Instructions for use will state clearly, “This kit is not intended for heat-processed foods using temperature higher than 70 °C.”

Besides these parameters, exercise tests were also carried out: drift, stability of single components of the kit, long-term stability of standards, microtiter plates, evaporation of liquid components, comparison with other kits, and turnover of the kit. In terms of the results of the exercise tests it is recommended that the samples be dispensed to the plate within 20 min and that the bottles be closed immediately after usage because of possible evaporation.

Interlaboratory Study

Eleven laboratories with routine daily ELISA experiences were included in this study. The participants received the Egg ELISA Kit-native with instructions and all samples. They were required to perform complete duplicate analyses of the samples from two weights (two extracts and two measurements of both extracts).

The absorbance values and the remarks on technical conditions (washing procedure, ELISA reader, centrifugation) from participants were required as well.

Sample Treatment

Laboratories received 5 g of each food sample. A 1 g portion of the sample was weighed, and 10 mL of extraction buffer was added. The sample was shaken for 60 min, and immediately centrifuged at 1800 g for 10 min. The supernatant was used for the assay directly without dilution. The supernatant was stored at a temperature of –18 °C.

Table 4. Results of the interlaboratory study: performance criteria (repeatability and reproducibility data)

Performance characteristics	Abbreviation	Sample No.							
		CS2	1	5	7	11	12	13	14
Mean value, mg/kg	<i>y</i>	3.72	2.18	42.3	65.4	6.29	1.82	7.98	0.80
Number of laboratories (after exclusion of outliers)	<i>n</i>	9	9	7	7	7	9	9	8
Number of outliers	<i>n</i>	2	2	4	4	4	2	2	3
Repeatability standard deviation	<i>s_r</i>	0.458	0.333	5.78	7.12	1.50	0.359	0.816	0.136
Relative standard deviation of repeatability	RSD _r , %	12.3	15.3	13.7	10.9	23.9	19.8	10.2	17.0
Repeatability limit	<i>r</i>	1.29	0.932	16.2	19.9	4.21	1.01	2.29	0.379
Reproducibility standard deviation	<i>s_R</i>	0.641	0.415	8.52	21.3	1.70	0.639	1.32	0.142
Relative standard deviation of reproducibility	RSD _R , %	17.3	19.0	21.1	32.6	27.0	35.2	16.5	17.8
Reproducibility limit	<i>R</i>	1.80	1.16	23.8	59.7	4.75	1.79	3.69	0.398
<i>r/R</i> ratio	<i>r/R</i>	0.717	0.803	0.681	0.333	0.886	0.564	0.621	0.952
Relative standard deviation of reproducibility from Horwitz equation	CV(R), %	13.1	14.2	9.1	8.5	12.1	14.6	11.7	16.5
HorRat	RSD _R /CV(R)	1.3	1.3	2.3	3.8	2.2	2.4	1.4	1.1

Determination

A 150 L volume of buffer for the first incubation was added into each well, and 50 L of standards, control samples, or samples was then added into corresponding wells. The plate was covered and incubated for 1 h at room temperature without shaking. The content of all wells was washed four times with 300 L of wash solution using the washer or repeating dispenser. The conjugate working solution (200 L) was immediately dispensed into all wells.

The plate was covered and incubated for 1 h at room temperature without shaking. The content of all wells was washed four times with 300 L wash solution using the washer or repeating dispenser. The TMB substrate (200 L) was immediately dispensed into all the wells. The plate was incubated for 10 min in the dark without shaking. A 50 L volume of the stop solution was added and shaken briefly; within 15 min, the reading was made at 450 nm.

Calculation

The calibration graph was constructed as the dependence of measured absorbance of corresponding calibration solutions, subtracted by the absorbance of zero standard (vertical axis) versus EWP concentration in the solution (horizontal axis—logarithmic scale). A logarithmic equation was used for EWP content calculation. An example of a calibration curve is shown in Figure 1.

Results and Discussion

Results and performance characteristics of the interlaboratory study are summarized in Tables 2–4. For samples out-of-scope on the calibration curve, i.e., samples with EWP content below the first calibration point (samples CS1, 3, 4, 8–10, 15, and 16) or above the highest calibration point (sample 2), performance characteristics were not calculated, and those results are not included in Table 4. Sample 6 revealed insufficient homogeneity and was excluded from statistical evaluation.

The satisfactory performance of the kit was verified by the HorRat, which were mostly between 1.1 and 2.5; only one ratio was higher than 3 (29).

LOD (0.43 mg/kg) and LOQ (1.4 mg/kg) of the kit were estimated from interlaboratory data as three times the value of the SD of reproducibility for LOD = 3 *s_R*, respectively, as 10 times the value of the SD of reproducibility for LOQ (LOQ = 10 *s_R*) on a level close to the first calibration point. Naturally, the LOQ (1.4 mg/kg) is slightly higher than the lowest point of the calibration curve (0.5 mg/kg), because it reflects the concentrations above which the presence of EWP is beyond reasonable doubt at a probability level of 95%. Cochran, Grubbs, and Mandel tests according to ISO 5725 were used for detection of outliers (30). Probability level = 1% was used for exclusion of a laboratory from further statistical evaluation.

Values of repeatability and reproducibility were calculated according to the procedure described in ISO 5725 (30) from

eight samples that were appropriate for reliable quantification (results summarized in Table 3). All repeatability values expressed as RSD_r are $<25\%$, and reproducibility values expressed as RSD_R are 35% . The RSD_r and RSD_R values, as well as HorRat (31) acquired in the interlaboratory study, fulfill the general criteria for ELISA methods laid down in *Codex Alimentarius* draft guidelines (32), and are in harmony with typical values published in community guidance for allergen ELISA methods (28).

Conclusions

Tracing of allergens in food has recently become quite important, as food allergens have been recognized as the cause of many symptoms of atopy in predisposed consumers. Some commercial kits for detection of food allergens are already available. However, the spectrum of food allergens is not fully covered, and confirmation of detected quantities is sometimes possible only with systems based on more than one kit.

The interlaboratory study of the developed ELISA kit confirmed that this kit is suitable for EWP quantification in a broad scale of food matrixes. All negative samples were determined as negative (less than LOQ estimated in the study) by all laboratories, and all positive samples were determined as positive by all laboratories as well. Performance characteristics (r , R , LOD, and LOQ) of the kit were determined within the interlaboratory study and are acceptable for routine use of the EWP test kit in food control laboratories.

Acknowledgments

The work was supported by projects of the Ministry of Agriculture of the Czech Republic, Research Plan of Ministry of Agriculture 0002702202 and SAFEFOODERA, 08125 Detection of Traces of Allergens in Foods. Collaborators included:

František Štumor and Pavla Dvorská, SEDIUM RD, Ltd, Pardubice, Czech Republic

Jan Plicka, Immunotech a Beckman Coulter Co., Prague, Czech Republic

Martin Kubík, Soňa Baršová, and Lenka Karšulínová, Czech Agriculture and Food Inspection Authority, Prague, Czech Republic

Hana Bulavová and Josef Brychta, State Veterinary Institute, Prague, Czech Republic

Stefania Iametti, Università degli studi di Milano, Milano, Italy

Jorge Antonio Guisantes Del Barco, Jorge Martínez Quesada, Esther Suñen Pardo, and Idoia Postigo Resa, University of the Basque Country, Basque Country, Spain

Kristine Takkinen and Marja-Leena Laukkanen, VTT Technical Research Centre of Finland, Espoo, Finland

Miguel Angel Pardo, Ana Baranda, Iñigo Martínez de Marañón, and Esther Jimenez, AZTI Tecnalia, Technological Park of Biskay, Basque Country, Spain

Lubica Piknová, Food Research Institute, Bratislava, Slovakia

Tomaz Langerholc and Avreljia Čenčič, University of Maribor, Hoče, Slovenia

References

- (1) Eggesbo, M., Botten, G., Halvorsen, R., & Magnus, P. (2001) *Allergy* **56**, 403–411
- (2) Kajosaari, M. (1982) *Acta Paediatr. Scand.* **71**, 815–819
- (3) Hill, D.I., Hosking, C.S., & Heine, R.G. (1999) *Ann. Med.* **31**, 272–281
- (4) Kanny, G., Moneret-Vautrin, D.A., Flabbee, J., Beaudouin, E., Morisset, M., & Thevenin, F. (2001) *J. Allergy Clin. Immunol.* **108**, 133–140
- (5) Cooke, S.K., & Sampson, H.A. (1997) *J. Immunol.* **159**, 2026–2032
- (6) Eigenmann, P.A., Huang, S.K., & Sampson, H.A. (1996) *Pediatr. Allergy Immunol.* **7**, 12–21
- (7) Elsayed, S., & Stavseng, L. (1994) *Int. Arch. Allergy Immunol.* **104**, 65–71
- (8) Holen, E., & Elsayed, S. (1990) *Int. Arch. Allergy Appl. Immunol.* **91**, 136–141
- (9) Honma, K., Kohno, Y., Saito, K., Shimojo, N., Horiuchi, T., Hayashi, H., Suzuki, N., Hosoya, T., Tsunoo, H., & Niimi, H. (1996) *Clin. Exp. Immunol.* **103**, 446–453
- (10) Honma, K., Kohno, Y., Saito, K., Shimojo, N., Tsunoo, H., & Niimi, H. (1994) *Int. Arch. Allergy Immunol.* **103**, 28–35
- (11) Kahlert, H., Petersen, A., Becker, W.M., & Schlaak, M. (1992) *Mol. Immunol.* **29**, 1191–1201
- (12) Mine, Y., & Zhang, J.W. (2002) *J. Agric. Food Chem.* **50**, 2679–2683
- (13) Szepefalusi, Z., Ebner, C., Pandjaitan, R., Orlicek, F., Scheiner, O., Boltz-Nitulescu, G., Kraft D., & Ebner, H. (1994) *J. Allergy Clin. Immunol.* **93**, 932–942
- (14) Bernhisel-Broadbent, J., Dintzis, H.M., Dintzis, R.Z., & Sampson, H.A. (1994) *J. Allergy Clin. Immunol.* **93**, 1047–1059
- (15) Cattaneo, A., Ballabio, C., Bertelli, A.A., Fiocchi, A., Galli, C.L., Isoardi, P., Terracciano L., & Restani, P. (2003) *Int. J. Tissue React.* **25**, 57–64
- (16) Marchal, R., Marchal-Delahaut, L., Lallement, A., & Jeandet, P. (2002) *J. Agric. Food Chem.* **50**, 177–184
- (17) Eigenmann, P.A. (2000) *J. Allergy Clin. Immunol.* **105**, 587–588
- (18) Kato, Y., Oozawa, E., & Matsuda, T. (2001) *J. Agric. Food Chem.* **49**, 3661–3665
- (19) Sakai, K., Matsuoka, A., Ushiyama, Y., Shimoda, T., & Ueda, N. (1998) *Arerugi* **47**, 1176–1181
- (20) Holen, E., Bolann, B., & Elsayed, S. (2001) *Clin. Exp. Allergy* **31**, 952–964
- (21) Shimojo, N., Katsuki, Y., Coligan, J.E., Nishimura, Y., Sasazuki, T., Tsunoo, H., Sakamaki, T., Kohno, Y., & Niimi, H. (1994) *Int. Arch. Allergy Immunol.* **105**, 155–161
- (22) Diaz-Amigo, C. (2009) *Food Anal. Methods, Towards a Comprehensive Validation of ELISA Kits for Food Allergens, Case 2—Milk Food Anal. Methods*, DOI 10.1007/s12171/s12161-0009-9094
- (23) Opinion of the Scientific Panel on Dietetic Products Nutrition and Allergies (2004) *EFSA J.* **32**, 1–197

- (24) European Directive 2003/89/EC, *Off. J. Eur. Union* **L308**, 15–18
- (25) European Directive 2000/13/EC, *Off. J. Eur. Commun.* **L109**, 29–42
- (26) *Decree No. 113/2005 Coll. (Czech Republic)* (2005) On The Method of Labeling Food and Tobacco Products, Date of entry into force: 21.3.2005, Ministry of Agriculture of the Czech Republic, Tesnov, Czech Republic
- (27) *The Immunoassay Handbook* (2001) 2nd Ed., D. Wild (Ed.), Nature Publishing Group, New York, NY
- (28) Abbott, M., Hayward, S., Ross, W., Godefroy, S.B., Ulberth, F., van Hengel, A.J., Roberts, J., Akiyama, H., Popping, B., Yeung, J.M., Wehling, P., Taylor, S.L., Poms, R.E., & Delehaut, P. (2010) *J. AOAC Int.* **93**, 443–450
- (29) European Commission Regulation (EC) No 333/2007, *Off. J. Eur. Union* **L88**, 29–39
- (30) International Organization for Standardization (1994) *ISO 5725:1994, Accuracy (Trueness and Precision) of Measurement Methods and Results*, Geneva, Switzerland
- (31) *Official Methods of Analysis* (2005) AOAC INTERNATIONAL, Gaithersburg, MD, Appendix D
- (32) Codex Alimentarius (2010) *Proposed Draft Guidelines on Performance Criteria and Validation of Methods for Detection, Identification and Quantification of Specific DNA Sequences and Specific Proteins in Foods*, ALINORM 10/33/23, Appendix III, Geneva, Switzerland