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Performance of ELISA and PCR Methods for the Determination of Allergens in Food: An Evaluation of Six Years of Proficiency Testing for Soy (*Glycine max* L.) and Wheat Gluten (*Triticum aestivum* L.)

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ABSTRACT: For the routine detection of allergens in foods, PCR and/or ELISA methods are employed. To assess the suitability of these methods, proficiency tests (PTs) could be used as a valuable instrument. It is a common practice to evaluate the results with respect to the experimentally obtained robust mean without considering the actual allergen content. In the present study, an overview is given of the results of allergen PTs for the determination of soy and gluten conducted by Dienstleistung Lebensmittel Analytik GbR (DLA). A total of 16 PTs were evaluated with respect to the comparison of PCR and ELISA performances and a new focus on the actually spiked values. The analytes were added in the ranges of 7.8−6264 mg/kg (gluten) and 184−5500 mg/kg (soy protein) in differently composed matrices such as pastry, infant food, and sausage meat. The evaluation of the PTs showed a widely reliable qualitative detection of both allergens by PCR methods. ELISA performances differed for soy and gluten. Although a high number of false-negative results occurred for the detection of soy, the qualitative detection of gluten was appropriate. Quantitative results showed obvious test kit-specific differences for the ELISA methods, but the limits of quantification were suitable for gluten determination. Both ELISA and PCR methods demonstrated their valuable contribution in food allergen determination.

KEYWORDS: *ELISA kits, food allergens, gluten, soya, PCR, proficiency testing, recovery*

INTRODUCTION

In 1984, the International Organization for Standardization (ISO) published guidelines for the first time with respect to the selection and implementation of proficiency tests (PTs) for chemical analytical methods. Even then, the intention was to provide assistance to certification and accreditation bodies in developing and operating PTs.[1]

The revised ISO-Guide 43:1997 "Proficiency Testing by Interlaboratory Comparisons" implemented the "International Harmonized Protocol for Proficiency Testing of Analytical Laboratories", established in 1993 by international organizations ISO, IUPAC, and AOAC, to outline the minimum requirements for the design, conduct, and interpretation of PTs.[2,3] The new International Standard ISO 17043:2010 defines a complete management system in addition to the described technical requirements for PT providers.[4]

When ISO/IEC 17025:2005[5] came into force, participation in PTs was mandatory, and it became an essential element of the quality management system for every accredited laboratory. In the field of food analysis, a broad range of PTs is available for nearly every practice-relevant analyte.

Since the Food Allergen Labeling and Consumer Protection Act (FALCPA)[6] and the introduction of mandatory allergen labeling for prepackaged foods by European Directive 2007/68/EG,[7] the challenges of allergen detection in food[8] and the conduct of allergen PTs have become more important.

Besides Dienstleistung Lebensmittel Analytik (DLA), other providers of food allergen PTs listed by the German PT agency "Koordinierungsstelle für Laboreignungsprüfungen (DKLL)" and the PT database "Eptis" are FAPAS and LVU-Lippold.[9,10]

DLA

DLA was founded in 2004 in Schleswig-Holstein (Germany). Since then, more than 220 laboratories from 29 countries have participated in the PTs. Nowadays, DLA offers about 50 PTs per year. Besides allergens, these PTs cover mycotoxins, toxic contaminants, nutrients, vitamins, minerals, and genetically modified organisms in foods.

Moreover, analyses of cosmetics and food contact materials are offered.

With six to seven PTs per year, DLA specializes in the conduct of food allergen PTs covering almost all allergens that have to be labeled (Table 1).[6]

Realization of Proficiency Testing. The implementation and evaluation of the DLA PTs were performed according to

Table 1. PT Program 2012—Allergens

PT	allergens	matrix
01/2012	egg and milk	sausage meat
02/2012	soy and wheat	"gluten-free" pastry
03/2012	β-lactoglobulin and gluten	infant food
04/2012	celery, mustard, and sesame	instant soup
05/2012	hazelnut and lupine	pastry
06/2012	peanut and almond	chocolate
07/2012	crustaceans and cashew	instant product
08/2012	lactose	"lactose-free" pastry

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the technical requirements of DIN EN ISO/IEC 17043 (2010) and DIN ISO 13528-2009.[4,11]

Organization. After registration, each participating laboratory receives two samples with different contents of the allergen to be determined and additional information with respect to conducting the PT. The participants should analyze the sample within 6 weeks. The methods are optional, and the results must be transmitted in written form. After the PT is over, evaluation should be conducted qualitatively and, if possible, quantitatively. A detailed report is sent to the participants. The data are presented in an anonymous form.

Production of the Samples. The selection of the sample material and its production should reflect realistic conditions of different food processing methods and composition of foods as they are common in commerce. It should be ensured that the participants can prove their ability to detect the analytes in differing matrices and levels of addition.

Therefore, matrices difficult to analyze because of a complex composition or thermal, chemical, and/or physical processing technique, as well as simpler matrices, will be used. For allergen PTs, two samples with differing allergen contents are provided. Usually one of the samples contains no allergen (a "negative sample") or the content is kept as low as possible. The homogeneity of the material is checked by analyzing five independent samples using ELISA methods. According to the recommendations of the AOAC Working Group, the Ministry of Health and Welfare (Japan), and DIN EN ISO 15633-1:2009, homogeneity is regarded as suitable when the repeatability standard deviation is <15%.[12−14]

Statistical Evaluation. The evaluation of the transmitted results is based on robust statistics according to DIN ISO 13528:2009. This statistical evaluation was adopted especially for the implementation of PTs.[11]

The robust mean, robust standard deviation, target standard deviation, z score, and standard uncertainty are calculated and used for the subsequent evaluation. Outliers will be determined by Mandel's H-statistic and eliminated in particular cases when necessary.[15] Practical experience showed that the precision requirements of the general model according to Horwitz[16] are not applicable to ELISA methods in a concentration range relevant for allergen detection.

Following the international recommendations of the AOAC Working Group, the Ministry of Health and Welfare (Japan), and DIN EN ISO 15633-1:2009, a relative target standard deviation of 25%, which could be deduced from data of precision experiments and from validation criteria, was set (DLA used 30% until 2009). This target standard deviation corresponds to the current capability of ELISA methods and is regarded by DLA as a realistic and desirable objective.[11]

In PTs it is assumed that the "true" value of a parameter is located in the center of several values, which normally spread around this value to a certain extent. Thus, the demand for a successful PT participation is to obtain an analytical result that is as close as possible to the center (the robust mean) of all results. The extent of deviation is established with the help of the z score, which is a measure of the difference between the laboratory result and the robust mean as a multiple of the target standard deviation. The criteria are considered fulfilled when the z score is ≤ 2.

Evaluation Report. After completion of the PT, each participant receives a report about the results and the statistical evaluation along with further information about the sample material and details from the participants about the analytical methods.

The statistical evaluation is performed with results from all methods. When there are at least five results from the same ELISA method (test kit), a separate statistical evaluation will be performed.

Evaluation of Method Performances and PT Overview. Basically, one should consider that the above-defined "true value" of the allergen amount could differ significantly from the actual amount that is actually contained in the PT sample, for example, by spiking a certain reference material. Such a disagreement is observed in particularly "difficult", for example, highly processed, matrices.

Therefore, in the present comprehensive PT overview, another evaluation in addition to the normal statistical evaluation in a PT report was carried out. The relative deviation between the medians of a specific method and the expected level of addition was calculated and referred to as "recovery rate" in the following sections. When there were at least five results from the same ELISA method, the standard deviation of the recovery rate was calculated. Below, the results are categorized according to the applied methods of PCR and ELISA and food matrices.

SOY ALLERGEN PROFICIENCY TESTS FROM 2006 TO 2011

From 2006 to 2011, a total of 76 laboratories participated in the soy allergen PTs, which were conducted each year. The matrices could be sorted in the categories of sausage meat and pastry. The spiking levels varied from 184 to 5500 mg/kg of soy protein. The used soy materials were flour (40% protein) and granulates (50% protein). The protein levels are average nutritional values given by the manufacturers. Soy flour was toasted by thermal treatment to a minimum of 100 °C for at least 30 min. Soy granulates are produced from the whole cleaned and toasted soybeans after crushing into a coarse meal and sieving. Table 2 gives an overview of the PT materials, including the allergen content.

Table 2. Composition of the Soy Samples of the PTs, 2006−2011

PT	matrix	level of addition of soy protein (mg/kg)
01/2006	soy flour in sausage meat	400
01/2008	soy flour in sausage meat	200
01/2007	soy granulate in pastry	5500
02/2009	soy granulate in pastry	138
02/2011	soy granulate in pastry	184
02/2010	soy flour in pastry	286

Qualitative Detection of Soy Using PCR and ELISA Methods. The following overview describes the qualitative agreement of results for the positive samples and the samples designated not to contain soy. For the purpose of better comparability, PTs with the same matrix and the same soy material were summarized in the categories of "soy flour in sausage meat", "soy granulates in pastry", and "soy flour in pastry". PCR and ELISA results were compared in these categories. Methods applied by the participants are listed in Table 3.

Soy Flour in Sausage Meat. The sausage meat samples for PTs 01/2006 and 01/2008 were manufactured using soy flour,

Table 3. ELISA and PCR Test Kits Used for the Determination of Soy in DLA PTs, 2006−2011[a]

abbrev	ELISA test kit	specificity	measuring range	intended matrices
AQ	AgraQuant, RomerLabs	soy trypsin inhibitor	1.7−42.5 mg/kg (unroasted) (LOD 0.68 mg/kg) 19−475 mg/kg (roasted) (LOD 7.6 mg/kg) (soy flour)	soy residues in a variety of food products
BK	BioKits, Neogen (formerly by Tepnel/ distributor Coring System Diagnostix)	soy protein (pAb)	0.35−7% (soy protein) (LOD 0.35%)	sausages
BK "high sensitive"	BioKits, Neogen (formerly by Gen-Probe/ distributor Coring System Diagnostix)	soy protein	1.25−20 mg/kg (soy protein) (LOD 0.3 mg/kg)	raw and cooked or processed foods, raw materials
ES	ELISA Systems, Transia	soy trypsin inhibitor	1−5 mg/kg (soy protein) (LOD 1 mg/kg)	
SA	Soy Alert ELISA Kit, Incura	soluble soy flour proteins	1−20 mg/kg (soyprotein) (LOD 0.4 mg/kg)	bakery products, cereals and pasta, milk and milk products, cocoa, chocolate, sauces, drinks, infant food
NL	NutriLinia	soy flour proteins	1−20 mg/kg (soy flour) (LOD 0.4 mg/kg)	
VT	Veratox, Neogen	soy flour proteins	10−100 mg/kg (soy flour) (LOD 1 mg/kg)	detection of soy flour

abbrev	PCR test kit	specificity	LOD/LOQ	intended matrices
SFA	Sure Food Allergen, Congen, r-Biopharm	soy DNA	<10 DNA copies ≤4 mg/kg (soy)	
FS	First Soya, GEN-IAL			
SK	Soya Kit, Incura	soy DNA	1 DNA copy	
AL	Alcum			

[a]Information according to the manufacturers' manuals.

Table 4. Qualitative Results: (A) Soy Flour in Sausage Meat, (B) Soy Granulates in Pastry, and (C) Soy Flour in Pastry

PT Material:	(A) Soy Flour in Sausage Meat				(C) Soy Flour in Pastry	
PT no.	01/2006	01/2006	01/2008	01/2008	02/2010	02/2010
method	PCR	ELISA	PCR	ELISA	PCR	ELISA
no. of participants	10	13	10	12	10	13
negative sample (no addition of soy): qualitative agreement	10 (100%)	13 (100%)	10 (100%)	10 (83%)	10 (100%)	13 (100%)
positive sample: qualitative agreement	10 (100%)	4 (31%)	8 (80%)	5 (42%)	10 (100%)	12 (92%)
PT Material:	(B) Soy Granulates in Pastry					
PT no.	01/2007	01/2007	02/2009	02/2009	02/2011	02/2011
methods	PCR	ELISA	PCR	ELISA	PCR	ELISA
no. of participants	6/7	12	10/11	10	11/10	16
negative sample (no addition of soy): qualitative agreement	5 (83%)[a]	10 (91%)	8 (80%)[a]	9 (82%)	9 (82%)	16 (100%)
positive sample: qualitative agreement	7 (100%)	11 (92%)	10 (91%)	8 (80%)	10 (100%)[a]	11 (69%)

[a]One participant without transmission of a result.

thus containing 400 and 200 mg/kg soy protein, respectively. The sausage meat ingredients (beef, 28%; pork, 28%; bacon, 24%; and preservatives) were minced with approximately 20% ice in a cutter. The sausage meat was filled in glasses, heated to a core temperature of 110 °C for 30 min, and stored at −18 °C until shipment.

In both PTs, 10 laboratories used PCR methods for qualitative soy detection, whereas 13 laboratories (01/2006) and 12 laboratories (01/2008) employed ELISA methods (Table 4A).

In PT 01/2006, both the negative and positive samples were identified correctly by 100% of the participants using the PCR methods. Five laboratories used the PCR kit Sure Food Allergen (SFA) and other in-house methods. The ELISA results showed larger discrepancies. A qualitative agreement of the positive sample was obtained by only one-third of the participants. Therefore, two-thirds of the results were false negative. The negative results were obtained using the test kit ELISA Systems (ES) (four of five) and Biokits (BK) (five of eight). There were no false-positive results.

In PT 01/2008, some more disagreements were obtained for both methods. Whereas the PCR methods showed an 80% agreement for the positive and 100% for the negative samples, the ELISA methods showed 42% agreement for the positive and 83% for the negative samples. A total of 7 of 12 laboratories (58%) could not detect soy protein in the positive sample, which was spiked with 200 mg/kg soy protein. The false-negative results were obtained using the test kits Veratox (VT) (one of one participant), ES (four of four), and BK (one of six) and one by an unknown ELISA method. All five positive ELISA results were obtained using the method from BK. To achieve this, some participants indicated a modification of the limit of detection of 3500 mg/kg soy protein.

Whereas the positive sample could be detected by the PCR methods, Soya Kit (SK), and in-house methods, one positive and one false-negative result were obtained using the SFA

method, and one false-negative result was obtained by the in-house method.

Soy Granulates in Pastry. The samples of PTs 01/2007, 02/2009, and 02/2011 were based on different pastry matrices (rusk, maize crisp bread, and cornflakes) with the addition of various amounts of spiked soy granulates (Table 2).

In the category "soy granulates in pastry" there were some more "false" results compared to the category "soy flour in pastry". The PCR method tended to be more reliable in the detection of positive samples, giving only 1 false-negative of 28 results (3.6%), whereas the ELISA methods gave 8 false-negative results of 38 results (21%) for the positive sample (Table 4B).

Soy was detected in the positive samples of 2007−2011 by the PCR methods SFA, FS, and SK with 100% agreement. Some laboratories did not indicate the applied method or used an in-house-method (one false-negative result for 02/2009).

A 100% agreement of qualitative results was obtained for the ELISA methods AgraQuant (AQ, n = 3 in 02/2009 and 02/2011), BK (n = 7 in 01/2007 and 02/2009), and NutriLinia (NL, n = 6 in 02/2011).

The highest number of false-negative results was obtained in PTs 02/2009 and 02/2011 using the ELISA methods. In total, 20% of participants (02/2009) and 31% of participants (02/2011) could not detect soy protein in the range of 138−184 mg/kg in the positive samples, whereas in PT 01/2007 with a soy protein content of 5500 mg/kg, only one false-negative result (8%) was obtained. False-negative results were obtained by applying the ELISA methods VT (one of one participant in 01/2007; two of four in 02/2011), and ES (two of five participants in 02/2009, two of three in 02/2011). Other participants using VT transmitted a qualitatively agreeing result for the positive sample (one of one in 02/2009; two of four in 02/2011) while indicating a content below the LOD of 10 mg/kg soy flour. The relatively high soy protein content of PT 01/2007 was detected positive by all seven participants using the ES test kit.

For the negative sample, a higher agreement was obtained using the ELISA methods as compared to the PCR methods, but it should be noted that the negative material is not certified to contain less than a certain level of soy protein. Thus, traces of soy protein may be contained in the designated "negative" samples. However, the ELISA methods BK and ES and an unknown method reported a total of three "false"-positive results in PTs 01/2007 and 02/2009. Using PCR, a total of five "false"-positive results were obtained using SFA (one result), SK (one result), and unknown methods (three results).

Soy Flour in Pastry. The PT sample of 02/2010 was a simple matrix of crushed and homogenized rice cakes with maize. The positive sample was spiked with 715 mg/kg toasted soy flour corresponding to 286 mg/kg soy protein (Table 2). The negative sample was spiked with wheat flour. The samples underwent no further processing such as baking, cooking, or roasting.

With one exception the qualitative agreement in this PT was 100% for both PCR and ELISA methods (Table 4C). Only one laboratory transmitted a false-negative result for the method ES (one of seven). The other ELISA methods BK (three participants), NL (two participants), and an unknown method revealed agreeing results. Similarly, PCR methods SFA (two participants), Alcum (AL) (one participant), and seven nonspecified methods showed qualitative agreement.

Quantitative Results. Because the target value ("true value") is normally in the center of all PT results, the homogeneity and comparability of results is especially important when several methods are applied. For example, PT 01/2007 demonstrates the diversity of quantitative results obtained using different ELISA-based test kits (Figure 2A). Often a separate statistical evaluation is necessary due to a bimodal or polymodal distribution of the results.[17] DLA performs a separate evaluation according to test kits in a normal PT report when at least five quantitative results are submitted. When only a lower number of results is available, a target value could not be established according to the referring test kit.

In the present evaluation all single results of the test kits are shown, for example, as in the dot plot diagram of PT 01/2007 (Figure 2A); whereas all four results of the ES method are in the range of 20 mg/kg soy protein, the results of the laboratories applying the test kit BK were about 200−300-fold higher. Therefore, the data showed apparently a bimodal distribution.

Hereafter the agreement between quantitative results of each soy PT in comparison to the actually added or contained allergen amount was assessed. Therefore, the laboratory results of the positive samples were considered separately according to each test kit applied. A "recovery rate" of the median of results was calculated for all of the applied test kits with respect to the spiked level.

Soy Flour in Sausage Meat. In PT 01/2006 only three of eight participants using the test kit BK were able to determine the added allergen by modification of standard and sample dilutions. The quantitative results varied considerably above the level of addition, giving a mean recovery rate of 262% (Figure 1A). From five laboratories using the test kit ES, only one participant obtained a positive result above the limit of quantification, specifying the result as >1 mg/kg soy protein. Therefore, the exact recovery rate is unknown (>0.25% of soy protein).

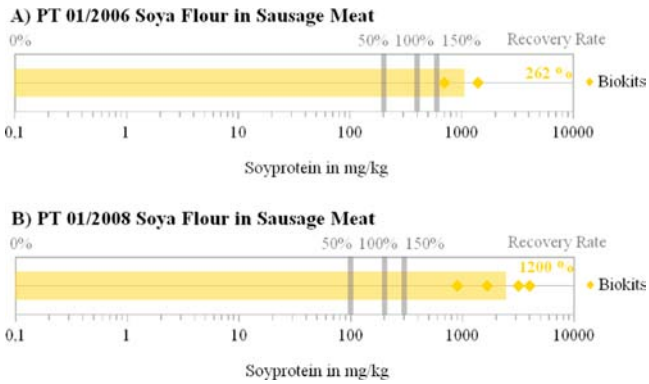

Figure 1. Dot and bar plots of median and recovery rates of data from test kit: soy flour in sausage meat.

In PT 01/2008 a total of 12 laboratories participated. Only 4 participants using the BK method gave a quantitative result again with modification of the LOQ (Figure 1B). The recovery rate was about 10-fold above the spiked allergen level with results varying about 4-fold. Another participant indicated a positive result below the limit of quantification (<3500 mg/kg soy protein).

Soy Granulates in Pastry. The recovery rates obtained using ELISA in PTs 01/2007, 02/2009, and 02/2011 are shown in Figure 2.

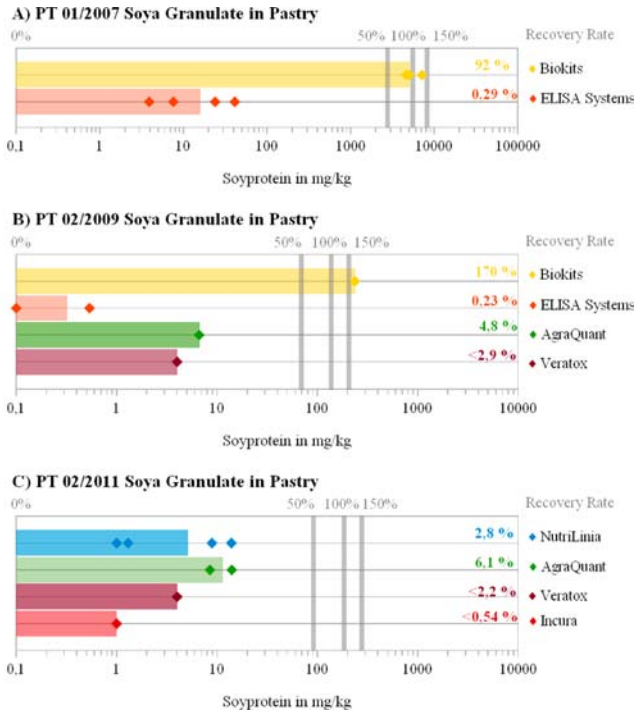

Figure 2. Dot and bar plots of median and recovery rates of data from different test kits: soy granulates in pastry.

There were 12 participants in PT 01/2007. The results of the laboratories obtained using the test kits ES and BK differed considerably (Figure 2A). The recovery of the spiked level (5500 mg/kg soy protein) of the four laboratories applying the test kit ES was only about 0.3%, whereas the three participants using the test kit BK detected 92% of the spiked level. One laboratory indicated a modification of the BK ELISA with respect to the calibration standards and sample dilutions. Another laboratory using the same test kit obtained a positive result below <7000 mg/kg soy protein.

A total of 11 laboratories participated in PT 02/2009 (Figure 2B). Overall, in comparison to PT 01/2007, very similar results were obtained. In one case, the modified test kit BK gave a recovery rate of 170%, and in the other case a positive result was indicated as <700 mg/kg soy protein.

Two of the four laboratories using the test kit ES designated the sample negative (<2.5 mg/kg soy) (Table 4B, qualitative results), whereas the two other participants determined only 0.2% of the spiked soy protein (138 mg/kg). One participant used the test kit AQ and received a recovery rate of 5%. One participant obtained a positive result, indicating <10 mg/kg of soy (<4 mg/kg soy protein) using the test kit VT. Therefore, the recovery rate was below 3%.

The positive sample of PT 02/2011 had a soy protein content of 184 mg/kg. Only 7 of 14 participants obtained a clearly positive result and reported a quantitative result (Figure 2C). With recovery rates of 2.8% (NL, four laboratories) and 6% (AQ, two laboratories), all positive results were at least 10 times below the spiked level. An additional three laboratories indicated positive results below the respective LOQ. The recovery rates were therefore below 2.2% (VT, two laboratories) and 0.54% (method Incura (SA), one laboratory).

Soy Flour in Pastry. There was only one negative result for the positive sample of PT 02/2010 with the matrix pastry (rice cakes). The 12 positive results were distributed in a similar range as shown in Figure 3: ES (six laboratories), BK (three laboratories), and NL (two laboratories). One additional result was obtained using an in-house method and was not considered here. The recovery rates were in the range of 3−5% of spiked soy protein for the test kits ES, NL, and the "high sensitive" test kit from BK applied for the first time in the soy PT series. The standard deviation (SD) for the recovery rate of ES results was ±2.1%.

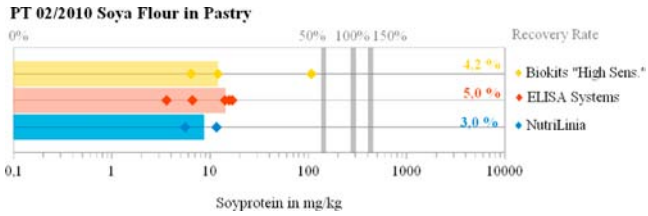

Figure 3. Dot bar plots of median and recovery rates of data from different test kits: soy flour in pastry (with a new "high sensitive" BK ELISA).

GLUTEN-ALLERGEN PROFICIENCY TESTS FROM 2006 TO 2011

A total of 10 allergen PTs with the parameter gluten were conducted by DLA from 2006 to 2011. A total of 169 participating laboratories performed qualitative and/or quantitative analysis using ELISA and PCR methods.

The PT samples contained gluten in the range of 7.8−6260 mg/kg. For spiking different gluten-containing materials wheat flour, spelt whole flour, wheat whole grain flakes, and wheat semolina were added. The food matrices were sausage meat, pastries, and infant semolina. Table 5 gives an overview of the PT materials including the allergen content calculated as gluten on the basis of the definition from the Gluten Intolerance Labeling Regulation (EU/41/2009).[18,19]

Table 5. Composition of the Gluten Samples of PTs 2006−2011

PT	matrix	level of gluten addition[a] (mg/kg)
01/2006	wheat flour in sausage meat	11
01/2008	wheat flour in sausage meat	34
02/2009	wheat flour in crispbread	139
02/2010	wheat flour in rice cakes	54
02/2011	wheat flour in cornflakes	39
02/2007	spelt whole flour and oat flour in infant semolina	6260
02/2008	wheat whole grain flakes in infant semolina	61
03/2009	wheat whole flour in infant semolina	122
03/2010	wheat semolina in sorghum infant semolina	25
03/2011	wheat flour and rice flakes in sorghum semolina	7.8

[a]The calculation is based on 90% gluten in wheat protein, following the definition of "gluten" from the Gluten Intolerance Labelling Regulation (EU/41/2009).[18,19].

Qualitative Detection of Gluten Using PCR and ELISA Methods. The following overview describes the qualitative agreement of results for the positive samples and the negative samples without the addition of gluten. PCR and ELISA results were compared in the following categories of the same matrices and the same gluten-containing materials: "wheat flour in sausage meat", "wheat flour in pastry", and "wheat/spelt whole flour and oat flour in infant semolina". The methods applied in PTs are listed in Table 6.

Wheat Flour in Sausage Meat. The sausage meat samples for PTs 01/2006 and 01/2008 were manufactured using 100 and 318 mg/kg wheat flour, respectively. The sausage meat ingredients (beef, 28%; pork, 28%; bacon, 24%; and preservatives) were minced with approximately 20% ice in a cutter and filled in glasses, heated to a core temperature of 110 °C for 30 min, and stored at −18 °C until shipment.

In PTs, no false-negative result was obtained using the PCR methods and only one false-negative result was obtained using the ELISA methods, whereas one "false"-positive result was obtained using the PCR and ELISA methods each (Table 7 A).

In PT 01/2006, a total of 14 laboratories employed the ELISA test kit Ridascreen (RS) and one test kit Transia Plate Gluten (TP). The false-negative result was obtained by one participant using the RS method.

With the PCR method Sure Food Allergen (SFA), one participant obtained a "false"-positive result. Two participants used the SFA method, one participant applied a PCR kit from Quiagen (QG), and another used an in-house-method.

In PT 01/2008 all participants obtained positive results for the spiked sample. The participants used the ELISA methods Biokits (BK) (3 laboratories), Veratox (VT) (1 laboratory), and RS (10 laboratories). One "false"-positive result was obtained using the ELISA method RS. Using the PCR methods of Incura (IC) (2 laboratories) and some in-house methods (3 participants), 100% agreeing results were obtained. Basically both PCR and ELISA methods were suitable for qualitative detection of the presence of gluten in sausage in the range of 11−34 mg/kg.

Wheat Flour in Pastry. The samples for PTs 02/2009, 02/2010, and 02/2011 were made from different pastries (rice cakes, cornflakes, and crispbread). According to the spiking level of wheat flour, the gluten content ranged from 39 to 139 mg/kg (Table 5).

The qualitative agreement for the positive samples of PTs was 100% for both PCR and ELISA results. With respect to the range, a reliable detection of gluten in pastry was ensured for both methods (Table 7B).

Among the ELISA methods, most often RS (approximately 80%) and BK (approximately 15%) test kits were used. Other ELISA methods were Gluten-tec (GT) (two participants) and Haven diagnostic (HD), AgraQuant (AQ), and ELISA Systems (ES) (one participant each). The PCR analyses were performed by the methods SFA, IC, and other not-specified methods and in-house methods.

For the "negative" samples some positive results were obtained using both PCR and ELISA methods primarily in PT 02/2011 with about one-third positives each. With one exception the ELISA results were <20 mg/kg, in agreement with the declaration of the sample as "gluten-free".[18] The "negative" sample of PT 02/2011 most probably contained a low background quantity of gluten. In the other PTs, 02/2009 and 02/2010, only one and two positive results were obtained, respectively.

Table 6. ELISA and PCR Test Kits Used for the Determination of Gluten in DLA PTs of 2006−2011[a]

abbrev	ELISA test kit	specifity	measuring range	intended matrices
AQ	AgraQuant, RomerLabs	gliadin	4−120 mg/kg (gluten) (LOD 0.6 mg/kg)	
BK	BioKits, Neogen (formerly by Tepnel/distributor Coring System Diagnostix)	ω-gliadin (Ab also specific for secalin from rye and hordein from barley)	3−50 mg/kg (gluten) (LOD 1 mg/kg) (since 2007)	
ES	ELISA Systems, Transia	ω-gliadin	2.5−25 mg/kg (gliadin) (LOD 1.25 mg/kg)	
GT	Gluten-tec, EuroProxima	gluten and gluten fragments (Ab against T-cell epitope of α-20 gliadin)	5−160 mg/kg (gluten) (LOD 2.5 mg/kg)	detection of intact proteins and hydrolyzed proteins
HD	Haven Diagnostic (later by Hallmark, Biocheck)	gluten	5−125 mg/kg (gluten) (LOD 0.5 mg/kg)	raw materials and processed foods
RS	Ridascreen, R-Biopharm (a, gliadin; b, gliadin FAST)	gliadin	(a) 5−80 mg/kg (b) 10−80 mg/kg (gluten) (LOD (a) 3 mg/kg. (b) 4 mg/kg)	for analysis of prolamins from wheat (gliadin), rye (secalin), and barley (hordein) in a gluten-free declared food
TP	Transia Plate Gluten, Transiaw	ω-gliadin	LOD 10 mg/kg (gluten)	
VT	Veratox, Neogen	gliadin (Ab also detects prolamines from rye and barley)	5−50 mg/kg (gliadin) (LOD 0.88 mg/kg)	
abbrev	PCR test kit	specifity	LOD	intended matrices
SFA	Sure Food Allergen, Congen, R-Biopharm	DNA from gluten-containing cereals	<5 DNA copies ≤0.4 mg/kg (gluten)	
IC	Incura	gluten-DNA from wheat, durum wheat, medium spelt, kamut, triticale, rye, oat, and barley	1 DNA copy	
QG	Qiagen			

[a]Information according to the manufacturer's manual.

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Table 7. Qualitative Results: (A) Wheat Flour in Sausage Meat, (B) Wheat Flour in Pastry, and (C) Wheat, Spelt Whole Grain, and Oat Flour in Infant Semolina

PT Material:	(A) Wheat Flour in Sausage Meat						(B) Wheat Flour in Pastry			
PT no.	01/2006	01/2006	01/2008	01/2008	02/2009	02/2009	02/2010	02/2010	02/2011	02/2011
method	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA
no. of participants	4	15	5	15	6	14	6	16	11	17
negative sample (no addition of gluten): qualitative agreement	3 (75%)	15 (100%)	5 (100%)	14 (93%)	5 (83%)	15 (100%)	5 (83%)	14 (88%)	7 (64%)	12 (71%)
positive sample: qualitative agreement	4 (100%)	14 (93%)	5 (100%)	15 (100%)	6 (100%)	15 (100%)	6 (100%)	16 (100%)	11 (100%)	17 (100%)
PT Material:	(C) Wheat, Spelt Whole Grain, and Oat Flour in Infant Semolina									
PT no.	02/2007	02/2007	02/2008	02/2008	03/2009	03/2009	03/2010	03/2010	03/2011	03/2011
methods	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA
no. of participants	3	16	2	19	3	23	2/1	15/14	5/4	20
negative sample (no addition of gluten): qualitative agreement	nd	nd	0 (0%)	16 (84%)	3 (100%)	23 (100%)	1[a] (100%)	14 (93%)	3 (60%)	19 (95%)
positive sample: qualitative agreement	3 (100%)	16 (100%)	2 (100%)	17 (89%)	3 (100%)	23 (100%)	2 (100%)	12[a] (86%)	4[a] (100%)	20 (100%)

[a]One participant without transmission of a result.

Wheat, Spelt Whole Grain, and Oat Flour in Infant Semolina. In 2007−2011 five PTs were performed using a matrix of infant semolina. For this purpose, different gluten-containing materials such as wheat semolina, whole flour, wheat whole grain flakes, spelt, and oat flour were mixed with infant cereal semolina of sorghum and rice. The sample with the highest gluten content had 6260 mg/kg gluten; the sample with lowest content had 7.8 mg/kg gluten (Table 5).

For the positive samples of the investigated matrices, there were 14 positive PCR results, giving a total of 100% agreement, and 88 positive ELISA results, giving 86−100% agreement (Table 7C).

For each of the PTs 02/2008 and 03/2010 two false-negative results were obtained using the ELISA methods. The positive sample of PT 02/2008 contained a spiked level of 61 mg/kg gluten. Despite the high content, two of the four participants using the test kit BK could not detect gluten. The other 15 participants all obtained positive results using the test kit RS.

In PT 03/2010 with an addition level of 25 mg/kg gluten two participants obtained false-negative results using the test kit RS.

In PTs 02/2008, 03/2010, and 03/2011 a total of five "false"-positive results were obtained using the ELISA methods for the nonspiked "negative" samples. The participants indicated mainly results <20 mg/kg. In 2008, two "false"-positive results were obtained using RS (of 14 results), and one of four results using the test kit BK. Only one positive result (37 mg/kg) was higher than the limit for labeling as "gluten-free" (20 mg/kg). In PTs 03/2010 and 03/2011 a false-positive result <20 mg/kg gluten was obtained using the test kit RS (1 of 14 results 03/2010 and 1 of 13 results in 03/2011). Using the test kits ES, GT, AQ, HD, and VT, no positive results were obtained for the "negative" samples.

Using the PCR methods false-positive results occurred in PTs 02/2008 and 03/2011.

Overall, with some limitations both PCR and ELISA methods were suitable for qualitative detection of gluten contents in infant semolina.

QUANTITATIVE RESULTS

As aforementioned for the soy PTs, a statistical evaluation of all results together is often not reasonable because of the multimodal distribution of results from different ELISA methods. As an example, the gluten results of PT 02/2009 (Figure 5A) showed deviating levels of results. Whereas the participants using the test kit BK determined results in the range below 10 mg/kg gluten, the results obtained using the RS method were approximately 30 times higher. The results of two other ELISA methods, AQ and ES, were located in between.

For the gluten PTs the recovery rates of the allergen content were calculated for each test kit, too. It was calculated from the median of all results from a respective test kit in relation to the added level of gluten.

Wheat Flour in Sausage Meat. In PT 01/2006, 14 participating laboratories applied the test kit RS (Figure 4A).

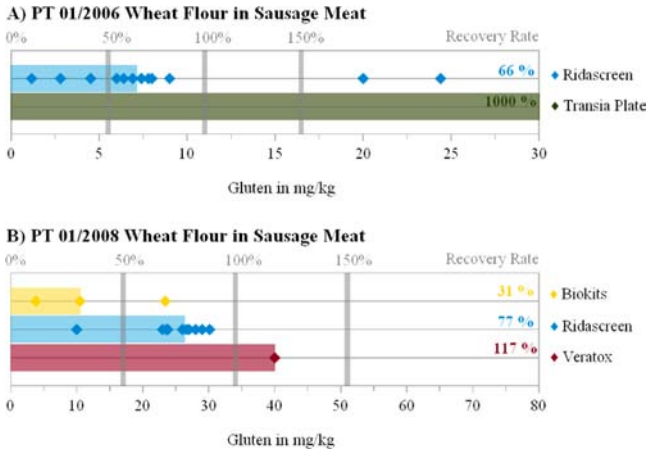

Figure 4. Dot and bar plots of median and recovery rates of data from different test kits: wheat flour in sausage meat.

Of these, 12 participants obtained a recovery rate of 66% (outlier corrected SD ± 23%) of the level of addition (11 mg/kg gluten). One laboratory submitted only a qualitative result (positive, >3 mg/kg). Another participant obtained a negative result (<10 mg/kg). One participant using the Transia Plate Gluten method (TP) obtained a result that was 10 times higher than the level of addition (Figure 4A).

In PT 01/2008, a total of 15 laboratories participated, 10 using the test kit RS, 3 using BK, and 1 using VT (Figure 4B).

All methods detected gluten in the positive sample. The mean recovery rates of the results obtained using RS (77%, SD ± 17%) and VT (117%) were in the range of 50−150% recommended by AOAC. The level of addition was 34 mg/kg gluten. The mean recovery rate of the results obtained using the test kit BK was 31% of gluten in the positive sample.

Wheat Flour in Pastry. The participants of PTs 02/2009, 02/2010, and 02/2011 predominantly applied the test kit RS (30 laboratories). Other test kits were BK (7 laboratories) and GT (2 laboratories) as well as ES, AQ, and HD (1 laboratory each).

In PT 02/2009 (Figure 5A) the mean recovery rate (128%, SD ± 62%) closest to the spiking level was obtained by seven participants using the test kit RS. The slightly higher recovery rate may be due to the fact that the antibody of the kit detects prolamins from rye and barley as well. The PT material crispbread contained, besides wheat, smaller amounts of rye, the exact level of which was unknown. However, the recovery rates in PTs 02/2010 and 02/2011 were higher, implicating a higher response to the target material by the RS test kit in the respective matrices of pastries. Both PT materials contained no other prolamin sources other than wheat flour. An additional four results in PT 02/2009 obtained using the test kit BK gave a recovery rate of only 6% of the level of addition. One laboratory determined 32% of the spiked gluten level by the test kit ES. Another participant used the test kit AQ with a recovery rate of 19% of the spiked gluten level.

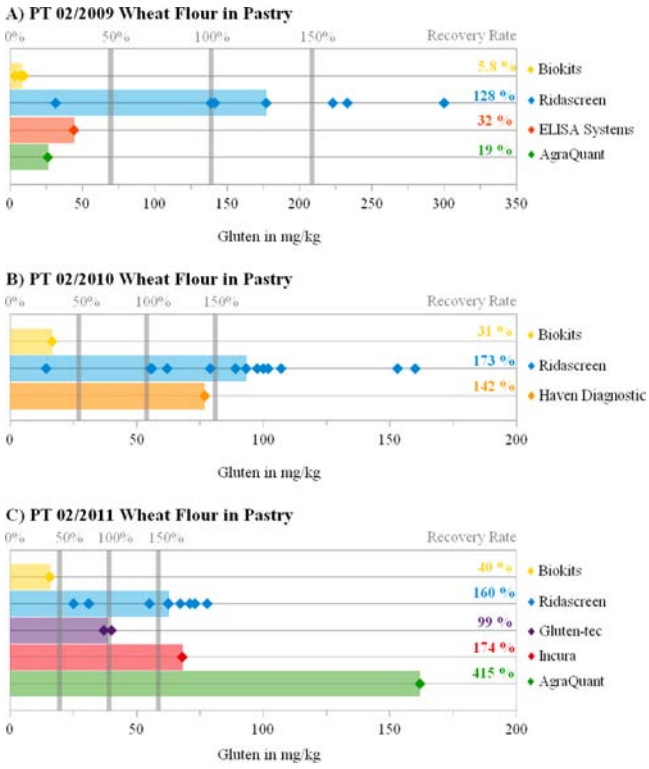

Figure 5. Dot and bar plots of median and recovery rates of data from different test kits: wheat flour in pastry.

In PT 02/2010 (Figure 5B) the mean recovery rate obtained by 13 participants using the test kit from RS was 173% (SD ± 73%) of the level of addition. This value was above the recovery range of 50−150% recommended by AOAC.[12] The result of one participant using the test kit BK corresponded to a recovery rate of 31%. A single result obtained by a laboratory applying the test kit HD gave a recovery rate of 142%, which was in the recommended range.

In PT 02/2011 similar levels of recovery were shown (Figure 5C). Again the recovery rate from the test kit RS (10 participants) was above 150% (160%, SD ± 50%), and both laboratories using the test kit BK obtained recovery rates below 50%. The two participants using the test kit GT found 99% of the added gluten amount. The single results of test kits IC and AQ gave recovery rates of 174 and 415%, respectively.

Overestimation of gluten due to traces in the PT sample matrices (as determined in the negative samples by a few participants) should be below 7 and 12% for the PTs 02/2009 and 02/2010 and below 20% for PT 02/2011 in relation to the spiked levels of the positive samples (data derived from DLA reports).

Wheat, Spelt Whole Grain, and Oat Flour in Infant Semolina. The results of PTs from 2007 to 2010 showed very similar recovery rates. The results obtained using the same methods showed constant but overall relatively low recovery rates over the years in the matrices of infant semolina (Figure 6).

The exception is PT 03/2011, in which e.g. for method RS a higher recovery rate (159%, SD ± 31%) was obtained in comparison to the low level of addition of 7.8 mg/kg gluten, which could be due to analysis near the limit of quantification. On the other hand, the participants indicated results for the negative sample matrix in the range from <1.5 to <10 mg/kg, which could eventually explain the higher recovery rates of the added gluten in the positive sample.

The recovery rate (94%) closest to the spiked level was obtained by three laboratories using the test kit GT in PT 03/2011. Also, acceptable recovery rates were obtained by one participant using the method Transia Gluten in PT 02/2007 and one participant using the test kit VT in PT 03/2009. The results obtained using the test kit RS showed a recovery rate (55%, SD ± 17%) within the recommended range of 50−150% only in PT 03/2010. In the other three PTs (without PT 03/2011), approximately 13 participants using the RS kit obtained recovery rates of 36 ± 13% (PT 02/2007), 41 ± 19% (PT 02/2008), and 30 ± 15% (PT 03/2009). A mean of three participants who used the test kit BK in the years 2007−2010 obtained recovery rates of only 3−5% of the added gluten amount. In PT 03/2011 one participant obtained a recovery rate of 67% using BK.

DISCUSSION

The PT results for the parameters gluten and soy protein showed differing suitabilities of the ELISA methods with respect to the target allergen, the degree of food processing, and specific test kit characteristics, which include extraction efficiency, calibration material, and antibody specificity. The observed differing levels of data obtained for the respective test kits (multimodal distribution of results) in the soy and gluten PTs from 2006 to 2011 often made a differentiated statistical evaluation necessary. In some cases, due to a low number of results for a test kit, even a separate evaluation in the respective PT report was not possible.

Although gluten could be reliably detected and quantified by most ELISA methods, these methods were not fit for the purpose of detection of soy protein. All ELISA test kits for soy applied in the PTs showed a high number of false-negative results, and recovery rates did not fulfill the recommenda-

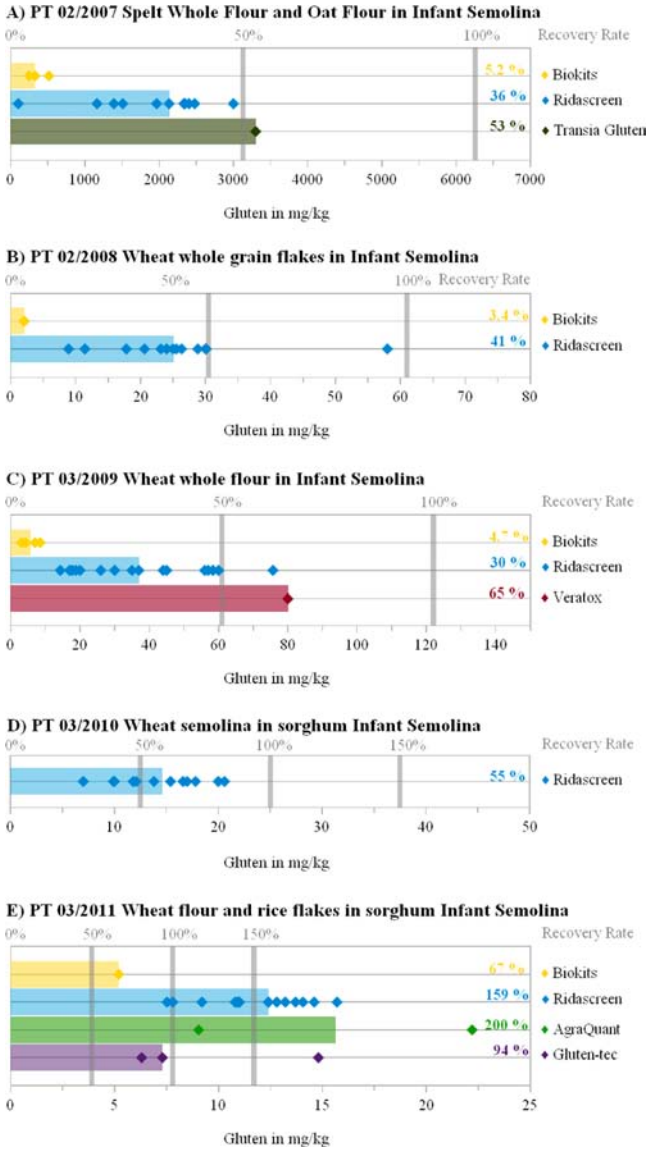

Figure 6. Dot and bar plots of median and recovery rates of data from different test kits: wheat, spelt whole grain, and oat flour in infant semolina.

tions.[12] Some ELISA methods were capable of detecting the soy protein added in a less processed state and in simpler matrices, whereas the recovery rates in more highly processed food matrices such as sausage meat were very poor. The effect of food processing on the recovery of food allergens in sugar cookies was recently discussed by Khuda et al.[20]

In some cases there is a lack of information about the suitability of the referring ELISA method for different food matrices. In other cases a method was applied for nonvalidated or even indicated as "not suitable for" food matrices. An example may be the BK test kit with a high LOQ of 0.35%, which is not sufficient for the detection of soy protein in the milligrams per kilogram range. Some participants modified the protocol to detect lower amounts and obtained predominately strongly differing quantitative results. Meanwhile, the manufacturer Gen-Probe developed a "high sensitive" sandwich-ELISA format with a suitable LOQ. The kit was used by participants in PT 2/2010. Another example is the kit VT, which was intended for the detection of soy flour by the manufacturer. Any further soy processing or food processing may lead to negative results. Regardless, the kit VT was used for the detection of soy protein in different states and matrices.

Soy. Most difficulties were observed for the detection of soy protein using ELISA methods. For qualitative detection of soy the PCR-based methods were much more reliable. Whereas only 5% false-negative results were obtained from the 57 submitted PCR results, one-third of 75 participants detected no soy protein in the positive samples using the ELISA methods (Table 8A). The highest number of false-negative results (69

Table 8. False-Negative Results for the Detection of (A) Soy and (B) Gluten in DLA PTs

PT matrix	PCR (%)	ELISA (%)
(A) soy PTs		
soy flour in sausage meat	10	64
soy granulate in pastry	3.6	21
soy flour in pastry	0	8
(B) gluten PTs		
wheat flour in sausage meat	0	3
wheat flour in pastry	0	4
wheat flour in infant semolina	0	0

and 58%) was observed for the ELISA methods for the detection of soy flour in sausage meat. In pastries the number of false-negative results were in the range of 8−32% for soy granulates and 8% for the detection of soy flour. From three false-negative PCR results two were obtained in the matrix of sausage meat and one in pastries.

For the quantification of allergens in foods using ELISA methods the AOAC Working Group recommends recovery rates in the range of 50−150%.[12] With the applied soy ELISA methods ES, BK, NL, and AQ, this recommendation could not be fulfilled even in simpler matrices with a high content of allergen in the range of 138−5500 mg/kg soy protein. One exception was seen for the method BK in PT 01/2007 (soy granulates in pastry). This test kit was developed for the quantitative determination of soy in sausages within a range of 0.35−7% of soy protein and was not intended for the detection of allergens in trace amounts. For the high level of addition of 5500 mg/kg soy protein, a recovery rate of 92% was achieved using the BK method (three participants). In PTs 01/2006, 01/2008, and 02/2009 with much lower allergen levels of 138−400 mg/kg soy protein, the participants applying the BK kit modified the measuring range to achieve a lower limit of detection. The results of the PTs with recoveries of 262, 428, and 1212% showed that a reliable quantification with the modified kit was not possible. In PT 02/2010 the "high sensitive" BK kit showed a lower recovery rate of 5.6% underestimating the allergen.

These findings are in agreement with the reported results of a comparative study of different methods for the determination of soy in eight different foods.[21] Results showed qualitative agreements of the commercial ELISA kit (BK, formerly Tepnel) and the PCR method for amounts >1400 mg/kg of soy flakes.

For the test kits NL and ES, clearly lower recovery rates were obtained for the more highly processed proteins from soy granulates than for the less processed soy flour proteins. The recovery rates were all below 10%. In the same range were the results of the test kit AQ (recovery rates of 6−12%) and VT

(positive below the LOQ) applied for the determination of soy granulates in pastry.

Therefore, in all matrices of the PTs from 2006 to 2011 the ELISA methods were not suitable for the detection of soy protein on a level that is relevant for allergic individuals (e.g., 10−100 mg/kg Soya Action level 2 VITAL Allergen Risk Assessment/25−250 mg/kg Soya EU-VITAL).[22] All applied ELISA-based test kits, which were intended for the detection in lower milligram per kilogram range, showed a dramatic underestimation of the soy allergen content.

Gluten. A total of 45 laboratories submitted PCR results and 170 laboratories submitted ELISA results for the parameter gluten in 10 PTs. The qualitative agreements of results were fair for both ELISA and PCR methods (Table 8B). There were no false-negative results for the PCR methods in all PTs. Using ELISA methods 98% of participants (165 of 170 results) detected gluten in the positive samples correctly. The five false-negative results were obtained for complex matrices such as pastry and sausage meat. In the category of "wheat flour in infant semolina" no false-negative results were seen. Both PCR and ELISA methods were generally suitable for the detection of different gluten amounts in infant food, pastry, and sausages in the range of 7.8−6260 mg/kg.

The quantitative ELISA results for gluten gave in general better recovery rates than for the soy ELISA methods. For gluten predominately some differences were observed regarding the specificity of antibodies applied in the test kits. The test kits BK, ES, and HD employed the so-called "Skerritt" antibody directed against ω-gliadin. The test kit RS used the "R5" antigliadin or "Mendez" antibody, which was recommended by Codex Alimentarius.[23]

Three other test kits worked with other antibodies developed against other distinct protein fractions or peptides.

The ELISA method BK was applied in mean by only three to four participants in general, achieving recovery rates clearly below 50%. With one exception in all PTs only a mean of 17% (3.4−40%) of the added gluten content was recovered. Therefore, the test kit BK was not suitable to fulfill the recommendation for a recovery of 50−150% for the determination of gluten in pastry, sausage meat, and infant semolinas. In PT 03/2011 one result with a recovery rate of 67% was obtained.

Mainly fair recovery rates were achieved by the participants using the two ELISA test kits (RS and GT) validated according to the AOAC guidelines. Participants applying the test kit RS achieved recovery rates in the range of 50−150% in four of the 10 PTs with matrices of sausage meat (01/2006; 01/2008), crispbread (02/2009), and infant sorghum semolina (03/2010). In the matrices of cornflakes (02/2010), rice cake (02/2011), and one infant semolina PT (03/2011), recoveries above 150% (173 and 160%) were observed, whereas the recoveries for three other infant semolina PTs were below 50% in 2007−2009. Immer and Haas-Lauterbach reported recovery rates of 84−109% in a recent collaborative study.[24]

With the test kit GT, 94 and 99% recoveries were obtained in the matrices of infant semolina (three participants) and cornflakes (two participants). The test kit uses a monoclonal mouse antibody directed against intact protein and protein fragments.[25]

The ELISA test kit AQ is based on a polyclonal antibody directed against prolamins of wheat, rye, and barley. The method was used by two participants in PT 03/2011 (infant semolina) and by one participant each in PT 02/2009 and 02/2011 (pastry). The respective recovery rates (200, 19, and 415%) were all out of the recommended range of the AOAC Working Group. In 2012, RomerLabs launched a new ELISA test kit "gluten G12" based on a monoclonal antibody directed against an α2-gliadin fragment.[26]

For some test kits only one or two results were submitted, allowing no reliable estimation of their suitability for the detection or quantification of gluten. The method ES was applied by one participant each in two PTs with recovery rates of 53 and 32%. For the test kits HD, Incura, Transia Plate, and Transia Gluten only one result was submitted each in different PTs. The recovery rates were 142, 174, 1000, and 53%, respectively. The method VT ("Veratox Gliadin") was used by one participant each in two PTs with recovery rates of 65 and 112%, respectively. This test kit was the predecessor of the test kit "Veratox R5 Gliadin" (Neogen) with a new extraction buffer and usage of an antibody similar to the R5 anti-gliadin antibody.[27]

In a recent investigation Sharma compared the immunoreactivity of six commercial ELISA test kits for gluten. The recovery rates were determined for maize flour samples spiked with wheat flour. For the Skerritt antibody-based test kits Biokits, Aller-Tek, and ELISA Systems, recovery rates were, respectively, 146%, approximately 1600%, and 395% of the spiked level, and for the R5 antibody-based test kit Ridascreen, the rates were 74%. The other methods Morinaga and AgraQuant achieved recovery rates of 126 and 294%.[28] Formerly, Thompson and Mendez reported some limitations of commercial test kits applying the anti-ω-gliadin antibody and the R5 antibody, respectively. The first ones were unable to accurately determine barley prolamins, whereas the latter overestimated barley hordein. Both methods could detect native and heated proteins but could not accurately quantify hydrolyzed gluten from wheat, barley, and rye.[29] Recently, a competitive ELISA using the R5 antibody was introduced by R-biopharm to detect gluten fragments.[30]

Multimodal distribution of ELISA results determined by different test kits in allergen PTs from FAPAS was also observed by Owen and Gilbert. They described the special problem of statistical evaluation of results in proficiency testing of allergen methods.[31] The systematic deviation of ELISA results could be due to the use of different extraction buffers, antibodies with different specificities, and different calibration materials.[31]

Recently, Sykes et al. described an attempt to transform a multimodal distribution of data from allergen proficiency tests into a symmetric distribution to evaluate these normalized results from different test kits together.[17] For this purpose PTs were performed with an additional PT sample. A total of three samples were analyzed by the participants: a spiked sample, a nonspiked sample, and another spiked sample called by the authors a "single-point-calibrant". Afterward, the quotient of the results for the two spiked samples was calculated and evaluated. The resulting data set showed an improved symmetric distribution of results quotients in contrast to the non-normalized data.[17]

The "normalized" results from different ELISA test kits were thus transformed into a "comparable" form and an evaluation of z scores was possible. On the basis of the described procedure the evaluation of a target range of the "real" allergen content in the respective food is not a criterion. Moreover, the z score for an individual laboratory result is not related to a target value expressed as an allergen content in milligrams per kilogram.

Instead, the internal correlation of the results for two samples containing different allergen levels is relevant for this kind of quality assessment.

The present evaluation of soy and gluten PTs showed general problems in the allergen analysis in foods and emphasized the special difficulties of the statistical evaluation of allergen PT results. These problems are relevant not only for the quality assurance of laboratory performances with respect to ISO 17.025 but also to the allergen risk management in the food industry to ensure adequate consumer protection.

Laboratories should be aware of the suitability of analytical methods for allergen detection and possible interpretation of respective results. In this respect, allergen PTs give valuable information not only for the performance of a single laboratory but for the applicability/suitability of ELISA and PCR methods for the determination of a specific allergen in a spectrum of food matrices.

Whereas both ELISA and PCR methods showed fair results for the determination of gluten, it was shown that the ELISA test kits are often not reliable for qualitative detection of soy in different food matrices. In contrast, soy PCR methods are much more reliable for excluding false-negative results. Therefore, in the case of soy detection, it could be useful to apply a combination of both methods, first, a qualitative test by PCR and, if necessary or demanded, a quantification by a suitable ELISA method to verify the allergen content.

More recent trends in allergen analysis in foods employ quantification by PCR methods and detection by multiplex PCR, biosensor chip PCR, and liquid chromatography−mass spectrometry. Köppel et al. demonstrated a qualitative accordance but a low correlation of quantitative results in a comparison of the determination of eight food allergens using tetraplex real-time PCR and ELISA methods.[32] Recently, the simultanous detection of eight allergens was demonstrated by Mustorp et al. using multiplex PCR and by Wang et al. by thin-film biosensor chips.[33,34] The perspectives and recommendations for the mass spectrometry methods were recently summarized by Johnson et al.[35]

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Notes

The authors declare no competing financial interest.

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