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Development of milk and egg incurred reference materials for the validation of food allergen detection methods

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Abstract

Background Effective allergenic risk assessment and management are important to limit the use of precautionary statements such as 'may contain' and to be able to protect allergic consumers. However, such approaches require reliable analytical tools for the detection of allergens in food. Very few validation data are available for the comparison of results obtained with different allergen detection methods. This is certainly due to the lack of harmonized validation protocols and of recognized reference materials. *Aims* The Monitoring and Quality Assurance Working Group on Food Allergens will provide incurred reference materials with egg and milk proteins at various concentrations. *Materials and Methods* The development of an incurred reference material for the analysis of milk and egg allergens in a baked cookie food matrix is described. *Results and Discussion* We present the results of the development of the incurred reference material and a pre-ring trial with two incurred reference materials for milk detection methods: cookies and soy-based infant formula. *Conclusions* The material produced seems to be suitable as reference material as well as for testing the performance of test kits. The forthcoming validation study according to the harmonized validation protocol will significantly and positively impact on future validation procedures.

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Introduction

Effective allergenic risk assessment and management are important to limit the use of precautionary statements such as 'may contain' and to be able to protect allergic consumers. However, such approaches require reliable analytical tools for the detection of allergens in food, in order to inform risk managers about the extent of carry over of allergenic ingredients on common processing lines, problems of cross-contact from dusts in factory environments and to monitor clean-up procedures. They are also required by those enforcing legislation to monitor food products for the presence of allergens in foods (Kerbach *et al.*, 2009).

While enzyme-linked immunosorbent assay (ELISA) is the most common method used for the detection and quantification of allergens in foodstuffs (Poms *et al.*, 2004b), very few validation data are available. However, it has been evident for some time that there are variations in kit performance, related to differences in antibody preparations used, extractions methods and calibrants (Poms *et al.*,

2005). There are also issues of sensitivity which are matrix dependent, with the best characterized example being peanut where different processing conditions can affect recoveries determined by ELISA (Poms *et al.*, 2004a).

In order to assess and compare the performance of methods provided by the various test kit manufacturers, it is necessary to have harmonized validation protocols and effective, validated, reference materials for allergen analysis, which are still not readily available. It is a major aim of the Monitoring and Quality Assurance (MoniQA) Food Allergen Working Group to provide both harmonized validation protocols and reference materials for ELISA-based test methods used to support food allergen management and official control of legal compliance. MoniQA is an EU-funded Network of Excellence working towards harmonization of monitoring and control strategies in the total food supply chain (Poms *et al.*, 2009).

A guidance protocol on method validation and testing the performance characteristics of quantitative food allergen ELISA methods was recently published under the auspices of the AOAC Presidential Task Force on Food Allergens and with the active contribution of the MoniQA Allergen Working Group (Abbott *et al.*, 2010).

The current lack of reference materials suitable for the development of allergen detection methodologies, particularly in different food matrices, must be urgently remedied in order to assess the output of different validation studies as well as to allow comparability between different methods (Kerbach *et al.*, 2009). The most important characteristics of a reference material for analytical quality control are homogeneity and minimum sample size, stability during transportation and storage, commutability and a measurement value, if possible with traceability properties and an uncertainty value (Poms *et al.*, 2006). Good commutability (according to International Standards Organisation, 2003 being a reference material as close/similar to the sample material to be assessed as possible) is of special significance for food allergen analysis, considering the matrix and processing effects that may hamper detectability of marker proteins or the allergen itself. In this context it is important to note that spiked samples may result in an artificially higher recovery than incurred samples.

An incurred sample is defined as a sample in which a known amount of the food allergen has been incorporated into the sampling before processing, mimicking as closely as possible the actual conditions under which sample matrix would normally be manufactured (Abbott *et al.*, 2010). The incurred material is introduced as an ingredient and undergoes similar processing to the other food ingredients (Poms *et al.*, 2005; Taylor *et al.*, 2009). Incurred materials can be difficult and expensive to produce as each type and dose of allergen requires its own production run with particular care taken to ensure no cross-contamination occurs. Homogeneity of incurred materials can also be difficult to ensure and must be considered at each stage of material processing. As the food matrix used will impact detection of the target it is necessary to use an incurred material with similar properties to that of the food being analysed, resulting in the necessity for many incurred materials to cover the broad range of typical food samples. Despite these difficulties, however, it is clear that in many cases spiked standards are insufficient for proper evaluation of allergen content in food, and that incurred materials must be developed. To this end we describe the development of an incurred reference material for the analysis of milk and egg allergens in a baked cookie food matrix. We also present the results of a pre-ring test with two incurred reference materials for milk detection methods: cookies and soy-based infant formula.

Materials and methods

ELISA test kits

Five test kits were selected to analyse the incurred cookies in an in-house evaluation: CER Groupe (in-house method), Casein Residue assay from ELISA Systems (Windsor, Queensland, Australia); Milk Protein ELISA Kit (Casein) from Morinaga (Sachiura, Yokohama, Japan); RIDASCREEN® FAST Casein from R-Biopharm (Darmstadt, Germany); BIOKITS Casein Assay kit from Tepnel Biosystems Ltd./ GenProbe (Deeside, Flintshire, UK). Results obtained with each test kit is presented only as 'A–E' to maintain commercial confidentiality. Veratox for Total Milk Allergen from Neogen (Lansing, MI, USA), was used in the pre-ring trial only, and CER Groupe was not included in that trial.

Most relevant information on the type of ELISA, range of determination and the standard calibrators is shown in Table 1.

Production of the incurred reference materials containing egg and milk

Cookie was the first foodstuff selected to develop an Reference Material Incurred for milk and egg detection methods. The recipe used to produce the cookies was based on the one published by Scaravelli *et al.* (2008). Then, those cookies were produced in order to determine the peanut content by real-time polymerase chain reaction. To avoid any traces of milk, butter was replaced with olive oil (Table 2). Several wheat flours were tested as they can

Table 1 Overview of ELISA test kits used in the study

Name of test	Format	Standard	LOQ (p.p.m.)	Range of quantification (p.p.m.)
Casein detection				
CER Groupe Casein Assay (in-house)	Sandwich	Casein	0.5	0.5–20
Casein Residue assay (ELISA Systems)	Sandwich	Skim milk powder	1.0	1.0–10.0
RIDASCREEN®FAST Casein (R-Biopharm)	Sandwich	Casein	0.5	0.5–13.5
BIOKITS Casein Assay kit (Tepnel)	Competitive	Whole milk powder RM8435 from NIST (expressed in casein)	1.6	1.6–25
Milk Protein ELISA Kit, Casein (Morinaga)	Sandwich	Milk protein	0.312	0.312–20
Veratox for Total Milk Allergen (Neogen)	Sandwich	Non-fat dried milk protein	2.5	2.5–25
β-lactoglobulin (BLG) detection				
CER Groupe BLG Assay (in-house)	Sandwich	BLG	0.25	0.25–5
BLG Residue assay (ELISA Systems)	Sandwich	BLG	0.1	0.1–1
RIDASCREEN® BLG (R-Biopharm)[1]	Sandwich	BLG	0.2	0.2–16.2
BIOKITS BLG Assay kit (Tepnel)	Competitive	BLG	2.5	2.5–40
Milk Protein ELISA Kit, BLG (Morinaga)	Sandwich	Milk protein	0.312	0.312–20
Egg detection				
CER Groupe Egg Assay (in-house)	Sandwich	Whole egg powder protein RM8415 (NIST)	2	2–50
Egg Residue assay (ELISA Systems)	Sandwich	Egg white protein	1	1–5
RIDASCREEN®FAST Ei/Egg Protein (R-Biopharm)	Sandwich	Egg white protein	1	1–27
BIOKITS Egg Assay kit (Tepnel)	Competitive	Ovomucoid (expressed in egg white protein)	0.5	0.5–10
Egg Protein ELISA Kit (Morinaga)	Sandwich	Egg protein	0.312	0.312–20

[1]Although RIDASCREEN® β-lactoglobulin is a *qualitative* assay for food samples, it has been used as a *quantitative* one.
ELISA, enzyme-linked immunosorbent assay; NIST, National Institute of Standards and Technology.

Table 2 Adaptation of the cookie recipe

Ingredients	Proportion (%)
Scaravelli's recipe	
Butter	19.6
Wheat flour	49.0
Dust sugar	18.4
Skimmed milk powder	5.9
Water	6.6
Sodium chloride	0.3
Sodium hydrogen carbonate	0.1
Ammonium bisulphate	0.1
Selected recipe	
Olive oil	16.0
Wheat flour	59.0
Dust sugar	19.0
Water	6.5
Sodium chloride	0.3
Sodium hydrogen carbonate	0.1
Ammonium bisulphate	0.1

contain casein as additives. The one selected was from Anco (Roeselare, Belgium), where casein was not detectable. Its proportion has been increased to compensate the removal of skimmed milk powder. Other ingredient proportions remained unchanged from Scaravelli's recipe.

As far as the baking method was concerned, we collaborated with the ITCA bakery school. The thickness of the cookies was set at 8 mm and the diameter of the punch was set at 60 mm. The weight of each cookie before baking was around 22 g. It is known that baking time has a significant impact on the detectability and quantitation of proteins. The cookie reference materials need to be representative of what is commonly available and consumed. Considering this, we decided on baking conditions of 17 min at 200 °C.

For the pre-ring trial, we focused on casein detection only. As basis for a second reference material a soy-based infant formula (obtained from Danone) was selected and incurred as described below.

Reference materials and spiking procedure

Pure reference materials were selected to be used for the production of incurred (matrix integrated) materials. For milk a non-fat milk powder RM 1549 and for egg spray-dried whole egg RM 8445, both from National Institute of Standards and Technology (NIST) were used. The flour base was incurred with the two compounds at two concentrations before processing the dough and baking the cookies in

order to obtain 100 or 1000 p.p.m. of each powder in the final dough mixture. A batch without milk and egg was also prepared, which served as a blank ('0' value) and as base for further dilution to final concentrations below 100 p.p.m.

Spiking was done in the wheat flour. Two spiking procedures were compared, direct and serial dilution. Direct spiking corresponds to the final concentration of milk and egg powder directly added to the wheat flour. Serial dilution spiking means serial dilution of a high concentrated wheat flour in each compound in order to obtain the desired concentration in the wheat flour at the end. The concentrations estimated by the CER Groupe's ELISA for the detection of casein, β-lactoglobulin (BLG) and egg in baked or non-baked cookies were compared according to the spiking procedure (data not shown). As there is little difference (CV = 7.4%) between the two procedures, direct spiking was chosen as standard procedure for any subsequent steps.

For the pre-ring trial, cookies and soy-based infant formula incurred with different final concentrations of non-fat milk powder RM 1549 were assessed. The final concentrations were 0, 8.3 and 16.7 p.p.m. milk powder what is corresponding to 0, 2 and 4 p.p.m. of theoretically expected casein concentration in each matrix, respectively.

Results and discussion

In-house evaluation of the cookies

A first evaluation study on cookies was done in-house with five different ELISA kit providers for the detection of casein, BLG and egg.

Non-baked cookie samples were analysed and compared with baked cookies in order to assess the processing influence on the allergen detection. As some results were not in the linear range of the calibration curve, a 10 times dilution of the 100 p.p.m. sample with the 0 p.p.m. extract was used to estimate the concentration of casein, BLG and egg in the incurred samples. Thus, four concentrations were assessed by ELISA: 0, 10, 100 and 1000 p.p.m. The estimated concentration, for which the signal was in the linear range of the test method's calibration curve, was used to calculate the concentration as it could be for the 1000 p.p.m. sample according to the respective dilution factor.

Results from the different kit providers are given on the same graph but the estimated concentration unit is specific to each kit. For egg, e.g., concentration for Tepnel is in egg white protein and for CER Groupe it is in NIST RM 8415 egg powder protein. Then, the sensitivity cannot be compared on the basis of estimated concentrations. To assess the sensitivity of kits we examined their ability to detect materials in baked cookies at the 10 p.p.m. level (10 times dilution of the 100 p.p.m. sample with the 0 p.p.m. extract). The assessment of the 'processing influence on detection' was calculated according to the following equation: (estimated concentration for baked cookies × 100%)/(estimated concentration for non-baked cookies) and was considered as the recovery after the baking process. The higher it is, the lower the kit detection is affected by the processing.

Moisture loss through the baking process was around 15%, corresponding to the difference of weight before and after the baking process. This loss was not taken into account when generating and documenting the quantitative results. The processing effects on the detection are therefore slightly under-estimated.

Figure 1 presents the estimated concentrations for the 1000 p.p.m. NIST RM 1549 non-fat milk powder incurred

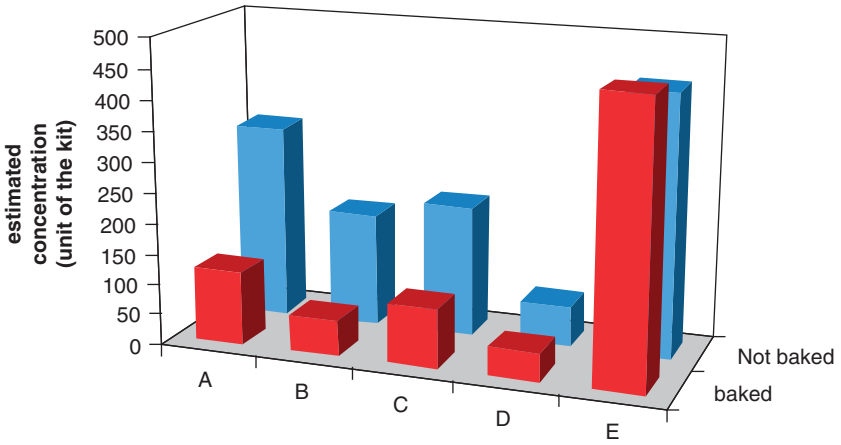

Figure 1 Analytical results for the 1000 p.p.m. non-fat milk powder (National Institute of Standards and Technology RM 1549) incurred cookies obtained with the different enzyme-linked immunosorbent assay test kits for casein detection.

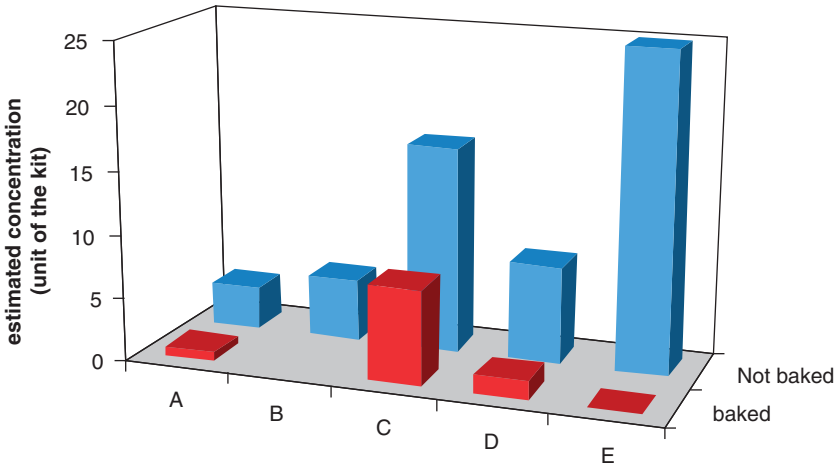

Figure 2 Analytical results for the 1000 p.p.m. non-fat milk powder (National Institute of Standards and Technology RM 1549) incurred cookies obtained with the different enzyme-linked immunosorbent assay test kits for β-lactoglobulin detection.

cookie assessed by the ELISA test kits targeted to detect casein. Each test kit has its own calibrators and its own quantification algorithm. Three assays were able to detect the sample at 10 p.p.m. in baked cookies whereas kit B and kit D were only able to detect casein at 100 and 1000 p.p.m. in baked cookies, respectively. Compared with the non-baked cookies, the recovery rate was generally around 40–50% – which was accounted to the effects of processing on the quantification of milk in cookie, except for kit E, for which the recovery was at 105%.

Figure 2 presents the obtained results after analysing the 1000 p.p.m. NIST RM 1549 non-fat milk powder incurred cookie using the ELISA test kits targeting BLG. For clarity, results from kit C and kit E were divided by 10. Again the concentrations were computed by using the calibrators and quantification algorithms provided with each test kit.

Even at 1000 p.p.m., kit B is not able to detect BLG in baked cookies. Only kit C is able to detect BLG at a concentration of 10 p.p.m. in baked cookies. Kits A and E were able to detect BLG at 1000 p.p.m. in baked cookies and kit D at 100 p.p.m. in baked cookies. All recovery values were well below 50% with the highest being for test kit C with 46%. Again, this relatively low recovery rate was attributed to the processing effects (baking process).

The estimated concentrations of 1000 p.p.m. NIST RM 8445 spray-dried whole egg powder incurred cookie by the ELISA kits for egg detection are shown in Figure 3. The concentrations were computed by using the calibrators and quantification algorithms provided with each test kit.

The detection signals for egg in baked cookies were very weak except for test kit C, which was able to detect egg also in the 10 p.p.m. sample. Moreover, it is the only assay that has a quite considerable recovery rate after the baking process at 61%. Test kit D was not able to detect egg even at 1000 p.p.m. in baked cookies. Test kits A, B and E were only able to detect egg protein at concentration levels of 1000 p.p.m. in baked cookies, but not at lower concentrations.

We see one reason for the relatively large discrepancies of results obtained with the various test kits in their different ways of computing the signals obtained and the calibrators used. Some providers give information on calibrators and unit conversion factors (e.g. ELISA Systems) but even with that, estimated concentrations differ from the expected amount. This highlights the need of having conversion table to reference materials to interpret the estimated concentration.

BLG and egg proteins are very sensitive to heating leading to an under-estimation of the presence of these allergens in the final product. It could be interesting to compare ELISA detection and allergic reaction with the developed cookies. The extent to which processing affects the ability of foods to induce allergic reactions is not known in most cases.

Two parameters are paramount in the ability of an ELISA assay to detect proteins: extraction and detection. Food processing can conceivably affect both of these. Proteins may precipitate or aggregate after a heating process and make covalent links, rendering them less amenable to extraction. Processing can also destroy epitopes on the protein. Conformational epitopes are more susceptible to be damaged following the denaturation of proteins, for instance by heat treatment and linear epitopes could be destroyed by hydrolysis. It should be noted that food processing might form neoallergen(s) by chemical modifications: glycosylation or oxidation may create new epitopes that could induce or increase allergic reaction.

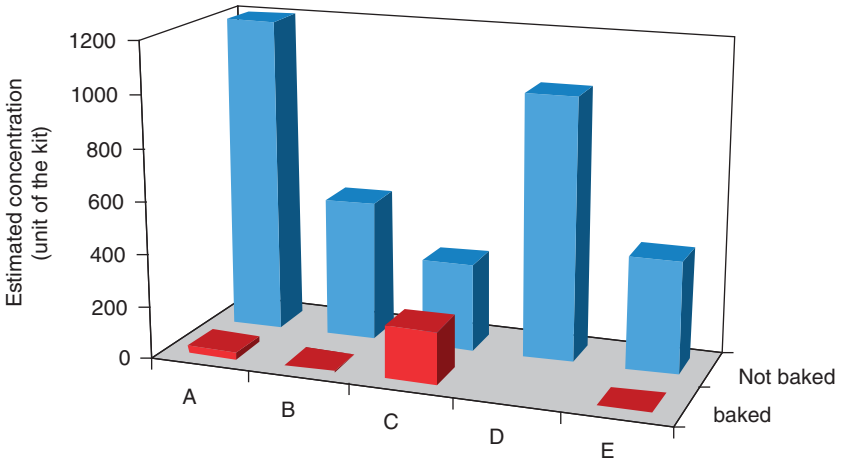

Figure 3 Analytical results for the 1000 p.p.m. spray-dried whole egg powder (National Institute of Standards and Technology RM 8445) incurred cookies obtained with the different enzyme-linked immunosorbent assay test kits for egg detection.

Food allergen detection methods should then be able to detect native and modified proteins in order to assess the presence of allergen in the foodstuffs whatever if the cross-contamination occurs before or after the processing. These results show the importance of having incurred reference material in order to compare and assess the ability of assays to detect the presence of food allergens.

Next steps have been focused on casein detection only. A second reference material has been selected: soy-based infant formula. That reference material was chosen to represent mildly processed material and cookies will represent highly processed material. Both are typical matrices for contamination with milk in a processing environment.

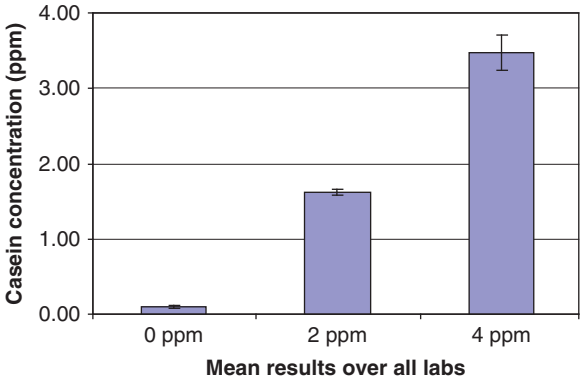

Figure 4 Average analytical results of various levels of casein content in soy-based infant formula incurred material obtained with kit1 by the participating testing laboratories.

The pre-ring trial

The produced reference materials, baked cookies and soy-based infant formula incurred with non-fat milk powder from NIST, were analysed for the casein content using several commercially available ELISA methods. The aim of the trial was to check if the materials are suitable as reference materials and if most of the available ELISA test kits are able to detect milk (used standard: NIST RM 1549) in the two matrices. Further the trial was intended to identify which contamination levels can be reliably detected and if these levels correspond to relevant concentrations for food safety management, even though no legal limits exist at this time. Based on these findings a validation study would be organized according to the harmonized validation protocol (Abbott *et al.*, 2010).

Five different ELISA test kits available at the market were used: Casein Residue assay from ELISA Systems, Milk Protein ELISA Kit (Casein) from Morinaga, Veratox for Total Milk Allergen from Neogen, RIDASCREEN®FAST Casein from R-Biopharm, BIOKITS Casein Assay kit from Tepnel Biosystems Ltd./GenProbe. As for the first evaluation, results obtained with each test kit are presented only as 'kit1–kit5' to maintain commercial confidentiality.

Three laboratories participated in the pre-ring trial and each lab received one coded sample of each concentration of each material: 0, 8.3 and 16.7 p.p.m. of non-fat milk powder RM 1549 incurred in the foods, which is corresponding to 0, 2 and 4 p.p.m. of theoretically expected casein content, respectively. These concentrations correspond to the lower range of quantification according to the information given by the test kit manufacturers. Three replicate determinations were preformed for each extracted sample.

For the soy-based infant formula incurred with milk powder the blank samples were all correctly identified using

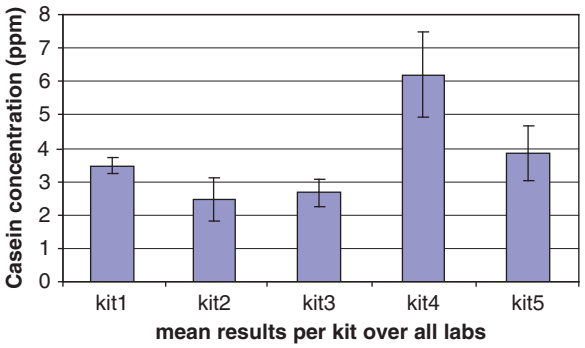

Figure 5 Average analytical results of casein content in soy-based infant formula incurred material at a 4 p.p.m. level obtained with the five test kits by the participating testing laboratories.

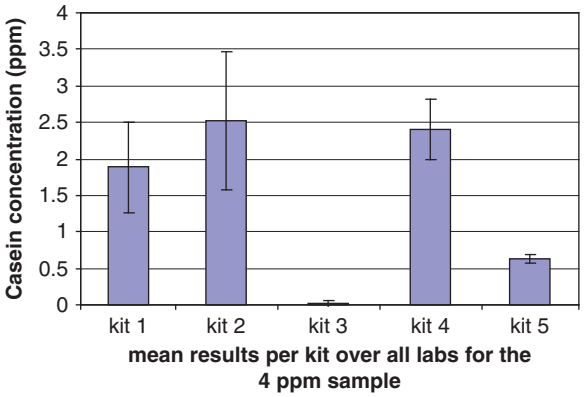

Figure 6 Average analytical results of casein content in cookie incurred material at a 4 p.p.m. level obtained with the five test kits by the participating testing laboratories.

any of the five test kits in all three labs. With one exception the three different milk levels were correctly recognized by all labs and test kits. As one example the mean results for one test kit obtained by the three testing labs are shown in Figure 4. The results of each lab for the blank samples were found clearly below the limit of detection. A mean of 1.62 p.p.m. casein was detected where theoretically 2 p.p.m. casein were expected and 3.5 p.p.m. where 4 p.p.m. casein were expected. Therefore all results were well within the expected ranges. The distributions of the single measurements obtained by the labs showed little variability for that test kit. As an example, the single values for casein for the 2 p.p.m. soy sample (theoretically expected casein value) ranged from 1.37 to 1.80 p.p.m.

The variation/deviation between the five kits observed is shown in Figure 5 displaying the mean values for the 4 p.p.m. sample over the all three labs. 2.5–6.2 p.p.m. casein was found when theoretically 4 p.p.m. casein was expected.

With one exception, all kits identified the increasing contamination levels for the incurred cookies. The variation/deviation between the five kits observed is shown in Figure 6, displaying the mean values for the 4 p.p.m. cookies sample over the all three labs. Taking into account only the four kits that detected correctly the incurred cookies, 0.6–2.5 p.p.m. casein was found when theoretically 4 p.p.m. casein was expected.

Conclusions

The testing labs obtained comparable results, leading to the conclusion that the material produced, based on the initial tests, seems to be suitable as reference material as well as for testing the performance of the test kits. We believe the results from this initial trial highlight the impact of the food matrix on allergen detection by ELISA and further emphasize the need for incurred materials which are representative of 'real-world' food matrices.

Taking into account the different concentration ranges to be determined using different test kits, a wider and higher range of concentrations than used for the first trial were chosen to produce the reference materials. Soy-based infant formula and cookies were incurred with calculated amounts of 0, 1, 2, 5, 10, 50 p.p.m. casein. The forthcoming validation study according to the harmonized validation protocol will significantly and positively impact on future validation procedures.

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