



Towards development of incurred materials for quality assurance purposes in the analysis of food allergens[☆]

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ARTICLE INFO

Article history:

Received 6 January 2010
Received in revised form 25 March 2010
Accepted 29 March 2010
Available online 4 April 2010

Keywords:

Food allergy
Enzyme-Linked Immunosorbent Assay
Method validation
Incurred samples

ABSTRACT

Food allergy and intolerance became very important problems in food safety and healthcare during the last few decades. Beside the pharmaceutical treatment of the symptoms, effective cure of these illnesses is the avoidance of the problematic food proteins. According to this reason, proper legislation is crucial for protecting sensitive people. In the European Union 14 allergenic components must be labelled which requires introduction of properly validated analytical methods for the appropriate quantification of allergenic proteins. The aim of our work is studying such parameters which may affect the analytical results, therefore have to be taken into account during the validation process. For investigating these issues, an incurred sample matrix was produced, namely a wheat flour based cookie, which contains allergenic proteins (milk or egg) in a dedicated amount. Using these samples the effects of food processing steps and the analytical performance of the applied Enzyme-Linked Immunosorbent Assay (ELISA) methods were studied. A major finding of our work is that heat treatment caused a large-scale decrease in the amount of measurable allergen content of the samples. The background of this phenomenon has not been clarified yet. Besides, the gathered data indicates that the performance of the ELISA method is highly related to the state of the sample matrix. These problems altogether must be taken into consideration for making a proper validation protocol and revealing their background also has a great importance in further evaluation of the analytical methods.

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1. Introduction

Food allergies and food sensitivities are individualistic adverse reactions to foods [1]. An increase of prevalence of these food induced hypersensitivity reactions is observed in recent years. Approximately 2–4% of adults and 2–8% of children is being affected by these illnesses [2–4]. The symptoms show a large variety from problems of the respiratory system, like sneezing and asthma, through symptoms occurring in the skin (e.g. urticaria and angio-oedema, etc.) and different gastrointestinal disorders (e.g. vomiting, diarrhoea, abdominal pain, etc.), to the very serious anaphylactic shock [5,6]. Certain proteins in foods are responsible for triggering these symptoms. 90% of food allergic reactions are caused by eight foodstuffs (the Big Eight) and their products: cereals (gluten), crustaceans, fish, milk, egg, peanuts, nuts and soybeans. According to the fact that today the only effective cure of these illnesses is the total avoidance of the sensitizing proteins, proper

labelling of allergenic components is crucial for ensuring the safety of allergic people [1]. In the European Union food manufacturers are obliged by directives 2003/89/EC [7] and 2006/142/EC [8] to label 14 allergenic compounds (the Big Eight and molluscs, celery, mustard, sesame, lupine and sulphur-dioxide) on the package of foodstuffs.

Due to the labelling obligation, proper quantification of allergenic proteins is necessary. To reach this goal, application of reliable and validated analytical methods for monitoring raw materials and food production has a great importance [9]. Nowadays, the most commonly used analytical methods for allergen analysis are immune-reaction-based ELISA (Enzyme-Linked Immunosorbent Assay) and LFD (Lateral Flow Device) methods [10]. The quality assurance of the analytical results is based on the systematic use of validated methods, reference materials and proficiency testing [11]. Today only a few data is available for the validation of immunological methods for allergen analysis. This situation is caused by several difficulties. On the one hand, comparison of results is critical because of the application of different validation protocols. On the other hand, due to the lack of proper reference materials, one of the most important assumptions of the validation and the quality assurance of the analytical results – according to ISO EN 17025 standard – is missing. In 2002, AOAC, in cooperation with

[☆] This article was written for the special issue of Analytica Chimica Acta for the 4th International Symposium on Recent Advances in Food Analysis.

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IUPAC and ISO, developed a harmonized protocol for method validation [12]. However, this protocol does not take some important issues into consideration, which are related to the qualification and quantification of allergens and may cause difficulties during the determination of these proteins [13]. One of these issues is possible interactions of allergenic proteins with other matrix components, like carbohydrates, polyphenols and tannins. Besides, food processing (mainly heat treatment) may result denaturation of proteins which can change their solubility and reactivity. Thermal processes can reduce solubility, allergenicity and antigenicity of the allergenic proteins. These effects and processes may alter the analytical results, as it has influence on the efficiency of extraction and the affinity of the allergens to the antibodies of the ELISA test kits, which are the basis of the detectability of these proteins. Besides, in spite of the above described effects of processing, the allergenic properties of the proteins will not reduce by necessity [14]. The affinity of allergenic proteins can also be affected by their origin as well (e.g. different species or geographical factors) [13]. For taking the specific problems of allergen determination into account, a guidance document on the validation on ELISA methods for food allergen analysis was created by EU FP6 funded Network of Excellence, MoniQA (Monitoring and Quality Assurance in the Food Supply Chain) and AOAC Presidential Task Forces [15].

In conclusion, it has the utmost importance to investigate the effects of food processing on the analytical results and the performance of analytical methods with samples in realistic food matrices. Revealing the background of these questions is one of the hot topics of the activity of the MoniQA project. The aim of our work within the MoniQA framework is the investigation of such parameters related to the analysis of allergenic proteins which may affect the analytical results and due to this reason must be taken into account during the validation process. The application of real, incurred food matrices instead of spiked samples gives more detailed and more exact information for method validation [11]. Due to this reason, investigation of the effect of food processing on the analytical results and studying the analytical performance of the applied methods were carried out with the help of an incurred food matrix with milk or egg proteins as major allergens. Incorporation of allergenic proteins in a representative processed food matrix can reveal some limitations of methods used.

2. Materials and methods

2.1. Production of the incurred sample matrix

For preparing the incurred sample matrix of wheat flour based cookies, a recipe by Scaravelli et al. [16] was adopted and modified. The main difference from the original recipe is the applied homogenizing method: powder mixing of the allergenic proteins with the dry components of the recipe was performed instead of mixing them into the fatty phase. The cookies were produced at two different allergen concentration levels: 0 and 100 mg kg⁻¹ milk powder (IRMM BCR-380R Whole Milk Powder) and 0 and 1000 mg kg⁻¹ egg powder (NIST RM 8415 Whole Egg Powder) concentrations were generated.

In the beginning of our work, different powder mixtures were made in order to produce the required allergen concentration. Firstly, a blank mixture was produced, which contained all of the dry components of the recipe without any allergen content. Secondly, a stock mixture was made, which contained all of the dry components of the recipe and a 100 fold amount of the necessary milk powder concentration or a 10 fold amount of the necessary egg powder concentration. The allergen concentration of the stock mixture was calculated by taking the diluting effect of margarine and water into account. Finally, a diluted mixture was produced which

is a blend of blank and stock mixtures in a proper ratio (1:100 for milk powder and 1:10 for egg powder). The allergen concentration of this mixture altogether with margarine and water is 100 and 1000 mg kg⁻¹ for milk and egg powder, respectively.

Production of the powder mixtures and kneading of the dough occurred by mixing in a valorigraph (FQA-205 METEFEM, Budapest) for 20 and 5 min, respectively. Three round shaped cookies with constant height and diameter were made from the raw dough. The leftover dough was freeze dried. The cookies were baked for 16 min at 180 °C.

2.2. Sample preparation and the analytical method

For the analytical examination, the cookies and the freeze-dried dough were ground in a sample mill (Cemotec 1090, Tecator). The equipment and the working area were cleaned with concentrated ethanol after each sample. Samples were taken from every powder mixture, ground raw dough and cookie. An indirect competitive ELISA kit (Tepnel BOKITS Casein Assay Kit, calibrated against NIST SRM8435 Whole Milk Powder) was used for analyzing the milk-powder-containing samples. A sandwich ELISA kit produced by R-Biopharm (RIDASCREEN Fast Ei/Egg Protein Kit, calibrated against NIST RM8445 Whole Egg Powder) was applied for determining egg proteins. Sample preparation and extraction were carried out according to the users' manual provided by the kit manufacturers.

2.3. The experimental design

In order to generate statistically representative results, five bakings were made at every concentration level and three cookies were produced at every baking. For investigating the effects of processing, three samples were taken from each step of the procedure (powder mixtures, raw dough, cookies). Applying this experimental scheme, it was possible to check the homogeneity of the sample matrix and to study the influence of dough-production and baking on the measurable protein concentration within the same experiment. Additionally, the analytical performance of the applied analytical methods could also be examined. The experimental design and workflow can be seen in Fig. 1.

The statistical evaluation of the data occurred by the examination of significance. Besides the performance of the analytical methods was also determined.

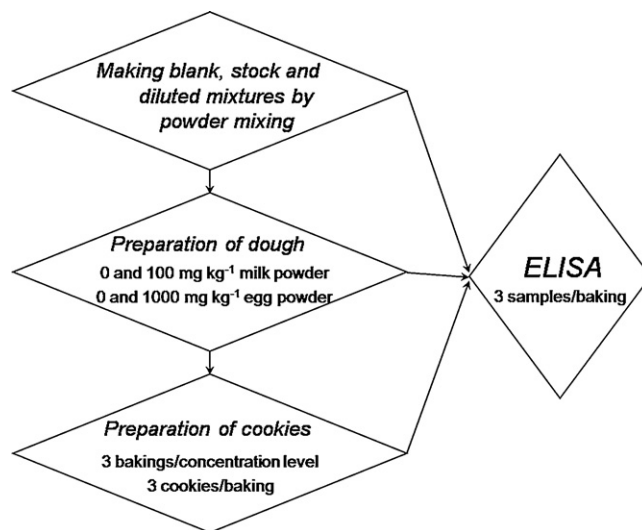


Fig. 1. The applied workflow and experimental design.

3. Results and discussion

3.1. Confirmation of allergen-free status of the blank materials

For proper declaration of the allergen content of the samples, it is very important to verify the status of raw materials (dry components, margarine) and samples (blank mixture, dough and cookie) which are not supposed to contain any allergenic protein. Besides, information can be obtained about the analytical response of the blank samples that is the baseline of the measurements.

The measured allergen concentrations were below the limit of quantification (1.6 mg kg^{-1} for casein and 1 mg kg^{-1} for egg proteins) in case of both kits and all of the examined blank samples. As a result, these samples were considered allergen-free. The data was not taken into account for further calculations as they were under the limit of quantification and due to this reason these values have an unpredictable uncertainty.

3.2. Study of the efficiency of the homogenizing process and the homogeneity of the sample products

In addition to the declared allergen amount, for generating statistically interpretable results homogeneity of the allergenic proteins in the produced samples is essential. Homogeneity was proven by the examination of the standard deviation of the measurements (Fig. 2). No significant differences were found between samples from a certain baking and from different bakings. The same result was shown within a certain ELISA measurement and between different measurements. According to these data, the homogeneity of the distribution of the allergenic proteins was adequate. As a consequence, proper interpretation of the analytical results is possible and it is proven that our simpler homogenizing method is properly applicable for this purpose.

3.3. The effects of food processing

After verification of homogeneity, the effects of processing steps on the analytical results were investigated. According to the results of ELISA measurements more effects could be detected (Fig. 3). On the one hand, raw dough contained a lower amount of allergenic protein than the diluted mixture but the difference did not exceed the expected ratio. On the other hand, a large-scale decrease in mea-

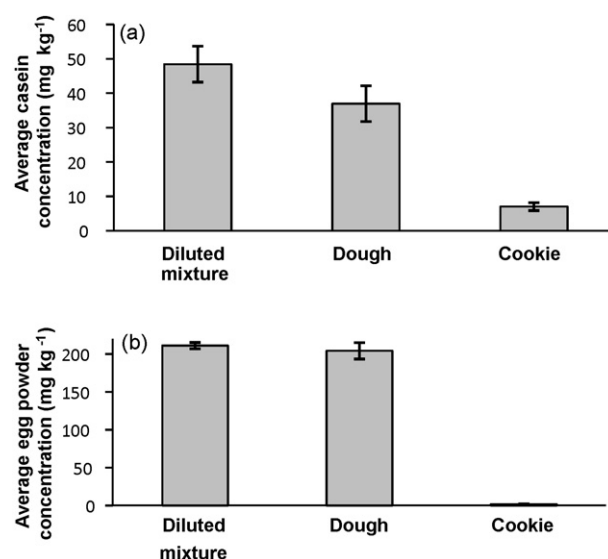


Fig. 3. Effect of processing on measurable casein (a) and egg protein (b) content.

surable protein amount was observed in cookies after baking. This phenomenon has not been clarified yet, revealing its background requires further examinations.

3.4. Study of the analytical performance of the applied methods

Proper reference materials and methods for allergen analysis are not available at the moment. Therefore, the analytical performance of the applied and widely used methods can only be estimated in different ways. For example, application of ring trials or spiked samples could be acceptable solutions for this purpose. But the first one is a relatively complicated process, while during the second solution the matrix effects are not taken into consideration.

In our experiments, the recovery values were determined in every processing step for estimating the trueness of the ELISA methods. Besides, studying the effects of processing on these values is applicable for checking the trueness of the applied analytical methods, too. Recovery was calculated for every sample but for estimating trueness, recovery values of the stock mixtures (Fig. 4) were examined only, due to the fact that the analytical results are altered by heat treatment.

The average recovery values are 94.46% and 78.57% for milk and egg stock mixtures, respectively. This data indicates that the trueness of determination of native proteins fits for the purpose of these experiments. Recovery values of cookies are much lower (36.37% and 2.16% for cookies with milk and egg powder, respectively) which may indicate the effects of heat treatment or the failure of the applied analytical method (e.g. inadequate extraction).

The repeatability of the measurements was investigated by using a few members of the ELISA kits' own standards as samples. For this purpose the chosen standards (1.6 , 3.2 and 6.4 mg kg^{-1} casein standards and 0.5 and 4.5 mg kg^{-1} egg standards) were placed randomly on the plate as they were samples. The concentrations of these standards were calculated from the calibration curves of the kits. Afterwards, standard deviations of these concentrations were determined and these deviations were compared with the deviations of the real samples (Fig. 5).

As the data shows, in most cases the deviations of the standards are comparable (0.09 – 0.94 and 0.76 – 1.99 mg kg^{-1} for milk and egg standards, respectively) or even higher than that of the samples (0.15 – 1.04 mg kg^{-1} for samples with milk powder and 0.11 – 0.54 mg kg^{-1} for samples with egg powder). According to this result, the main part of the deviation of the samples is originated

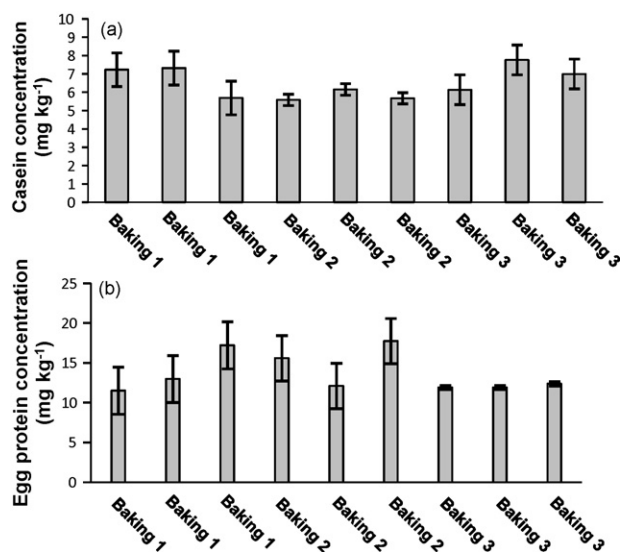


Fig. 2. Homogeneity of the distribution of measured protein content in cookies with milk (a) and egg (b) powder content from three different bakings.

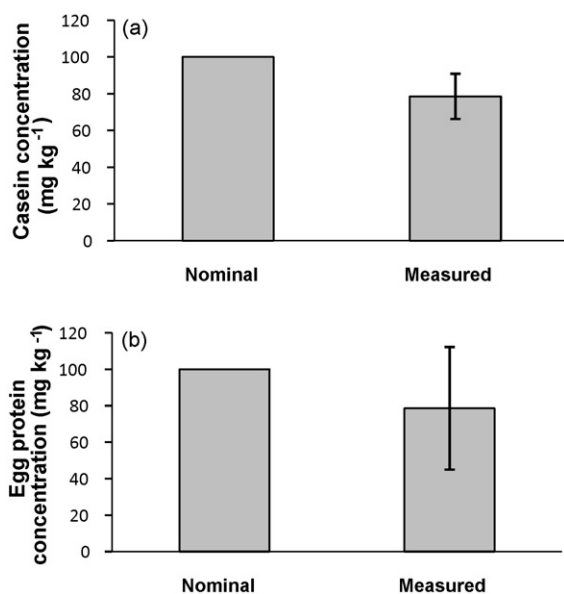


Fig. 4. Recovery of casein (a) and egg protein (b) concentration in stock mixture, on a 100 mg kg⁻¹ nominal concentration base.

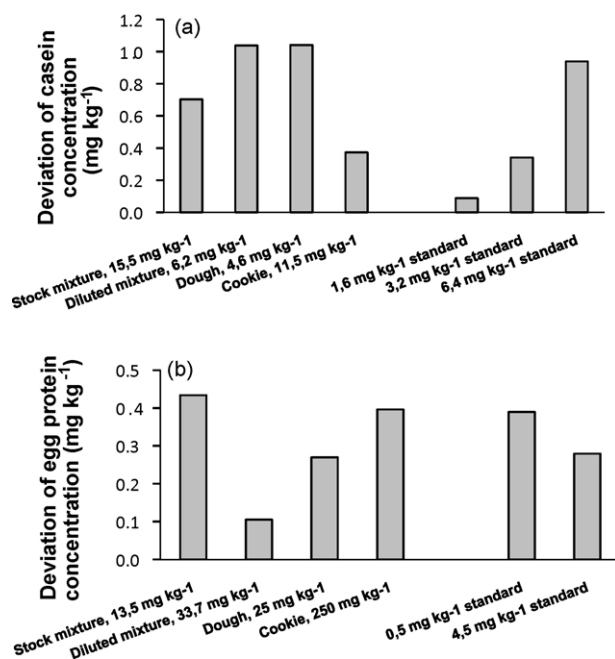


Fig. 5. Comparison of standard deviations for estimating repeatability of the casein (a) and the egg (b) ELISA kits.

from the uncertainty of the applied analytical method. In conclusion, failures causing larger deviations during sample production and preparation were avoided; measurements can be carried out with satisfactory repeatability and the incurred samples can be properly used.

4. Conclusions

Several important questions emerge in connection with the application and validation of methods for allergen qualification and quantification. One of them is the effect of food processing on measurable allergen content. The most important result of our experiments is that a large-scale decrease of measurable protein amount was observed in every case where heat treatment was

involved. According to previous studies, a decrease in detectability could be expected [14]. This phenomenon has not been clarified yet and can have different explanations. For instance, the heat denaturation of proteins, minor or major modifications in their structures or their reactions with other food components may cause decreased solubility or reactivity in immune-analytical and/or enzymatic reactions. Some possible explanation could be found in different studies. Denaturation temperature of some milk proteins (β -lactoglobulin and α -lactalbumin) was determined between 60 and 80 °C and it was also described that over 80 °C casein may react with other milk components [17]. Besides it was also proven that thermal treatment of egg white proteins for 90 °C and 10 min caused a significant decrease of RAST binding intensity of these proteins [18].

As for the effect of dough preparation, the observed difference between the measurable protein content of raw dough and diluted mixture was caused by the diluting effect of margarine and water added to this mixture to produce the dough. This data indicates that the first step of dough processing (addition of fatty phase, hydration, dough development processes) has no effect on the measurable allergen content, there is no protein degradation, as it was expected.

The effects of these issues on the analytical performance of the ELISA method are highly important. Neglecting these problems during the development of analytical methods and validation protocols may cause such large-scale errors in the low concentration range which mean a potential danger for allergic people. Due to this reason, revealing the background of these questions is essential for ensuring the safety of sensitive people properly.

Application of incurred reference materials should be used for studying the problematic issues mentioned above. Another major result of this study is that our model product is a satisfactory first step for the development of reference materials, as it is applicable for the above mentioned quality assurance purposes. Production of this material is possible because proper homogeneity of the allergenic protein distribution was successfully implemented. These materials mean a great help in development of validation protocols and evaluation of immunoanalytical and other types, e.g. spectroscopic or molecular biological methods.

Acknowledgements

This work was carried out with the financial and professional support of MoniQA Network of Excellence (FOOD-CT-2006-036337). Further financial support was provided by the fellowship for PhD students awarded by the government of Hungary. The ELISA measurements were carried out with the personal and scientific support of Dr. Éva Gelencsér and Éva Valentin at Hungarian Central Food Research Institute and Dr. Bert Pöpping and Dr. Sandra Kerbach at Eurofins Analytik GmbH in Hamburg, Germany.

References

- [1] S.L. Taylor, S.L. Hefle, *Food Technol.* 55 (9) (2001) 68–83.
- [2] S. Taylor, in: S.J. Koppelman, S.L. Hefle (Eds.), *Detecting Allergens in Food*, Woodhead Publishing, Ltd., Cambridge, 2006, pp. 2–20.
- [3] D.K. Sohi, J.O. Warner, *Pediatr. Child Health* 18 (7) (2008) 301–308.
- [4] C. Ortolani, M. Ispano, J. Scibilia, E.A. Pastorello, *Allergy* 56 (Suppl. 67) (2001) 5–8.
- [5] B. Bohle, S. Vieths, *Methods* 32 (2004) 292–299.
- [6] S.H. Sicherer, H.A. Sampson, *J. Allergy Clin. Immunol.* 117 (2) (2008) S470–S475.
- [7] Directive 2003/89/EC of the European Parliament and of the Council, *OJEU* 46 (2003) 15–18.
- [8] Commission Directive 2006/142/EC, *OJEU* 49 (2006) 110–111.
- [9] R. Krska, E. Welzig, S. Baumgartner, *Anal. Bioanal. Chem.* 378 (2004) 63–65.
- [10] A.J. van Hengel, *Anal. Bioanal. Chem.* 389 (2007) 111–118.
- [11] R. Poms, H. Emons, E. Anklam, in: S.J. Koppelman, S.L. Hefle (Eds.), *Detecting Allergens in Food*, Woodhead Publishing, Ltd., Cambridge, 2006, pp. 348–356.
- [12] AOAC Official Methods of Analysis (2002) Appendix D pp. 1–12.

- [13] S. Kerbach, A.J. Alldrick, R.W.R. Crevel, L. Dömötör, A. DunnGalvin, E.N.C. Mills, S. Pfaff, R.E. Poms, B. Popping, S. Tömösközi, *QAS* 1 (1) (2009) 50–60.
- [14] R.E. Poms, E. Anklam, *J. AOAC Int.* 87 (6) (2004) 1466–1474.
- [15] M. Abbott, S. Hayward, W. Ross, S.B. Godefroy, F. Ulberth, A.J. Van Hengel, J. Roberts, H. Akiyama, B. Popping, J.M. Yeung, P. Wehling, S.L. Taylor, R.E. Poms, P. Delahaut, *J. AOAC Int.* 93 (2) (2009) 442–450.
- [16] E. Scaravelli, M. Brohée, R. Marchelli, A.J. van Hengel, *Eur. Food Res. Technol.* 227 (2008) 857–869.
- [17] L. Monaci, V. Tregoat, A.J. van Hengel, E. Anklam, *Eur. Food Res. Technol.* 223 (2006) 149–179.
- [18] M. Yoshinori, M. Yang, *J. Agric. Food Chem.* 56 (2008) 4874–4900.