

Identification of the factors affecting the analytical results of food allergen ELISA methods

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Abstract In order to support patients' safety, the analytical methods should be able to quantify the allergenic proteins in food products. Analytical performance of the currently used ELISA methods is not always appropriate, particularly in case of processed foods. A possible way to investigate the sources of analytical errors is the utilisation of model food matrices that mimic the technology and behaviour of real food products. Consequently, factors affecting the analytical results were identified, their contribution to the whole analytical error was determined, and the underlying phenomena were interpreted in this study. Heat-treated model matrices incurred with gliadin, milk, egg, or soy proteins were produced and analysed with commercially available ELISA test kits. The data were evaluated with statistical tools. Results clearly show that the type of the food matrix, the level of processing, and the analytical methods (i.e. the type of the ELISA kit) are the three main factors that play a significant role in the uncertainty. The developed laboratory methodology seems to be suitable for generalising the investigation of other allergens, methods, matrices, and the scientific background of the phenomena.

Keywords Food allergens · Coeliac disease · Model food products · ELISA · Statistical evaluation

Introduction

Hypersensitivity reactions triggered by food components are a major food safety problem. Eight food groups are responsible for 90 % of these reactions. All of these foods are capable of inducing IgE-mediated food allergy, besides gluten proteins can induce coeliac disease as well. In both disorders, the only effective treatment is the avoidance of the problematic proteins in the patient's diet [1–3].

Certain food proteins are responsible for provoking hypersensitivity reactions (food allergy, coeliac disease) [4]. In the allergen activity of proteins, their immunogenicity and immunoglobulin-binding capacity play a decisive role. They can be influenced by the chemical and physical properties (stability, size, etc.) and the immunological properties (affinity, nature of epitopes) of the proteins as well. Foods are basically colloidal dispersions of biopolymers and their complexes and can contain several macro- and micro-components. Typical macro-components of foods are lipids, carbohydrates, and water, besides the proteins, while a wide range of salts, additives, colouring agents, vitamins, metal ions can also occur as micro-components. Chemical reactions between the food components can affect the techno-functional and nutritional value of the product and the impact related to the hypersensitivity reactions as well. In addition, it can be stated that in most cases, foods are not consumed raw; they undergo very diverse and numerous processing steps. In foods, proteins can interact with other proteins and non-protein components as well, depending on the applied technological operations, medium, etc. In the course of different processing operations, epitopes can also change, which could potentially affect the adverse impact of proteins. The behaviour of proteins during the production process and in the presence of other protein and non-protein components raises temporarily unclear health, food safety, and analytical issues [5–8].

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There are a number of methods available for detection and quantification of allergens. At the same time, methods that meet all the requirements of the field are currently not available, so their reliability is limited. In research and routine allergen analysis, the most widely used methods are immunoanalytical methods (ELISA, LFD), DNA-based techniques, and mass spectrometry methods. Several requirements have to be fulfilled by the analytical methods suitable for the determination of components triggering hypersensitivity reactions. The high degree of analytical performance (specificity, sensitivity, precision, and accuracy) is obviously needed, since allergens can cause serious food safety problems even in low concentrations [2]. Besides, analytical methods should be quick, easy to use, and cost-effective and they also have to work reliably in case of differently processed food products, with various compositions [2]. The analysis of food allergens is further complicated by the shortcomings in the regulatory environment. Except gliadin/gluten in foods for coeliac patients, the generally accepted regulations for allergens define only the components to label and do not declare threshold levels. This means that zero tolerance is in effect for these proteins, which cannot be guaranteed with today's analytical capabilities. A part of the problem is that clinically approved allergen threshold levels have not been established yet, which is another very challenging area. From the viewpoint of chemical analysis, the zero value itself is not interpretable. The uncertainty of the available methods should have to be taken into consideration for the regulation in a way that is equally applicable for all stakeholders of allergen analysis [9–11]. Allergenic proteins are often present only in small amounts in foods and their homogeneous distribution cannot be assumed. During the analysis, the most important phase of the sample preparation, besides homogenisation, is the extraction of the target proteins. The solubility of certain proteins can be diverse even in native state, but in processed foods, the effects of processing steps also should be taken into account. Processing may induce protein denaturation, aggregation, and secondary and tertiary structural modifications which also affect solubility. Furthermore, other components of food matrices can interact with the investigated proteins which can also influence the extractability. Therefore, the choice of proper buffers and extraction procedures is essential [12, 13].

Foods often contain more than one allergen component, so a relatively new direction in method development is focused on techniques for the analysis of multiple allergenic components. The already developed methods enable simultaneous detection of even seven allergen components, but quantification is not possible in every case [14–17]. A further difficulty is the development of extraction protocols which are able to effectively extract all of the different proteins [18].

The specificity of the immunoanalytical methods is assured by the applied antibody. During development of antibodies, structural modifications of target proteins due to food processing have to be taken into account. It can happen that the antibody produced against native proteins cannot recognise the target protein after processing [19]. In the development of immunoassay methods, the use of calibration materials is essential. The materials used for calibration in commercially available ELISA methods are usually not known, and different manufacturers apply different calibrators. In addition, for calculation of the final results, sometimes a number of correction factors (i.e. calculation from gliadin content to gluten, milk protein to milk,) have to be used. For determining these calculation factors, the calibration materials serve as the benchmark. However, for both plant and animal proteins, genetic factors greatly affect the amount and the ratio of expressed proteins, and environmental factors (climate, temperature, soil components, etc.) may have an effect on the protein composition as well [20, 21].

A number of ELISA tests are available for the analysis of allergens; however, as these test kits may differ significantly in terms of the applied antibodies, target proteins, calibrators, and extraction procedures, they provide considerably different results for the same analyte [22–24].

One possible solution of the problems outlined above is the detailed understanding of the behaviour of the allergenic proteins and epitopes. This can be done by examining the proteins directly using protein isolates or protein fractions. However, isolates or purified protein products are not able to model the behaviour of proteins in real food matrices. As it was stated above, the proteins are susceptible to changes in the environment and capable of forming many interactions with other protein or non-protein components. In order to study the behaviour of proteins in real foods, well-characterised, processed model materials are necessary [25–28].

However, during the development of such model products, a number of factors should be considered. The first one is the material used as the allergen source. The use of protein isolates allows examination of one or more specific proteins (e.g. gliadin isolates), but does not model components used in the manufacturing of food products (e.g. wheat flour), and in many cases, well-characterised or even certified protein isolates are not available. During the model product development, special attention should be paid to the concentration of the allergenic sources. The expected analytical performance characteristics of methods and the existing, regulated gluten threshold levels should be taken into account. The method of protein addition into the matrix is also important. In case of simple spiking, only the native protein content is measured, and the effect of processing is modelled only when allergens are added before processing [29, 30]. It can be clearly seen that the creation of products modelling real foods is a challenge, but these model products can be suitable for use as

a reference material. During the development of reference materials, a number of requirements should be met. One of these is the defined amount of allergens including uncertainty data. It is also necessary to ensure the homogeneous distribution of the analyte in the matrix, and the storage and delivery conditions should be defined as well. The shelf-life of the material should also be verified with short- and long-term stability studies. There are two types of reference materials. The first one includes the protein isolates or protein sources (e.g. wheat flour) which can be used for preparation of the other type, namely reference materials in matrix (spiked or incurred). At present, the available isolates or protein sources for allergen analysis are very limited. Typically, materials (milk powder, egg powder, soy flour, wheat flour) are available which have certified composition or special components, but these are usually not standardised for allergen analysis [24, 31, 32]. Several research groups deal with the development of RMs. One is the Prolamin Working Group which has created a gliadin isolate with excellent solubility, homogeneity, and stability properties [33]. Besides, only a few incurred RMs exist. Milk-, egg-, soy-, and gliadin-containing IRMs (incurred reference materials) were developed by Dumont et al. [34] and our research group [25, 26, 35, 36]. In addition, in the scientific literature, only a few studies are available about development of multi-component matrices. Gomaa et al. [37] have created a processed matrix, which contains milk, soy, and egg proteins. In addition, Khuda et al. [38, 39] developed IRMs with milk, egg, and peanut allergens in sugar biscuit and dark chocolate matrix.

During our previous studies gluten-free flour-based model food matrices were developed, which contain one or more allergen sources (milk powder, egg powder, soy flour, gliadin). With the help of these products, the effects of matrix components (presence of more allergen sources) and processing steps (mainly heat treatment) were investigated with ELISA methods [36]. The main goal of the present work was setting up a methodology suitable for estimating the factors affecting the analytical results of ELISA measurements with the help of the developed real food matrices and with statistical tools.

Materials and methods

Production of model matrices

Processed food model sample products were developed with amaranth flour as raw material. As allergen sources, whole milk powder (IRMM BCR-380R), whole egg powder (NIST RM 8415), soy flour (NIST SRM 3234), and a gliadin isolate (PWG Gliadin, German Research Center for Food Chemistry, Freising, Germany [33]) were used. Model products were made containing one allergen source individually or

four allergen sources simultaneously. The theoretical concentration was 50 ppm for each allergen source. The procedure was the same as published previously [36]. Briefly, the dry components and the allergen source(s) were homogenised. Then, dough was formed with the addition of margarine and water. The dough was shaped and baked. All main product of the key technology steps (powder mixture, dough (in freeze-dried form)) and baked cookies were investigated.

Analytical methods

For the analysis, four sandwich ELISA methods were applied: AgraQuant Casein, AgraQuant Egg white, AgraQuant Soy, and AgraQuant Gluten G12 (all manufactured by Romer Labs, Tulln, Austria). Sample preparations and the analytical tests were carried out according to the manufacturer's instructions. The reagents used were provided by the manufacturer excluding ethanol which was of analytical grade and was obtained commercially (96 V/V% ethanol, MOLAR Chemicals). The measured data were evaluated using the BioRad Microplate Manager 6 software, and the presented concentrations refer to ppm whole milk powder, whole egg powder, soy protein, and gliadin.

Statistical evaluation

The analytical results were statistically evaluated with the investigation of means, standard deviations, and analysis of variance using Statistica 12.1 software (StatSoft Inc., Tulsa, USA). The significance of differences was studied using *t* tests. For statistical evaluation, the results of our previous study [36] were also used. The experimental factors were raw material (gluten-free or amaranth flour), number of allergen sources (single or multi), level of processing (powder mixture, raw dough, and baked cookie), and applied ELISA methods (test kits "A" or "B"). On every level of every factor, 3–5 replicates were measured. For the complex evaluation of the effects of experimental factors, 2-, 3- and 4-factor ANOVAs were used. All four factors can have several levels, of which a few were highlighted in this study; thus, factors were regarded as random factors. The homogeneity of variance and the normality of distribution were checked in all examined cases.

Results and discussion

Amaranth flour-based model matrices

Homogeneity and recovery

In case of every model matrix, the homogeneity of analytes was checked. The results (Table 1) show that the

Table 1 Recovery (%) of the analyte concentration on a 50-ppm nominal concentration base

Sample	Milk		Egg		Soy		Gliadin	
	Single	Multi	Single	Multi	Single	Multi	Single	Multi
Powder mixture	220 (2)	239 (13)	62 (10)	43 (1)	104 (15)	107 (8)	87 (9)	104 (15)
Raw dough	159 (14)	159 (10)	52 (6)	35 (7)	92 (15)	107 (13)	70 (6,5)	69 (13)
Baked cookies	43 (16)	38 (4)	0 (0)	0 (0)	4 (11)	5 (6)	50 (10)	65 (7)

Values in brackets are CVs %

distribution of allergen sources is homogeneous in all steps of the production. The presence of four allergen sources did not affect the homogeneity, which means that the developed production procedure is suitable for addition of several different allergens.

The recovery values of every measurement were also evaluated. It could be observed that the measured concentrations were significantly lower than the theoretical concentrations (50 ppm). The background of this phenomenon is not clear at the moment, but it should be searched in the physico-chemical properties of the flour. The amaranth flour has high protein and lipid content. Consequently, it is possible that the allergenic proteins formed chemical interactions with other components, which altered their physical properties, including solubility and extractability of allergens, or modified the affinity to the antibodies applied by the ELISA tests.

Comparison of single- and multi-component matrices

The effects of presence of more than one allergenic component were investigated through the ELISA results (Fig. 1). It is clearly seen from the results that differences could be observed between the results of single- and multi-matrices. However, it can be stated that in case of recovery values and standard deviations, the values are changing randomly and there are no apparent trends. This statement is similar to our former results obtained with previously developed gluten-free flour-based products [36]. The results show that in case of milk- and soy-containing model matrices, no significant differences could be observed between the single- and multi-matrices. In contrast, in all of the model products containing egg the difference is significant, while in case of gliadin, only the investigation of baked cookies gave significant difference.

The effects of food processing

With the investigation of powder mixtures, raw doughs, and baked cookies, the effects of food processing were also evaluated. A continuous decrease could be observed throughout the process, mainly in the baked cookies compared to the raw dough. In matrices containing milk, the measurable milk protein concentration of raw dough is

approximately 30 % lower and of the baked cookie is 80 % lower. In model products containing soy and egg, the decrease in the measured protein concentration in the raw dough is only 10–20 % lower, while the decrease in baked cookies is 100 %. In contrast, the measurable concentration of gliadin is decreasing by 20 % (raw dough) and 40 % (cookie) only. These results strengthen the fact that the applicability of currently available kits is limited in case of processed foods.

Comparison of gluten-free and amaranth flour-based products

In this study, the analytical results of newly developed amaranth flour-based products and the previously developed gluten-free flour-based model matrices [36] were compared (Fig. 2) for the investigation of matrix effects originated from the modification of the applied raw material flours. The results show that the measured concentrations of amaranth flour-based samples are lower, except for doughs and cookies containing soy. With two exceptions (cookies containing milk and dough containing soy), a significant difference could be observed in all cases. Another finding of this study is that in case of the amaranth flour-based samples containing native proteins (powder mixture and raw dough), the measured concentration values approach well the theoretical concentrations, though they are somewhat lower. The only exceptions are the matrices containing milk. Deviations between the replicates of model matrices with milk or egg allergens were random; no trends could be observed. In contrast, in case of the amaranth flour-based products containing soy and the gluten-free flour-based products containing gliadin, the standard deviations are slightly higher.

An error occurring during the preparation of model products can be excluded; the homogeneity of the matrices was appropriate on the bases of the evaluation of the standard deviations. The amaranth flour has higher protein and lipid content; both components are present in a ten times higher level compared to the gluten-free flour. Consequently, it is possible that the two types of matrices led to different physical properties, solubility, etc. of the investigated proteins and/or modified affinity to the antibodies.

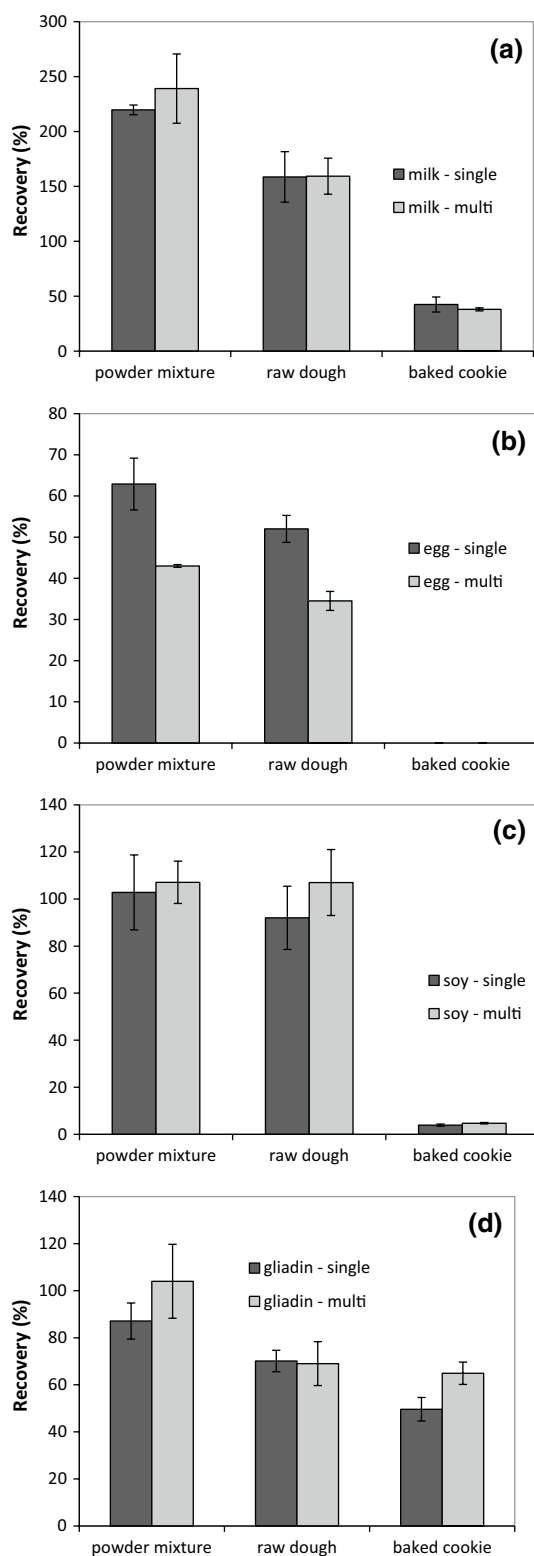


Fig. 1 Comparison of single- and multi-component sample matrices with the determination of **a** whole milk powder, **b** whole egg powder, **c** soy flour, and **d** gliadin concentration. Missing bars mean that the measured concentration was below the limit of detection

Identification of main sources of analytical errors with statistical tools

An experimental design was established, and statistical evaluation (ANOVA) was utilised for the identification of error sources of the applied ELISA methods and for estimating their extent. With the assessment of these factors, the estimation of factors originated from the food matrix (matrix composition, processing) could be carried out considering the factors originated from the analytical method (applied ELISA kit, analytical uncertainty). The statistical methodology was built up with continuous extensions of the investigated factors.

Two-factor ANOVA

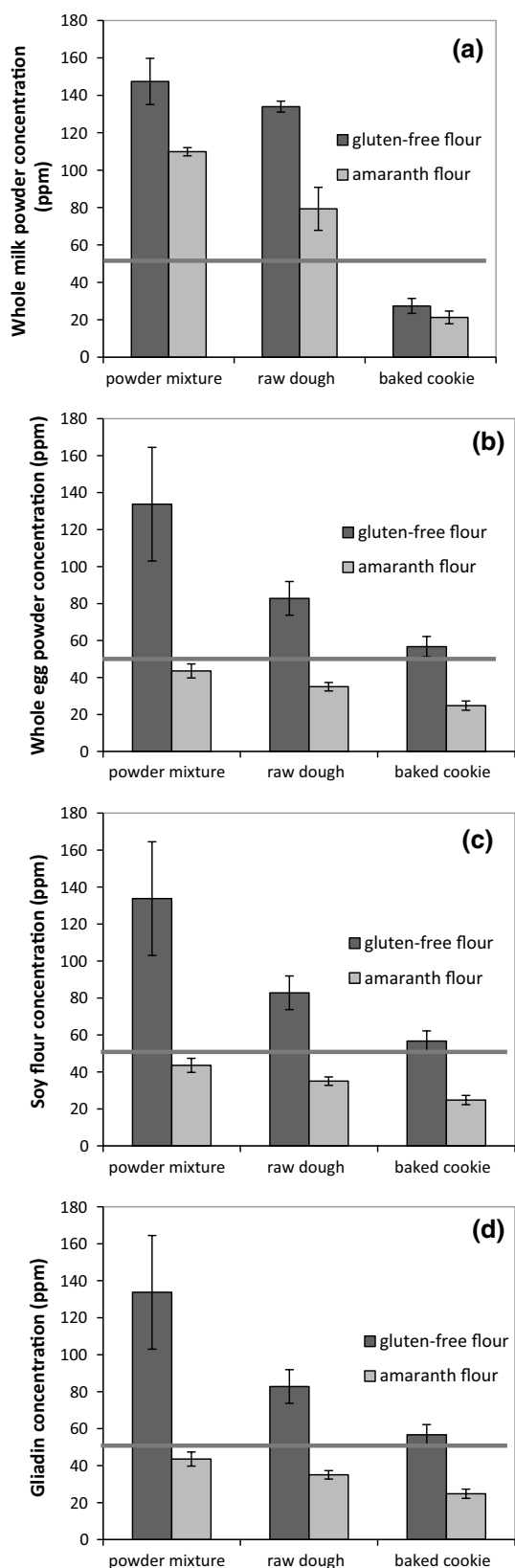
A two-factor ANOVA analysis was used for the investigation of matrix effects (factors: raw material, number of allergen sources). Besides, the extent of the effects was also compared on different levels of the other factors (processing and applied ELISA kit). During the analysis, determination of deviation components was carried out. With the deviation components, the extent to which the examined factors (in this case, the use of different raw materials and the simultaneous presence of several allergen components) influenced the analytical result and the analytical errors could be identified. 'Error' components of pie charts represent the intra-group variation. The intra-group variation is estimated from replicate analytical results.

Based on the analysis of powder mixtures (Fig. 3), it can be said that the variation in the measured concentrations is mostly affected by the choice of raw material. It verifies the experiences that the ELISA results of powder mixtures made with gluten-free flour or amaranth flour differ highly. This effect leads to a large difference in the measured protein values, which overweighs the observed distinction between single- and multi-component matrices. Furthermore, the interaction of the two factors also plays a role in the deviation of the results (except for the powder mixtures containing soy flour). So the impact of the raw material depends on the number of the used allergen sources.

The trends for dough and cookies (data not shown) are the same for the powder mixtures. The only difference is the interaction of two factors in the cookies. The extent of the interaction is increasing, but this can also be affected by the processing steps (see Sect. 3.3.2).

Three-factor ANOVA

Since the effects of the processing performed on the ELISA results were investigated in the single- and



multi-allergen-containing matrices with gluten-free flour and amaranth flour also, it was possible to perform a complex statistical evaluation of the effects of these three

Fig. 2 Comparison of gluten-free flour and amaranth flour-based sample matrices with the determination of **a** whole milk powder, **b** whole egg powder, **c** soy flour, and **d** gliadin concentration. Horizontal lines represent the theoretical concentration. Missing bars mean that the measured concentration was below the limit of detection

factors (Fig. 4). In case of matrices containing milk, it can be observed that the variation in the results is mostly affected by the level of processing and the raw material used for the production. The data set could not be transformed because of the zero values of cookies, so the percentages are approximate estimates. For model products containing egg allergens, a similar trend could be observed as for the milk. However, differences can be seen in the powder mixture and dough made with amaranth flour; the measurable protein concentration is significantly lower than in case of matrices produced with gluten-free flour. Consequently, in the distribution of deviation components, the raw material plays a greater role as well as the interaction of the processing level and the raw material. In case of soy, the factorial ANOVA results clearly show that neither the number of allergen sources nor the raw material has a great impact on the analytical result. The highest deviation components are the level of processing and the interaction of the processing and raw material. Finally, for gliadin the level of processing and the used raw material caused the highest differences in the measured concentrations.

Four-factor ANOVA

The measured concentrations of food allergens by ELISA methods can depend on the applied test kits as well. During our investigations, analytical measurements were taken using two ELISA kits for each allergen. Thus, the factorial analysis of variance could be completed with the applied kit as the fourth factor (Fig. 5).

For milk proteins, it can be observed that the applied ELISA and the level of processing have the greatest impact on the outcome. Thus, the effect of the applied kit decreased the effect of raw material. According to the four-factor ANOVA in matrices containing egg powder, the variation in the results is affected mostly by the processing, the raw material, and their interactions. So, the applied ELISA as the fourth factor is a less influencing factor than in case of matrices containing milk. However, the interactions related to the ELISA tests (ELISA kit*processing level, ELISA kit*single/multi, ELISA kit*processing level*single/multi) have great impact on the analytical results. For model products containing soy, it was found that the variation in the measured concentrations is mostly influenced by the level of processing and the related interactions. However, in case of these three allergens, the measurable concentrations of baked cookies were zero in almost all cases, which

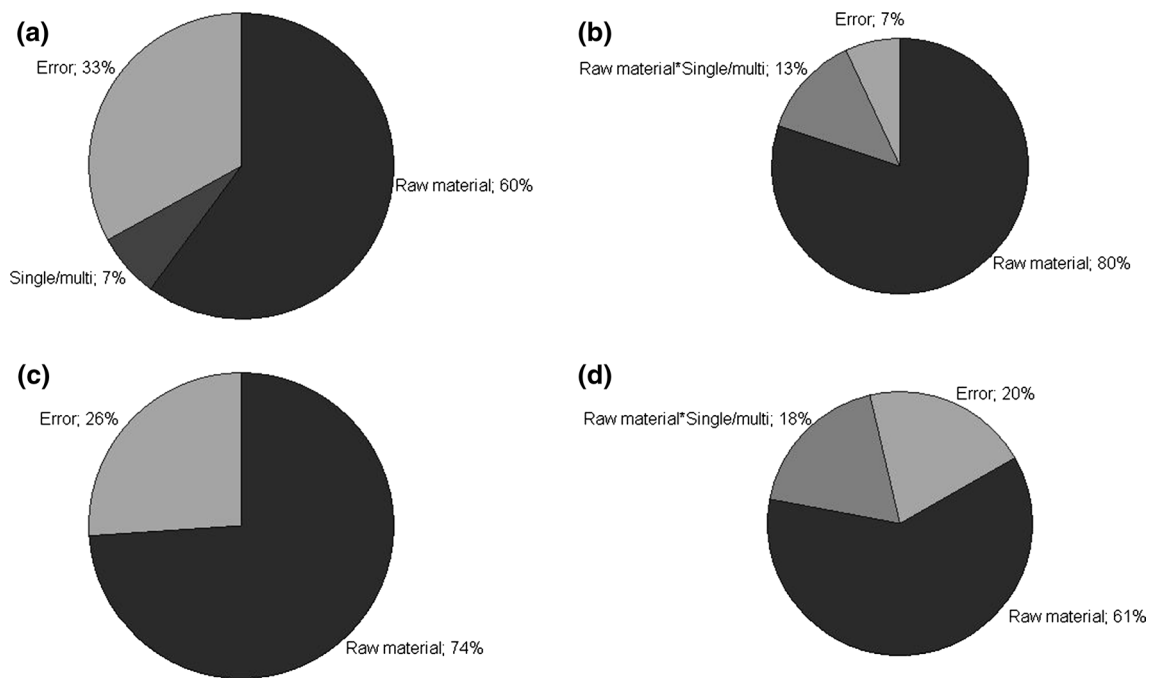


Fig. 3 Contribution of two factors (raw material and number of allergen sources) and their interactions to the SD of measured concentrations in model products containing **a** whole milk powder, **b** whole egg powder, **c** soy flour, and **d** gliadin

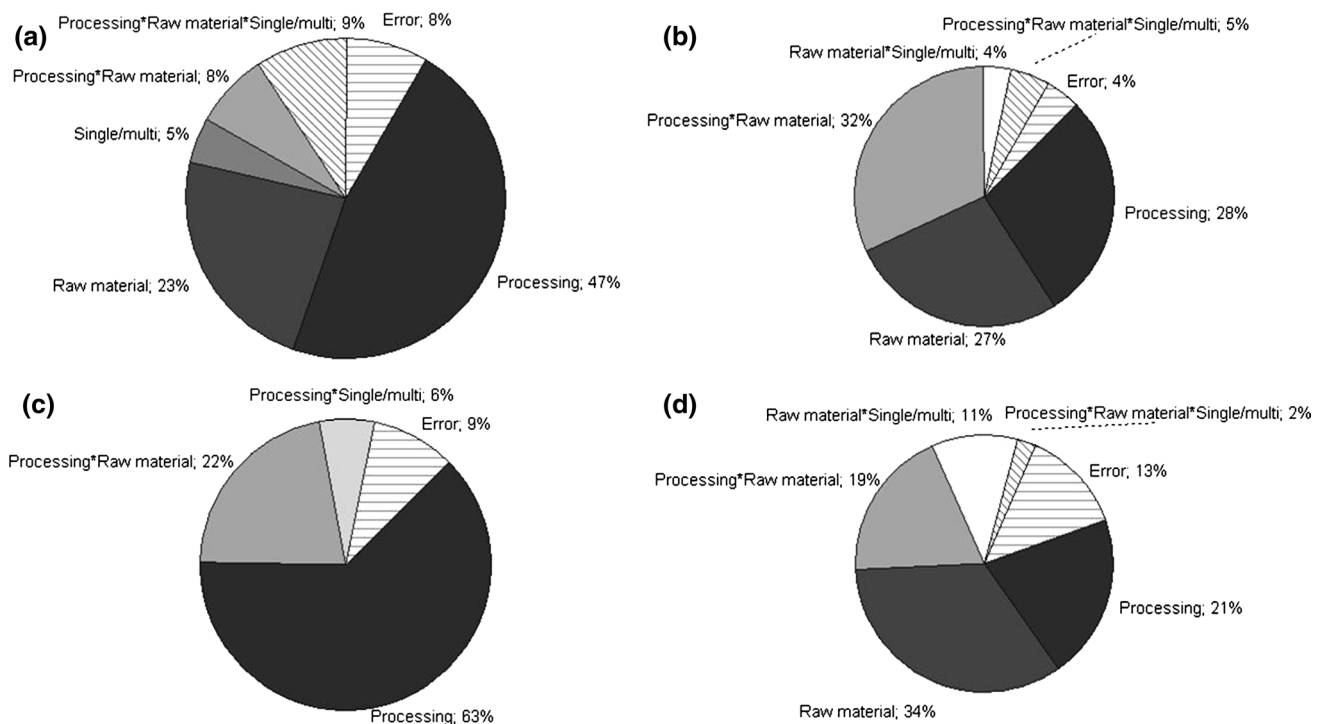


Fig. 4 Contribution of three factors (raw material, number of allergen sources, and level of processing) and their interactions to the standard deviation of measured concentrations in model products containing **a** whole milk powder, **b** whole egg powder, **c** soy flour, and **d** gliadin

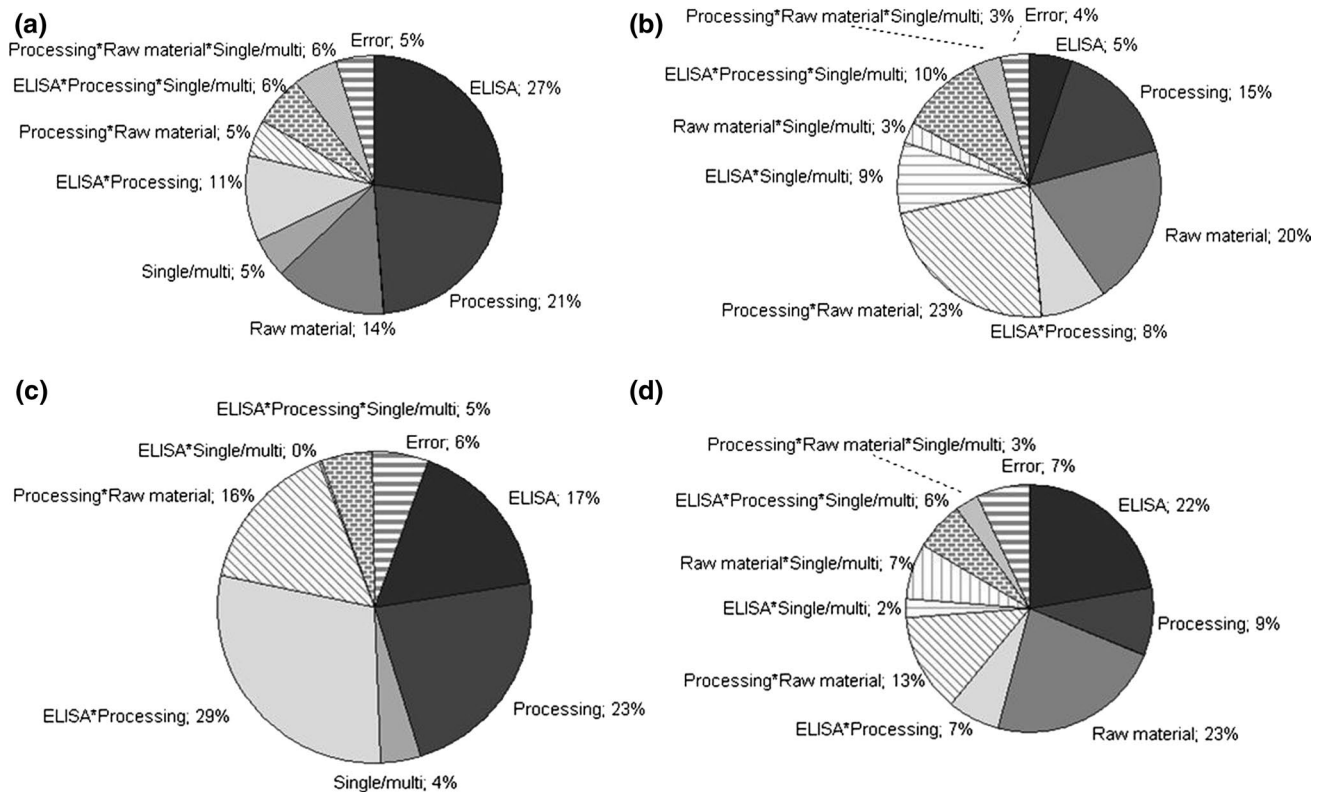


Fig. 5 Contribution of the four factors and their interactions to the standard deviation of measured concentrations in model products containing **a** whole milk powder, **b** whole egg powder, **c** soy flour, and **d** gliadin

Table 2 Percentage distribution of standard deviations related to the measured concentrations

Deviation component (%)	Milk	Egg	Soy	Gliadin
Kit	27	5		27
Processing	21	15	24	20
Raw material	14	20		27
Single/multi	5		3	
Kit * processing	11	8	38	
Kit * raw material				
Kit * single/multi		9	4	
Processing * raw material	5	23	21	
Processing * single/multi				1
Raw material * single/multi		3	1	8
Kit * processing * raw material				
Kit * processing * single/multi	6	10	6	8
Kit * raw material * single/multi				
Processing * raw material * single/multi	6	3		4
1 * 2 * 3 * 4				
Analytical error	5	4	3	6

means the extent of the effect of processing is extremely high. So, the applicability of this statistical model for heat-treated matrices is questionable because of the inadequacy

of ELISA methods. In contrast, for gliadin the measured concentrations differ significantly from zero even in baked cookies. At the same time, the results of the four-factor ANOVA were similar; the deviation of concentrations was mostly affected by the applied ELISA, the raw material, and the level of processing.

Statistical evaluation of the results from the same experiment (Table 2) shows that the level of processing has a great impact on the analytical results in case of every tested allergen. Besides, in milk-containing matrices, the applied ELISA kit and the interaction between the processing and the kits also have proved to be the most influential. In contrast, the analytical results of model products containing egg were affected by the raw material and its interaction with the processing. In case of soy, the largest proportion of the deviation components was the level of processing and the related interactions, while for gliadin, beside the processing level, the applied kit and the raw material gave the highest effect.

According to the experimental results, it can be concluded that the determination of all four examined allergen components is affected by the matrix components and the processing steps; however, the extent of the effects varies in case of the different analytes.

The application of routinely used ELISA methods for the determination of food components triggering

hypersensitivity reactions and utilisation of the results are limited by several known but less explored phenomena. The choice of the target protein, the sample preparation methodology, and the using of conversion factors are partially or completely different in the ELISA kits made for the determination of the same analyte. Besides, most test methodology does not take into account the characteristics of the examined matrices and the effects of processing. Since proteins as target molecules are sensitive to environmental factors, this can affect the reliability of the analytical results.

Conclusion

From the complex evaluation of the result, the following—partly generalisable—assertions can be drawn. The recovery values for each matrix were affected by the applied raw material and the level of processing, but in varying degrees. The simultaneous presence of more allergen sources had no significant effect on the measurable concentration, which is understandable because of their low concentration. The effects of the production process (in this case, heat treatment) can be observed in case of every analyte. Depending on the heat stability of the examined protein sources, the effects are different, but it is very clear that the recovery values are affected significantly and the heat treatment has the highest effect on the accuracy of the investigated ELISA tests. In the background of this phenomenon, not only protein–protein, but also protein–non-protein interactions can play important roles, which resulted drastic modification in structure and solubility of target proteins [6, 35]. Thus, primarily the chemical reactions and the formed products have to be identified in order to find a solution for the protein extraction from heat-treated food samples. Unfortunately, it can be concluded that most of the currently used sample preparation protocols used of the commercially available ELISA kits are not suitable to solubilise processed proteins, consequently to correctly determine the allergens from heat-treated food products. In case of food matrices containing multiple allergens, the situation is more complicated. The structure, physical and chemical properties, chemical reactions, and solubility of proteins are very different; therefore, it is difficult to evaluate the same sample preparation (extraction) protocols for every target component. So, on the one hand, the development of multi-component reference materials is more than desirable, because of better modelling of real food matrices and simplifying the analytical protocols and validation. On the other hand, the multi-allergen incurred materials require the same sample preparation protocols for all target proteins, which has not been solved yet. The heat denaturation of measured proteins (and also the whole food matrices)

causes a drastic reduction in recovery values of ELISA tests, meaning that the accuracy of these methods can be very poor in case of heat-treated samples. The key word of the solution is the improvement in the efficiency of protein extraction processes.

Furthermore, it could be observed for every analyte that the different ELISA kits provide different analytical results. During the analysis of the same incurred model matrices, significant differences were observed even in case of native allergen proteins (powder mixture and dough). The precision of all investigated kits is fine, but their other analytical parameters—like accuracy—are different and poor in case of processed samples. These results draw attention to the importance of the harmonisation of analytical methods, the improvement in the analytical performance, and method validation. The evaluated incurred food models and the statistical methodology presented here seem to be appropriate for the identification of the main sources of analytical errors and for the estimation of the extent of their effects on the analytical results. During our work, only a few types of model products with given characteristics (two raw materials, one or four allergens, three technological steps) were investigated with only two types of ELISA methods. Therefore, the results cannot be generalised, but the complex procedure methodology can be extended with the involvement of other levels of the investigated factors or other factors to study their effects on the analytical result.

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Conflict of interest The authors declare that they have no conflict of interest.

Compliance with Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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