

Investigation of incurred single- and multi-component model food matrices for determination of food proteins triggering allergy and coeliac disease

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Abstract For detection and quantification of food proteins triggering hypersensitivity reactions, reliable, validated analytical methods are necessary. At present, the most commonly used methodology for this purpose is ELISA. The lack of incurred reference materials (RM) complicates the fulfilling of method validation. Today, a well identifiable research direction is the development of methods (immuno-analytical, PCR and MS methods) suitable for multi-component analysis. As a consequence of these R+D efforts, the development of multi-component RMs is reasonable. Our work presented here is focusing on the development and investigation of multi-component model food matrices that can be suitable for supporting the validation processes. On the basis of the relative incidence of hypersensitivity reactions, four components—wheat, milk, egg and soy—were selected for our preliminary work. The target proteins were determined in single- and multi-component matrices with different ELISA methods, and the results were compared by statistical evaluation. The main direction of our investigation was to identify the effects of the potential interactions of these proteins on the analytical results, and the influence of food processing was also investigated. The results showed that the presence of multiple allergenic components caused much less uncertainty in the analytical results than analytical and technological steps, such as sample preparation, repetition or even heat treatment. However, probably due to the relatively complex matrices, some discrepancies from the expected outcome could also be observed.

Keywords Food allergens · Coeliac disease · Multi-component model food matrix · ELISA

Introduction

Food-related hypersensitivity reactions can be divided into the categories of immune-mediated food allergy and non-immune-mediated food intolerance [1, 2]. According to FAO, eight food components are responsible for more than 90 % of these food-related disorders [3]. The “Big Eight” includes cereals containing gluten, crustaceans, egg, fish, peanuts, soybeans, milk, tree nuts and sulphites [4]. Consumption of food stuffs involving these components—even in trace amounts—may result abnormal reactions in susceptible individuals. Except for sulphites, the symptoms are triggered by innate food proteins. For these consumers, the only effective way of therapy is the total avoidance of the offending food components in their diet. In order to provide reliable information for patients to facilitate safe food choices, food manufacturers are obligated to label the presence of allergenic compounds on the package of foodstuffs. Labelling instructions vary around the world [5]; according to the corresponding EU directives, 14 components appear on the list of common food allergens [6, 7].

As a consequence, there is a need of sensitive, reliable analytical methods that are able to detect and quantify the allergenic components, even in trace amounts. Several methods [immunoanalytical methods (ELISA), PCR, MS techniques] are available for the qualitative or quantitative determination of the triggering proteins or other proteins that indicate the presence of the offending food. However, the conditions of validation are imperfect because of the lack of reference methods and reference materials. Currently the method-of-choice for the quantification of

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food allergens and gluten are the immunoanalytical-based ELISA methods because of their high specificity, sensitivity and easy handling [8].

There are a number of ELISA test kits for the determination of food proteins triggering hypersensitivity reactions; however, the results provided by different methods for the same sample show a considerable variation [9–12]. For the patients' safety, understanding the background of the altering analytical results and basically to determine and improve the analytical performance is of utmost importance. Development of ELISA methods is quite challenging because a large number of factors has to be considered [8].

Selection of the proper antibody is crucial in case of immunoanalytical methods. Mostly, commercially available ELISA methods are using different antibodies for the same target component. Different antibodies are specific for different amino acid sequences and in many cases ELISAs are applying polyclonal antibodies detecting more than one target epitopes. The materials applied for calibration are also various. Besides, to calculate the final concentration from the measured absorbance data, a number of factors have to be applied, such as correction with the dilution factor, conversion of the amount of the target protein to total protein or total commodity content. The value of these conversion factors depends on different things such as the types of protein sources, species and varieties (as genetic factors) or the year, location and condition of growing (as environmental factors). Therefore, the determination of the exact values of the conversion factors is difficult, and it is also an important source of analytical error [2, 8, 13, 14].

Another important question is sample preparation. It has to be able to solubilise all of the target proteins without destructing their structure and/or changing the availability of epitopes. Selection of the right extraction solution and procedure is therefore essential. The different ELISA kits and methods contain (partly) different extraction buffers for the same proteins. Sample preparation protocols can also differ; the most frequent deviations are the extraction time, incubation temperature and the concentration of the used buffers [15–18].

A further considerable factor in terms of quantification of allergens and gluten is matrix effect. Food products contain a number of micro and macro components that can affect the structure and availability of the measured proteins. Formation of complexes or resistant aggregates during processing cannot be excluded. These modifications may lead to masking of the target epitopes or the formation of new epitopes. The matrix components can reduce the affinity of the epitopes to the antibody, which can prevent their recognition. Additionally, these factors may also influence the solubility of the target molecules resulting lower efficiency of extraction procedures and therefore decreasing the reliability of the analytical results [19–21].

Finally, the analytical determination of these proteins is rendered even more challenging by the possible effects of food processing. The most commonly applied processing steps are different types of thermal treatment. Heating can cause denaturation, aggregation or polymerisation of proteins and can induce their interactions with other food matrix components. These alterations may affect solubility properties of proteins, which can modify the extractability of the target molecules. Solubility properties of food proteins can also be changed by high pressure, pH changes, enzymatic hydrolysis and chemical modifications. In addition, processing steps can modify the immunoaffinity of target proteins as well [22–25].

Another limiting factor of method development and validation in this field is the lack of commercially available incurred reference materials. Application of reference materials during the process of analytical method development is necessary because they can ensure the metrological traceability of an analytical measurement and the determination of the accuracy and other parameters of analytical performance of methods. Reference materials have to fulfil several essential requirements, such as defined concentration, homogeneity, stability and characteristics of their physical, chemical and biological behaviour [26]. The development, validation or even standardisation of ELISA methods would be improved by incurred reference materials (RM) because with these most of the aforementioned matrix effects could be taken into consideration and would become possible to investigate. Recently, several research studies have been focusing on the development of incurred reference materials [11, 27–32]. The effects of different factors on the analytical results are also being studied in complex model matrices. In addition, development of multi-component methods is identified as a new direction of allergen analytical research efforts [33–35]. The number of studies dealing with the impact of the simultaneous presence of multiple allergenic or coeliac-toxic proteins in a food sample is currently very limited though. To date, published studies are describing the production of multi-component matrices and the investigation of the effects of heat treatment [36, 37]. Thus, the development of multi-component reference materials seems to be a reasonable goal for the near future.

The main objective of this study was to investigate, identify and estimate the analytical errors originated from the presence of more than one allergenic or toxic protein, to compare them with the errors caused by other matrix effects and thermal treatment, and to understand the background of the identified phenomena. To achieve these goals, multi- and single-component incurred food model matrices using four different protein sources—gluten, milk, egg and soy—in defined concentration level were developed and investigated. Every specific protein component

and the potential cross-reactions were studied with two different ELISA methods. The analytical results obtained with single- and multi-component matrices were compared and evaluated by statistical methods. (Remark: We would like to emphasise here that the currently applied ELISA methodology for gliadin determination considers gliadin, a coeliac-toxic protein, which may result ambiguity in the nomenclature applied in this work. To avoid this, we will refer to gliadin as an allergenic protein too in order to make the presentation of the results clear. This is also reasonable because besides being a coeliac-toxic protein, gliadin also plays a role in wheat allergy and can be used as an indicator of the presence of wheat in food products.)

Materials and methods

Experimental design

In order to achieve our goals, model matrices containing a single or multiple sources of allergenic proteins were produced and the effects of the simultaneous presence of four allergens on the analytical results were examined. The flow chart of the experimental design is shown in Fig. 1. This design made it possible to investigate the effects of processing in all three key stages of the production process: in the basic powder mixtures, in the raw dough and in the baked cookies as well. This way—similarly to our previous studies [27, 28], we could perform the comparative study of random analytical errors resulting from different

sources, such as sample preparation, repetition, heat treatment and effects of other matrix components.

Production of model matrices

The matrices are processed food products, namely baked cookies. The commodity of the model products is a commercially available gluten-free baking mix (Mix Schär C, Dr. Schär Srl/GmbH, Burgstall, Italy), and they also contain sugar, salt, baking soda, margarine and water. 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, [31]) were used. Matrices were produced containing single allergen sources (milk or egg or soy or gliadin) and multiple allergens with the simultaneous application of all four components.

The matrices containing milk or egg or soy individually were produced according to the recipe and method of Bugyi and co-workers [28], while for the production of gliadin-containing model matrices, a different production procedure [27] was applied. For preparation of multi-component food matrices (Fig. 1), the two aforementioned production procedures were combined [27, 28]. Briefly, the dry components and the allergen sources were mixed in the first step to form a powder mixture. With the addition of margarine and water to this mixture, a dough was formed. Afterwards, the raw dough was shaped into cookies and was baked. 50 ppm of the standard protein sources (milk or

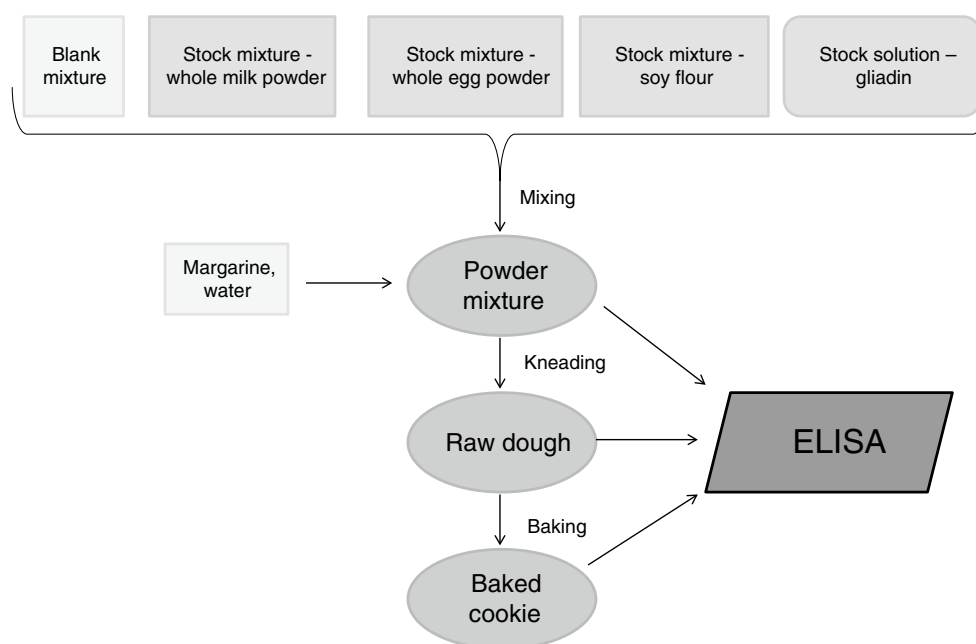


Fig. 1 Workflow of multi-component model matrix production

Table 1 The applied ELISA tests and their performance characteristics

Allergen	Manufacturer/ELISA test	Limit of detection (ppm)	Range of quantification (ppm)	Expressed result
Milk	R-Biopharm/Ridascreen Fast Milk	0.7	2.5–67.5	Milk protein
	Romerlabs/AgraQuant Casein	0.04	0.2–6	Casein
Egg	R-Biopharm/Ridascreen Fast Ei/Egg Protein	0.1	0.5–13.5	Whole egg powder
	Romerlabs/AgraQuant Egg white	0.05	0.4–10	Egg white protein
Soy flour	R-Biopharm/Ridascreen Fast Soya	0.31	2.5–20	Soya protein
	Romerlabs/AgraQuant Soy	0.016	0.04–1	Soy-trypsin inhibitor
Gliadin	R-Biopharm/Ridascreen Gliadin	1.5	2.5–40	Gliadin
	Romerlabs/AgraQuant Gluten G12	2	4–200	Gluten

egg powder, soy flour or PWG gliadin isolate) was added to each single or multiple model matrix in different ways, as described by previous studies [27, 28].

The freeze-dried raw dough and the baked cookies were ground (Retsch AS 200, Basic) and stored in sealable plastic bottles until analysis. For the analytical measurements, samples were taken from each step of the procedure (powder mixture, raw dough and baked cookie).

ELISA methods and statistical evaluation

For the analysis, eight sandwich ELISA methods were applied (Table 1), two different test kits for the investigation of each individual allergenic compound. The most important criterion of the ELISA kit selection was the representation of the variety of antibodies using different epitopes for the same target component. For the investigation of the possible interactions between the allergenic proteins and the antibodies of the other ELISA methods, the powder mixtures containing single allergen compounds were tested with the six other ELISA kits as well. Sample preparations and the analytical tests were carried out according to the manufacturer's instructions enclosed to the kits. The reagents used were provided by the manufacturers excluding ethanol which was of analytical grade and was obtained commercially (96 V/V % ethanol, MOLAR Chemicals). For the presentation of results, kits were coded by letters A and B because the goal of this study was to identify and highlight the possible effects of different factors on the analytical results and not the evaluation and comparison of the accuracy of the results obtained with different methods.

The analytical results of the ELISA measurements were calculated using the BioRad Microplate Manager 6 software. As the reporting units are different in each kit, the measured data were converted to the same unit according to the manufacturer's instructions. Hence, the presented concentrations refer to ppm whole milk powder, whole egg powder, soy protein and gliadin. The data were evaluated by the investigation of means, standard deviations and significance tests (*f* test, *t* test).

Results and discussion

Homogeneity study

Production procedure of single-component model matrices was developed previously [27, 28], and the reproducibility of the production was achieved. Nevertheless, in case of multi-component matrices, four different materials—i.e. protein sources—with partially different physicochemical properties had to be homogenised. The distribution of target proteins within the single- and multi-component matrices was investigated with relevant ELISA methods. Homogeneity was examined by the statistical evaluation of the standard deviation of the replicates (Fig. 2). In case of every sample matrix and every allergenic protein, no significant differences could be observed between the four replicates. Standard deviation measured in multi-matrices (which is the result of random errors originated from the analytical method and homogeneity) is not higher than that of single-component products. Moreover, this standard deviation is of the same extent as that of the standards applied in the ELISA kits. Thus, random error deriving from the homogenising process does not increase the uncertainty of the analytical results, so the model products can be regarded as homogeneous matrices.

Examination of cross-reactivity

Beside the homogeneous distribution of the proteins in question, another critical point of the further applicability of the model matrix containing multiple allergen sources is the potential cross-reactivity of these proteins with the antibodies of the non-specific ELISA methods. Thus, powder mixtures containing a single allergen source were investigated with the ELISA methods designed for the determination of the other three analytes. In these experiments, only the powder mixtures containing single native proteins were used, in order to eliminate the influence of food processing on the analytical results (see “Investigation of the effects of food processing steps”).

In case of egg white protein ELISA kits, no cross-reaction with the other proteins was detected. In contrast, soy

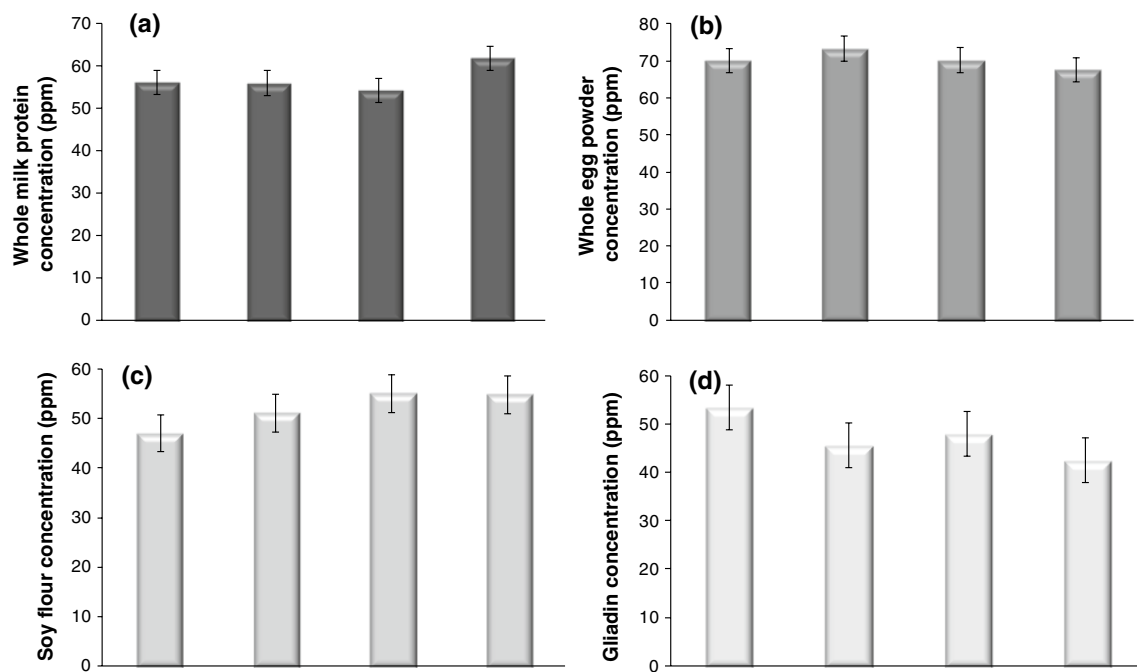


Fig. 2 Distribution of the measured analyte content in multi-component matrices with **a** whole milk powder, **b** whole egg powder, **c** soy flour and **d** gliadin content. Bars are representing the four measured parallels

ELISA tests showed absorbance values above the limit of detection (LoD) in all other matrices. The milk and gliadin ELISAs 'B' gave analytical signals with other protein sources below the LoD. The milk ELISA 'A' measured concentrations above LoD in all mixtures, while gliadin ELISA 'A' showed detectable signal in egg and soy containing powder mixtures.

As a conclusion of the cross-reactivity study, it can be stated that in all cases the detected analytical signals were below the limit of quantification (LoQ); therefore, in all measurements, the detectable signals were not possible to be taken into consideration as a correction factor during the calculation of final concentrations. However, in some cases, values were above the LoDs. It must be confirmed whether these analytical signals are a result of cross-contact of materials in the applied processing or they are originated from other compounds of the utilised matrix components. These examinations are necessary to identify the existence of cross-reactions between the examined analytes and antibodies specific for other allergens. Any analytical signal originated from theoretical failure (calculation problems) or cross-contamination causes analytical errors that have to be excluded.

Comparison of the results of single- and multi-component model matrices

After confirming homogeneity of the model products, a comparative study was carried out between the single- and

multi-component model matrices (Fig. 3). The PWG gliadin is an isolate; the other applied protein sources were certified powders with a complex chemical composition. Every single investigated proteins sources and the model food matrices are also containing many different proteins and other reactive components. Therefore, the determination of the effects of the targeted proteins on the measured analytical results is not possible directly. Otherwise, our incurred material model real food products where the investigated proteins are embedded in a real food matrix in which and different interactions and chemical reactions can take place. Thus, our model system is not suitable to identify the individual effects, but seems to be applicable to estimate the analytical uncertainty originated from the coexistence of more than one measured components. For these purposes, both the measured absorbance values and the calculated concentrations of the analysed components (egg, milk, soy or gliadin powders) were determined, compared and statistically evaluated. The main reason of the utilisation of the absorbance values in the evaluation process is that in some cases, the measured concentration in the baked cookies was lower than the limit of quantification, so the comparison of these samples was carried out using the absorbance values only.

The recovery values were studied and compared in all types of matrices (Table 2). In most cases, recovery values in unheated model products (powder mixtures and raw dough) were significantly higher than the theoretical

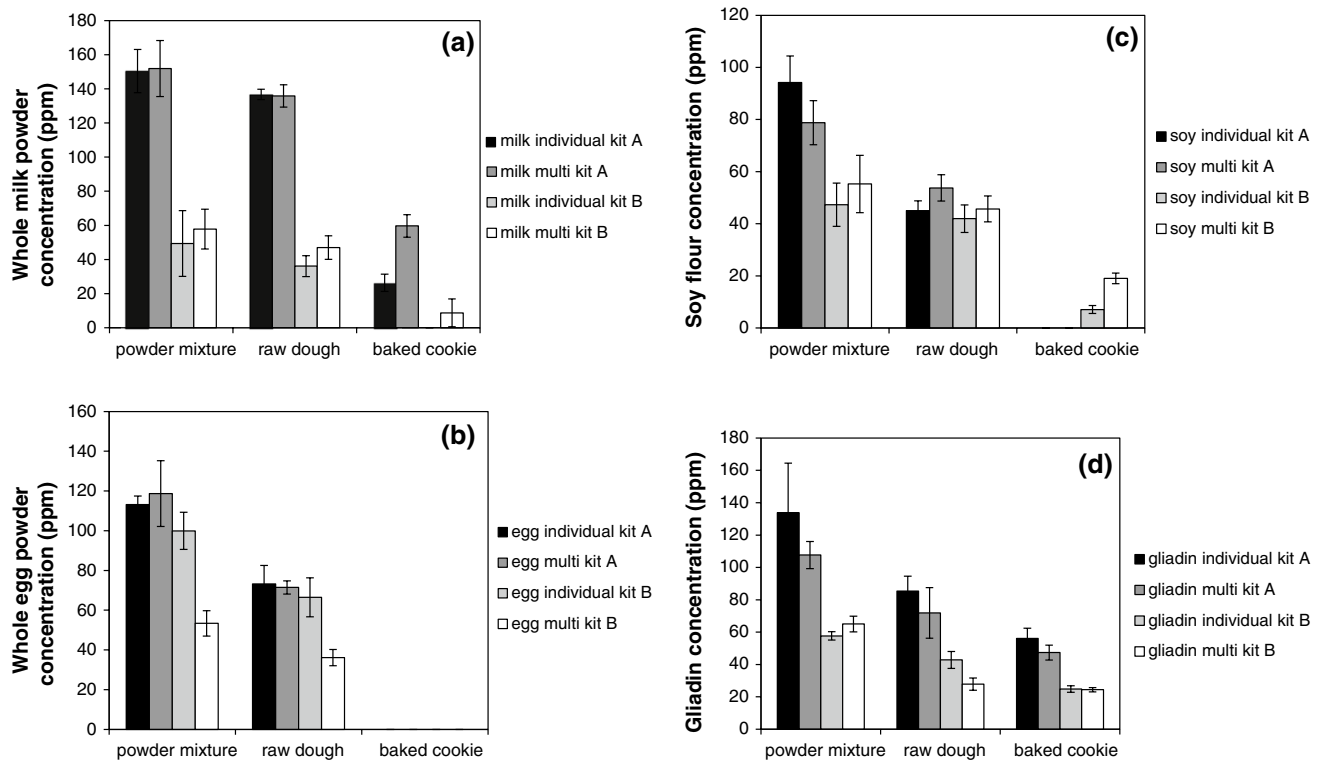


Fig. 3 Comparison of individual and multi-component sample matrices with the determination of **a** whole milk powder, **b** whole egg powder, **c** soy flour and **d** gliadin concentration. Missing bars mean that the measured concentration was below the limit of detection

Table 2 Recovery (%) of the analyte concentration on a 50 ppm nominal concentration base

Sample	ELISA kit	Milk		Egg		Soy		Gliadin	
		Single	Multi	Single	Multi	Single	Multi	Single	Multi
Powder mixture	A	301 (8)	304 (11)	226 (4)	237 (7)	188 (11)	158 (10)	268 (22)	215 (8)
	B	99 (4.8)	116 (20)	200 (9)	107 (12)	95 (10)	111 (6)	115 (4.5)	130 (7.5)
Raw dough	A	273 (2)	272 (5)	147 (12)	143 (5)	113 (8)	108 (9.5)	166 (11)	144 (21)
	B	72 (15)	88 (15)	133 (13)	72 (11)	84 (12)	91 (11)	124 (12)	80 (13)
Baked cookies	A	53 (14)	119 (11)	0 (0)	0 (0)	0 (0)	0 (0)	112 (11)	95 (10)
	B	0 (0)	18 (16)	0 (0)	0 (0)	14 (21)	38 (10)	120 (8)	63 (5)

Values in brackets are CV %

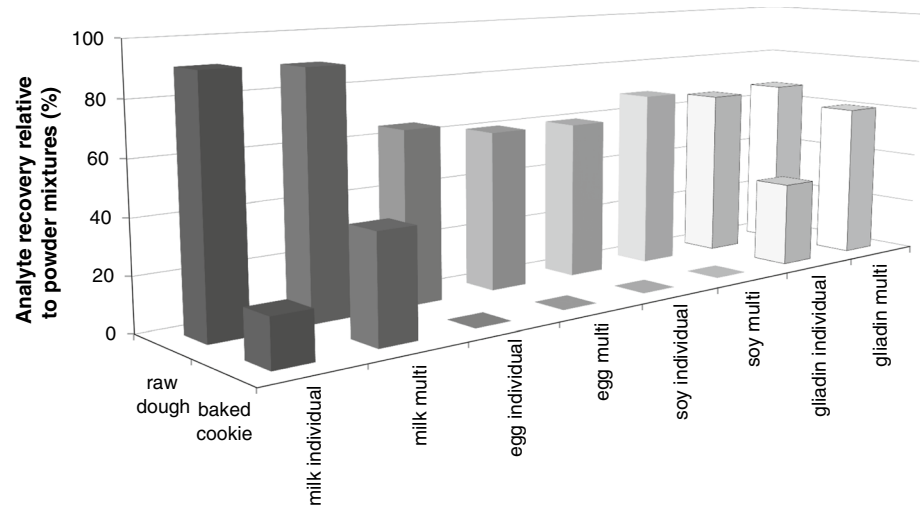
concentrations, as opposed to baked cookies, in which these values were lower. Besides, it also can be stated that significantly different results can be measured with the different ELISA kits for a certain sample.

In case of milk, the measured concentrations do not differ significantly in single and multi-powder mixtures and neither in raw dough (Fig. 3a). On the other hand, milk ELISA 'A' provides differences in baked cookie samples, and the calculated concentration of multi-component cookies is significantly higher than that of cookies containing only milk powder. In case of ELISA 'B', the results for cookies are below the limit of quantification, so inferences could not be drawn from the concentrations. Based on the

evaluation of the absorbance data, the difference between single- and multi-cookies appears to be significant; however, it must be highlighted that these data fall into a less reliable category.

Investigation of egg powder content in model matrices with ELISA 'A' did not show significant differences between single- and multi-component materials in case of any sample types (powders, dough and baked cookies) (Fig. 3b). In contrast, according to the results of ELISA 'B', powder mixtures and raw doughs differ significantly, in multi-matrices lower concentration could be measured. Single- and multi-allergen-containing cookies gave the same absorbance values, but below the LoQ.

Fig. 4 Changes during food processing measured by ELISA tests ‘A’. Bars are representing the extent of decrease in the raw dough and in the baked cookies as a percentage of the corresponding data measured in the powder mixture of raw materials



In case of soy, there were no significant differences in the evidence between the single- and multi-model matrices, only ELISA ‘B’ shows deviation for baked cookies (Fig. 3c). Concentration in multi-cookies is two times higher than in single soy content cookies. For baked cookies with soy ELISA ‘A’, only absorbance data are available, which show no significant differences between single- and multi-component cookies.

According to the investigation of gliadin powder mixtures and baked cookies, the individual and multi-matrices do not differ significantly (Fig. 3d). In case of raw dough, ELISA ‘B’ shows significantly lower concentration for multi-matrix than for single-component raw dough.

The experienced phenomena may be explained by several reasons. The complex matrices contain much more macromolecules and smaller components, providing an environment for more complex chemical reactions forming protein–protein interactions, protein–lipid or protein–carbohydrate complexes. The physical properties of multi-component matrices can also be different, forming local emulsions or molecular surfaces, which can also affect the physical behaviour, e.g. the solubility of components. The proteins can be denaturated during hydration or heating processes, and their higher structures can also be changed causing partly different physical and chemical properties in multi-matrices. Furthermore, the formation of new epitopes cannot be excluded either. Understanding the background of the observed phenomena and identifying those key effects that might influence the analytical results significantly are essential from the point of view of developing multi-component matrices as real incurred reference materials.

In summary, in some cases, the measurable concentration differs in multi-component matrices compared with single-component samples. Moreover, the rate and the direction of the alterations in the measured values are different. Therefore, it is possible that the measured effects are

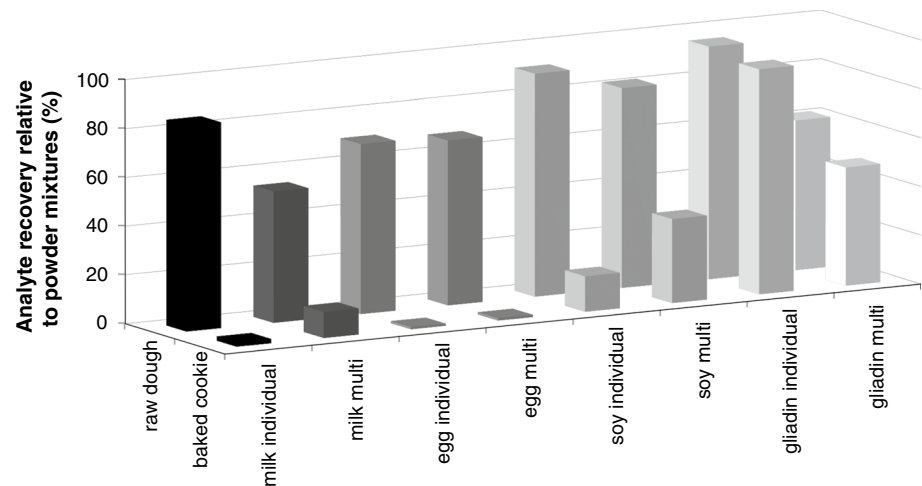
random and non-regular and reproducible phenomena. The extent of the measured differences from the nominal values is similar in both single- and multi-matrices. Similar statements can be made in case of repeatability values. Therefore, it seems that the utilisation of more than one protein sources is not increasing the extent of the regular and random errors in any cases.

Investigation of the effects of food processing steps

Besides the factors mentioned above, food processing has a non-negligible effect on the analytical data. Based on samples from different steps of the model matrix production, a decrease in the measured protein concentration could be observed throughout the processing steps in case of all four compounds (Figs. 4, 5). The observed decrease in raw dough compared to the powder mixtures is not significant, but the extent of decrease is extremely high in baked cookies. These phenomena have already been described in many previous studies [20, 21, 25, 37–39]. However, the extent of the denaturation and therefore the caused analytical errors are highly dependent on the investigated proteins and even on the applied ELISA kits. In case of milk powder, 80 and 60 % decreases were measured with ELISA kit A in single- and multi-component-baked cookies, respectively. Results of milk ELISA kit ‘B’ showed a higher level of decrease: a 90 % loss of milk powder content was measured in multi-cookies, while in case of the single-compound matrix, the measured concentration was below the LoQ, and thus, the loss was virtually 100 %. In all experiments where egg powder were measured in heat-treated model matrices, the analytical signals were below LoQ; therefore, the egg protein content of baked cookies is not detectable with either ELISA tests.

Examination of matrices containing soy with ELISA kit ‘A’ showed that soy proteins could not be detected in case

Fig. 5 Changes during food processing measured by ELISA tests ‘B’. Bars are representing the extent of decrease in the raw dough and in the baked cookies as a percentage of the corresponding data measured in the powder mixture of raw materials



of either single- or multi-allergen cookies. In case of soy ELISA kit ‘B’, the measurable concentrations of individual and multi-allergen-containing cookies are decreasing with 90 and 60 %, respectively. Target protein of ELISA kit ‘A’ is soy-trypsin inhibitor, a heat-sensitive protein that can give an explanation for the undetectable content.

The analytical experiences with gliadin sources are described in detail in previous papers. Our results in this study are in harmony of the previously published data [24, 27, 36, 37]. The measurable gliadin concentration in single-baked cookies is decreasing with 70 % and in multi-baked cookies with 40 % according to gliadin ELISA ‘A’, while with ELISA ‘B’, the measured values of both individual and multi-matrices showed a decrease of 60 % compared with the powder mixtures. Similarly, the measurable gliadin concentration in multi-baked cookie is reduced to 63 %.

To sum it up, it can be seen that continuous decreasing can be observed throughout the process mainly in the baked cookies compared to the raw dough. The extent of these changes is a function of the measured proteins, the applied ELISA methods and the investigated matrices. The hypothetic reasons of the effects of heat treatment are also described in previous studies [15, 19, 20]. Different ELISA tests are measuring different epitopes, and thus, it is possible that the structure of these epitopes changes differently, which has an effect on the affinity to the applied antibody. On the other hand, it can also occur that the sample preparation protocols were developed for native proteins and are not capable of recovering the proteins with modified structure. Gliadin seems to be the most heat-stable protein, while the other measured proteins are absolutely unstable after heat treatment. During heat treatment, the proteins can undergo structure modifications, such as denaturation or aggregation. The extent of these changes depends on the duration and temperature of the treatment, the stability of the protein in question and the environment in which the protein is located. Furthermore, heating may have an

influence on the interactions between allergenic proteins and other matrix components. Due to the reasons detailed above, it can be concluded that estimating and predicting the decrease in measurable protein content and the potential functional changes caused by heat treatment is complicated in real food matrices. But our multi-component model matrix may provide a helpful tool for the estimation of these effects.

Conclusion

As an overall conclusion, it can be stated that the production of a multi-component incurred model matrix is technically feasible. The applied procedure is reproducible, and the homogeneity of the end products is satisfactory.

However, results of commercially available ELISA test kits show phenomena that have to be further investigated and taken into account during characterisation of the reference material candidate. In one hand, some of the examined protein sources gave analytical signal in non-specific ELISA tests, though neither of these signals appeared to have analytical relevance. The background of this phenomenon has to be still clarified as inadvertent cross-contamination cannot be excluded. However, food processing has a truly considerable impact on the measurable protein concentration of variable extent, depending on different factors, such as the target molecule or the applied ELISA method. It suggests that behaviour of epitopes—and proteins containing these epitopes—differs during food processing steps [14, 15, 17, 20].

Another issue that introduces a further source of analytical uncertainty is that in case of most ELISA tests, the final concentrations have to be calculated from the measured absorbance using certain conversion factors. In many cases, the final results have to be recalculated into the concentrations of the complex food components (e.g. from casein

to whole milk powder or from gliadin to gluten). In these cases, conversion factors are used, which are usually provided by the kit manufacturers. Generally, the way of calculating these converting factors are not known and/or they contain generalisations, such as gliadin/glutenin ratio or constant protein content [14, 40]. Thus, it is likely that protein sources used by the manufacturers are different from the ones used for these experiments, and this may imply different protein characteristics.

Protein content and distribution of protein fractions in raw materials depend on various factors. The primary factor is the genetic characteristics of the protein source. Besides, the environment may also affect the expression of proteins. In case of plants, growing conditions play an important part in protein expression, while for animals, the raising/feeding circumstances can be determinant factors. Furthermore, during food production, raw materials can be prepared with different techniques (e.g. extraction, precipitation, spray drying, freeze drying and heat treatment). The various ways of application of allergen-containing raw materials may result in different protein characteristics. As a result of these effects, the measurable concentrations may differ in the single and multi-matrices and it can lead to increased uncertainty of the analytical results.

The comparison of the different effects shows that heat treatment and the choice of the ELISA method have the highest effect on the analytical results. Considering these effects, the deviation observed in multi-matrices compared with single-component RM candidates is inconsiderable. So it can be concluded that the developed model matrix (baked cookie which contains four allergenic compounds) can be suitable for using as a reference material. On the other hand, the applied analytical method can be a limiting factor, since the majority of the examined ELISA methods are not or only restrictedly suitable for quantification of processed food matrices. This also means that the quantification of target molecules from real food matrices is prone to analytical errors. The analytical results can deviate from the real value, which is a critical problem from food safety aspects. Besides, the experienced phenomena might hold significance in respect of multi-method development works too. The analytical uncertainty may increase in the course of investigation of real food matrices using multi-methods calibrated with single-component reference materials.

These outcomes highlight the main theoretical (selected epitopes, calculation mode of the final concentrations of components from the measured analytical signals of target proteins/epitopes) and practical (sample preparation, solubilisation, homogeneity, effects of heat treatment) problems of analytical methods and difficulties of RM production. Reference materials can help to improve the conditions of analytical measurements of allergenic and coeliac-toxic proteins or the raw materials containing these proteins.

However, to solve the above-mentioned problems, a new approach of method development must be established.

In order to support the safety of patients and to improve the labelling regulations and the analytical method development, it is essential to characterise the possible interactions of allergens and other critical proteins with matrix components and to understand their behaviour and their changes during food processing. Behaviour of proteins during food processing should be investigated with separation techniques using model matrices without any protein content except for allergen sources.

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

Compliance with Ethics Requirements 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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