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Improving the conditions of the analytical methodology for the quantification of food allergens

Summary of Ph.D. thesis

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1. INTRODUCTION AND GOALS

Beside the environmental factor-dependent microbiological, biological, physical and chemical hazards, certain natural food components can also be a food safety risk for particular consumer groups. These natural components- mainly proteins- are able to trigger hypersensitivity reactions (e.g. allergies, intolerances) in the human body. The clinical backgrounds of the different types of disorders are significantly different, mostly only their symptoms can be treated. Due to this reason the most effective cure of these illnesses is the avoidance of the triggering proteins by a special diet.

Supplying the affected consumers with safe raw materials and food products requires the cooperation of many stakeholders (food manufacturers, control laboratories and authorities). The basis of this cooperation and the solution of the complex problems must be provided by the collaboration of multiple scientific fields.

Clinical research must determine the mechanisms of hypersensitivity reactions together with the identification of the triggering proteins and epitopes and threshold levels. Besides, excessive chemical investigation of these proteins and epitopes is also necessary just like understanding the effects of food processing on the structure, functional properties (e.g. solubility) and biological activity (e.g. immunoreactivity) of proteins. These pieces of information are essential for establishing the related legislation. For the proper controlling of foods a methodology capable of the reliable determination of the triggering proteins is necessary (Gendel et al. 2008)[1] . A validated analytical methodology is a key for the control of the food chain, for operating food safety and allergen management systems and for providing reliable information for the consumers.

Currently the method of choice in this field is the relatively easy-to-perform and specific, immunoanalytical based ELISA (Enzyme-Linked Immunosorbent Assay) method. The applicability of this methodology is limited partly because of the limitations of the validation of the commercially available test kits. This is a result of the lack of reference materials and methods and other related factors. The most

[1] Gendel S., Buchanan R., Dennis S., Acheson D., Assimon S. A., Beru N., Bolger P., Carlson D., Carvajal R., Copp C., Falci K., Garber E., Harden E., Kane R., Kvenberg J., Luccioli S., Park D., Raybourne R., Troxell T., Vierk K. Approaches to establish thresholds for major food allergens and for gluten in food. *Journal of Food Protection*. **2008**; 71 (5): 1043-1088.

important factors are the genetic and environmental variability of the triggering proteins, the insufficient data on thresholds and the properties of the proteins and the imperfect description of the effects of food processing on these molecules. These factors are all influencing the development of both the analytical methods and the reference materials (Kerbach et al. 2009)[2].

Several international research groups are working on the solution of these problems including our Department as a member of the Food Allergen Working Group of the MoniQA (Monitoring and Quality Assurance in the Food Supply Chain) Network of Excellence funded by the 6th Framework Programme of the European Commission. Connected to the activity of the Working Group, the goals of my PhD work are the following:

- Development of new reference materials containing three major allergens, milk, egg and gluten proteins in a processed food matrix.
- Determination and comparison of the analytical performance of the commercially available ELISA methods using the developed reference materials, explaining the experienced phenomena. The novelty of our work in this field is the application of reference materials modelling real food matrices. This way method validation and the investigation of parameters influencing the analytical data can be implemented in better-defined circumstances.
- Comparison of analytical data obtained by the determination of native and processed proteins, thus estimating the analytical uncertainty and molecular changes of proteins caused by food processing

The application of reference materials creates the possibility of estimating the scale of factors having the most significant influence on the analytical results (e.g. properties of samples, systematic and random errors originated from the application of ELISA kits, uncertainty caused by technological effects). This information contributes for the better interpretation of the analytical results and for the harmonization of analytical methodology and the related legislation.

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[2] Kerbach S., Alldrick A. J., Crevel R. W. R., Dömötör L., DunnGalvin A., Mills E. N. C., Pfaff S., Poms R. E., Popping B., Tömösközi S. Managing food allergens in the food supply chain- views from different stakeholder perspectives. *Quality Assurance and Safety of Crops and Foods*. **2009**; 1 (1): 50-60.

2. MATERIALS AND METHODS

The basis of the reference material development is the recipe of a model product (cookie) made by Scaravelli et al. (2008)[3] for the development of PCR methods for the determination of peanut residues. The two main steps of our experiments were the modification of the recipe and the development of methods for homogenizing milk, egg and gliadin proteins in the model product. The experimental steps and the related results are described in section "Results".

For studying the reference materials we established an experimental design that is also suitable for investigating the effects of food processing on the analytical results. The baked model products, together with the mixture of raw materials and doughs coming from the previous steps of the production process were analyzed by ELISA methods.

Furthermore, with the help of the gliadin reference material we iplemented a comparative study of seven commercially available ELISA test kits (AgraQuant Gluten Assay (Romer Labs), BIOKITS Gluten Assay Kit (Tepnel), Gliadin ELISA (ELISA Systems), HAVen Gluten-Check ELISA kit (Diagnostic Innovations), RIDASCREEN Gliadin (R-Biopharm), Veratox Quantitative Gliadin Test (Neogen), Wheat protein ELISA kit (Gliadin) II (Morinaga)).

The ELISA measurements and the evaluation of the analytical data were carried out according to the users' manual provided by the manufacturers. The obtained results were statistically evaluated by analyzing average values, standard deviations and t-tests.

3. RESULTS

3.1. DEVELOPMENT OF REFERENCE MATERIALS CONTAINING MILK AND EGG PROTEINS AND GLIADIN

As it seemed to be expedient to produce a sample matrix that could be used universally for the reference material development in case of any allergenic components, firstly it was necessary to modify the original recipe for making an allergen-free model product. Modifications primarily occurred for eliminating components that are interfering with the future materials (e.g. milk powder) without

[3] Scaravelli E., Brohée M., Marchelli R., van Hengel A. J. Development of three real-time PCR assays to detect peanut allergen residue in processed food products. *European Food Research&Technology*. **2008**; 227: 857-869.

negatively affecting the consistency of the product and at the same time keeping the production process reproducible.

Besides we had to develop such homogenizing methods that are able to ensure the homogeneous distribution of milk, egg and gliadin proteins added to the sample matrix in low concentration levels. In the case of milk and egg proteins we accomplished this goal with powder mixing, while in case of gliadin- after choosing the best gliadin isolate experimentally- gliadin was blended in a powder mixture as an ethanol solution.

As a result of the raw-material development and the homogenizing experiments we established production protocols for the preparation of milk, egg and gliadin reference materials.

3.2. Characterization of the Reference Materials and Their Production Process

For evaluating the reliability of the reference material production, homogeneity of the samples and the determination of the uncertainty between different batches (the error of the production of independent batches) are very important. Using these two factors it is possible to estimate the random error originated from the application of the sample matrix. To reach this goal we created an experimental design for each model product making 3-3 parallel batches according to the previously mentioned production protocols. The applied concentration levels were the following:

- Milk: 0, 100 ppm milk powder
- Egg: 0, 1000 ppm egg powder
- Gluten: 0, 10, 50 ppm gliadin

The samples were analyzed multiple times by ELISA method. The analytical data show that the distribution of the analyte within the developed reference materials is homogeneous both within and between batches implying that standardization of the production procedure is feasible. Besides, the data makes it possible to estimate the error of the application of the model products in case of a certain ELISA method.

3.3. INVESTIGATION OF THE PERFORMANCE CHARACTERISTICS OF THE ELISA METHODS USING THE REFERENCE MATERIALS

For the further steps of the work, the reference materials made it possible to compare the performance of the currently used analytical methodology on a more correct scientific basis and to interpret –at least on a hypothetical level- the physico-chemical phenomena behind the obtained results. Besides, it also became possible to estimate the individual analytical uncertainty, the systematic and random errors of the applied methods and to identify their sources. For this we studied the accuracy and precision (repeatability and reproducibility) of the applied methods.

We estimated **accuracy** by calculating the recovery of the analytes. The data indicated that recovery values did not reach the nominal value and that recovery obtained from the baked cookies was lower than those in the mixture of raw materials containing the proteins in their native form. Recovery values are influenced by several factors, e.g. the method of calculating the theoretical concentration of the samples, food processing and the applied analytical method.

To determine **repeatability** of the methods, we used certain units of the ELISA calibrator standard solutions as samples (together with the reference materials) and analyzed their allergenic protein content by ELISA. The investigation of the standard deviation (SD) of the data showed that SD values of the samples from any step of the reference material production process (powder mixture of raw materials, raw dough, cookie) is comparable with the SD values of the standard solutions, or in certain case, even lower. In one hand, it means that the homogeneity of our reference material is appropriate; on the other hand it proves that the main source of the uncertainty of the analytical data is the method itself. Thus, during the production of the model products and the implementation of the measurements such random errors did not occur that would increase significantly the uncertainty of the results. It is important to note that the SD values of the standard solution show a dependence on concentration, the SD values are growing with increasing concentration.

The comparison of repeatability and **reproducibility**, in case of milk and gliadin ELISAs the results turned out to be as expected, SD values of reproducibility were higher than those of repeatability, the uncertainty of the data increased. As for the rest of the data, the SD values did not differ significantly or the reproducibility SD was lower. This is partly caused by the reliability of the methods and by the applicability of the developed materials.

3.4. Comparative Study of Commercially Available Gluten ELISA Kits

With the help of the gliadin reference material we had the chance to carry out a comparative study of seven commercially available ELISA kits designed for the quantification of gluten. The novelty of this experiment is the application of a reference material made of a realistic food matrix. This is advantageous for designing such experiments that are able to characterize the applied methods and to compare their performance by estimating random errors with a real food matrix, this way modelling the daily analytical routine. Complex evaluation of the data is helpful for identifying the critical points of immunoanalytical methods and the extent of errors caused by them. (In case of milk and egg ELISAs we could not afford to implement such a study.)

The data of this study showed that the results obtained by measuring a certain sample varied considerably among the kits which can be a significant food safety risk. The variability of the measured gliadin concentrations is mainly caused by the application of different antibodies and extractions methods and the possible difference between the protein composition of the reference material and that of the calibrators of the kits. The latter highlights the lack of standardized calibrating materials and the problem of their production (e.g. using protein extracts of more complex materials, like flours). It is also observable that random errors of the methods do not differ significantly. Thus, the differences of antibodies and sample preparation processes affect mostly the recovery values (the accuracy of the methods). This fact would not have been identifiable without the reference material.

In case of our research this picture becomes more complicated because another factor must be taken into account, namely the quantification of gliadin in a processed food matrix and the effect of processing on the analytical data.

3.5. Effects of Food Processing on the Analytical Results

Most foodstuffs are consumed in a processed form, thus it is important to obtain information on the effects of processing on the proteins triggering the hypersensitivity reactions and to find out how they influence the analytical results. These data are also important for the development of reference materials in a processed matrix and for method validation as well.

For the related experiments, we took samples from all steps of the reference material production (raw material mixture, dough, and cookie); this way we created the possibility to study the effects of certain processing steps on the analytical data. We

found that the measurable allergenic protein content in the dough is lower than that of the mixture of raw materials which corresponds to the diluting effect of margarine and water added to the latter. Thus, dough formation does not affect the measurable allergen content. However, the measured allergenic protein concentration of the cookies were significantly lower that is definitely a result of heat treatment. It can be concluded that heat treatment has a significant influence on the results provided by the immunoanalytical methods which should be carefully taken into account during method development. Basically, heat treatment can affect the analytical results in two ways. In one hand, protein modifications can change the solubility of the proteins, damaging the efficiency of the extraction. On the other hand, changes of the protein structure can reduce the affinity of the proteins towards the antibodies affecting the analytical detectability. Besides, the interaction of these effects can also occur. However, the background of these phenomena are not properly described, mostly there are only hypotheses available. Understanding the affecting factors requires molecular level studies.

3.6. CONCLUSION

With the development and application of the new reference materials, it became possible to estimate the errors of the ELISA methods by comparing the data to a known nominal concentration. This error indicates the limitation of the applicability of the ELISA method. The current legislation, which does not even provide uncertainty limits for the existing threshold levels, ignores this limitation. This way, food manufacturers have to face such requirements that cannot be justified with the current methodology. It calls the attention to the necessity of harmonization in the field of analytical method development.

4. THESES

1. We developed three reference materials containing milk, egg and gliadin proteins in known amount in a processed food matrix (2, 3).
2. We standardized the production procedure of the reference materials and created a production protocol to each of them. We determined the analytical uncertainty originated from the production process (3).
3. With the application of the reference materials we determined certain performance characteristics of the applied ELISA methods. Applying realistic food matrices is a

step forward compared to the previously applied unprocessed model matrices. With the help of the new materials, performance characteristics of the currently available ELISA methods can be determined more precisely, just like the analytical uncertainty of the methods and the phenomena occurring during the analysis of real food samples can be studied more effectively (1, 3).

4. The reference materials made it possible to carry out a comparative study of commercially available gluten ELISA kits that was implemented with a reference material in a realistic food matrix for the first time. We observed that the results of the analysis of a certain sample varied among the kits. During this experiment we identified several factors that can affect the analytical results (e.g. applied antibody, extraction method). We also found that these factors are mostly influencing the accuracy of the measurement and not its precision (1).
5. We studied the effects of heat treatment on the analytical results with the help of reference materials for the first time. We found that heat treatment reduces the measurable allergenic protein content. Thus, heat treatment influences the solubility and immunoaffinity of the proteins (or both) through changing their structure (1, 3, 4).
6. During this work we determined the analytical errors of the applied ELISA kits which provide a possibility for the harmonization of the legislation and the analytical performance. Acceptance of this harmonization would improve the handling of the related food safety problems for all stakeholders (1).

5. APPLICATION POSSIBILITIES

The developed reference materials can be used as standard materials for method development and method validation. Besides, as the analyte went through all steps of food processing, errors of the ELISA methods can be modelled more reliably using these materials and the limitations of the methodology can be estimated more precisely. This information can be used for refining official thresholds and for determining the future trends of analytical method development. With the application of the reference materials, factors causing the variability of the analytical data can be identified better (e.g. target molecules, antibodies, calibrators, effects of processing) that could be useful for improving the methodology. Improving the analytical methods together with the improvement of the results of clinical research could result in a more precise allergen policy helping the work of food manufacturers, this way enhancing the safety of consumers and widening the range of the products available for them.

6. PUBLICATIONS

Publications related to the Ph.D. thesis:

1. Bugyi Zs, Török K, Hajas L, Adonyi Zs, Popping B, Tömösközi S. Comparative study of commercially available gluten ELISA kits using an incurred reference material. *Quality Assurance and Safety of Crops&Foods*. (accepted, to be released: first issue of **2013**) (IF (2011): 0,642)

2. Bugyi Zs, Török K, Hajas L, Adonyi Zs, Diaz-Amigo C, Popping B, Poms R, Kerbach S, Tömösközi S. Development of incurred reference material for improving conditions of gluten quantification. *Journal of AOAC International.* **2012**; 95 (2): 382-387. (IF: 1,199)

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Other publications:

- Bugyi Zs, Török K, Hajas L, Tömösközi S. Egy mindennapi élelmiszer-alapanyag, a búza. Barát vagy ellenség? *Élelmiszer Tudomány Technológia*. **2012**; 4: 5-9.
- Török K, Bugyi Zs, Hajas L, Adonyi Zs, Tömösközi S. Az élelmiszerallergének mérésének lehetőségei ma- kihívások, megoldások, a fejlesztés irányai. *Élelmiszervizsgálati Közlemények*. **2011**; 57 (2): 83-91. (IF: 0,040)
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- Tömösközi S, Bugyi Zs. Harmonizációs törekvések az élelmiszer-analitikában. Nemzetközi együttműködés a MoniQA Kiválóság-hálózat keretében. *Élelmiszer Tudomány Technológia*. **2010**; 2: 32-33.

Oral presentations:

10th European Cereal Scientists and Technologists Workshop, Helsinki, Finland, 23-25 May 2011

Zsuzsanna Bugyi, Kitti Török, Lívia Hajas, Zsanett Adonyi, Sándor Tömösközi: Development of incurred reference material for gluten quantification

340th Colloquium of Central Food Research Institute, Budapest, 24 September 2010

Bugyi Zsuzsanna, Török Kitti, Hajas Lívia, Adonyi Zsanett, Tömösközi Sándor: Allergénanalitikai módszerek fejlesztése és érvényesítése- K+F együttműködés nemzetközi kiválóság-hálózatban

2nd MoniQA International Conference, "Emerging and persisting food hazards: Analytical challenges and socio-economic impact", Krakow, Poland, 8-10 June 2010

Poster competition presentation: Zsuzsanna Bugyi, Kitti Török, Lívia Hajas, Zsanett Adonyi, Sándor Tömösközi: Development of reference material for gluten quantification (Best poster award)

9th Young European Cereal Scientists and Technologists Workshop, Budapest, Hungary, 25-27 May 2010

Zsuzsanna Bugyi, Kitti Török, Lívia Hajas, Zsanett Adonyi, Sándor Tömösközi: Quality assurance in gluten analysis- Development of reference material for gluten quantification

8th Young European Cereal Scientists and Technologists Workshop, University of Tuscia, Viterbo, Italy, 3-5 August 2009

Zsuzsanna Bugyi, Sándor Tömösközi: Determination of allergenic proteins in wheat flour based model systems- a scientific cooperation within MoniQA Network of Excellence

Posters:

14th ICC Cereal and Bread Congress and Forum on Fats&Oils, Beijing, China, 6-9 August 2012

Zsuzsanna Bugyi, Kitti Török, Lívia Hajas, Tamás Langó, Sándor Tömösközi: Improving the conditions of analytical methodology for the quantification of proteins responsible for hypersensitivity reactions triggered by wheat (Best poster award nyertes)

2nd MoniQA International Conference, "Emerging and persisting food hazards: Analytical challenges and socio-economic impact", Krakow, Poland, 8-10 June 2010

Zsuzsanna Bugyi, Kitti Török, Lívia Hajas, Zsanett Adonyi, Sándor Tömösközi: Development of reference material for gluten quantification (Best poster award nyertes)

6th Workshop on Food Allergen Methodologies, Toronto, Canada, 9-12 May 2010

Zsuzsanna Bugyi, Kitti Török, Lívia Hajas, Zsanett Adonyi, Sándor Tömösközi PhD: Development of reference material for gluten quantification

Conference for PhD students, Budapest, 4 February 2010

Zsuzsanna Bugyi, Sándor Tömösközi, Judit Nagy, Kitti Török, Lívia Hajas: Determination of allergenic proteins in wheat flour based food model systems-a scientific cooperation within MoniQA Network of Excellence

4th International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, 4-6 November 2009

Zsuzsanna Bugyi, Sándor Tömösközi, Judit Nagy, Kitti Török, Lívia Hajas: Determination of allergenic proteins in wheat flour based food model systems-a scientific cooperation within MoniQA Network of Excellence

10th International Gluten Workshop, Clermont-Ferrand, France, 7-9 September 2009

Zsuzsanna Bugyi, Annamária Kovács, Zsuzsa Őri, Sándor Tömösközi: Study of effects influence the results of allergen determination in wheat flour based model system

Conference for PhD students, Budapest, 4 February 2009

Bugyi Zsuzsanna, Nagy Judit, Tömösközi Sándor: Allergén fehérjék vizsgálata élelmiszer modellrendszerekben