

Standardization in allergen determination

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Abstract An overview is given of sensitization and allergic reaction on food, along with minimal eliciting doses for selected allergens (milk, egg, peanut), followed by the legislative situation in the European Community, the USA, Canada, Australia/New Zealand and Japan. The main focus of the paper discusses methods for food allergen determination (chromatography, DNA-based, antibody-based) and their limitations. Special emphasis is given on antibody-based systems, which are at the moment the most advanced and validated methods available. Examples are given of the current status of standardization for egg, milk, nuts, prolamins, glutelins and for clinical validated reference samples from the EU project EuroPrevall. Recommendations for ongoing activities are also included.

Keywords Allergen · ELISA · Standardization · Calibrant · Reference materials

Sensitization and allergenic reaction

Eating is not only necessary to sustain life, but it also enhances the quality of life for most individuals. The availability of a wide variety of food with different textures and flavours leads to an enjoyable experience for consumers. Unfortunately, some individuals react in an adverse, immune-mediated way to certain foods. The reactions can range from mild symptoms to life-threatening

anaphylactic shock. Although the reason for sensitization of certain individuals is not clearly understood, the immunological mechanisms of an allergic reaction are well-characterized and could be divided into immunoglobulin E (IgE)-mediated (immediate) reactions and cell-mediated (slow) reactions.

Under normal conditions, IgE is involved in protecting the body from parasitic infections. For unknown reasons, environmental antigens (proteins) from pollen, dust mites or food induce binding of specific IgE to mast cells at the first contact (sensitization). When the same antigen enters the body again, these sensitized mast cells will bind the antigen on their surface IgEs causing degranulation and releasing mediators such as histamine, which are responsible for the immediate effects of allergic reactions [1].

Cell-mediated allergic reactions appear after 6–24 h and the underlying mechanisms are not nearly as well understood. The best described example for this kind of reaction is coeliac disease. This is characterized by severe damage of epithelial cells in the small intestine, which is caused by the prolamins and glutenin fraction of proteins from wheat, rye and barley. Peptides, released from these proteins, enter intestinal epithelial cells and are deamidated by transglutaminases, enhancing T-cell reactivity. Subsequently, atrophy of villi is observed, nutrients are not properly absorbed and, along with the ongoing inflammatory process, severe malabsorption characterized by diarrhoea, anaemia and muscle cramps occurs [2].

Minimal eliciting doses

The amount of an allergen required to provoke an allergic reaction is dependent on the patient, the day, the cultivar and the processing stage of the food. As a consequence,

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Table 1 Lowest provoking doses for peanut, egg and milk (given as mass of protein or food) determined by double-blind placebo-controlled food challenge (DBPCFC) in varying numbers of patients (data selected and re-arranged from [3])

Allergen	Food	Lowest provoking dose		Patients (<i>n</i>)
		Mass of protein (mg)	Mass of food	
Peanut	Ground peanut	0.25	1 mg	74
	Peanut flour	2	4.3 mg	14
	Ground peanut	40	160 mg	5
	Ground peanut	66	265 mg	9
	Peanut butter	100	400 mg	10
Egg	Whole egg (raw)	0.13	1 mg	38
	Egg white	0.2	2 mg	19
	Whole egg (raw)	0.65	5 mg	7
	Egg white	26.5	265 mg	8
	Whole egg powder	200	400 mg	25
Milk (cow)	Milk formula	1.5	0.1 mL	56
	Milk	15	0.5 mL	31
	Milk	30	1 mL	6
	Non-fat dry milk	67	2 mL	66
	Non-fat dry milk	140	400 mg	21
	Milk	180	5000 mg	3

only a range of allergen concentrations can be given. A test that minimizes the above-mentioned influences is known as the double-blind placebo-controlled food challenge (DBPCFC). Using this approach, the minimal eliciting dose is determined, not only for one person but within a group of patients over a range of doses (distribution). Table 1 gives some examples from the literature for peanut, egg and milk using different numbers of patients, showing a broad range of minimal eliciting doses on comparing different experiments [3].

Crevel et al. [4], however, highlighted that there are different protocols used for DBPCFCs and recommended a protocol, which is already in use within the European Community-funded Integrated Project EuroPrevall. Using this standardized protocol a basis for further calculations is created. Madsen et al. [5] described three possible approaches and gave strengths and weaknesses for each approach. The most promising seems to be the probabilistic approach (Fig. 1) first described by Spanjersberg et al. [6]. This methodology can be used to estimate the proportion of an allergic population that may suffer from a reaction due to the presence of a certain level of (undeclared) allergen in a food product [5].

The main problem in setting a dose for all consumers is to try to find a compromise between the percentage of reacting patients and the concentration occurring in processed food. A risk of 0% is only possible when the industry is able to produce food “free of allergens”. This is either not possible or the associated costs are far too expensive for customers; therefore, legislative authorities need to hazard the consequences of this residual risk.

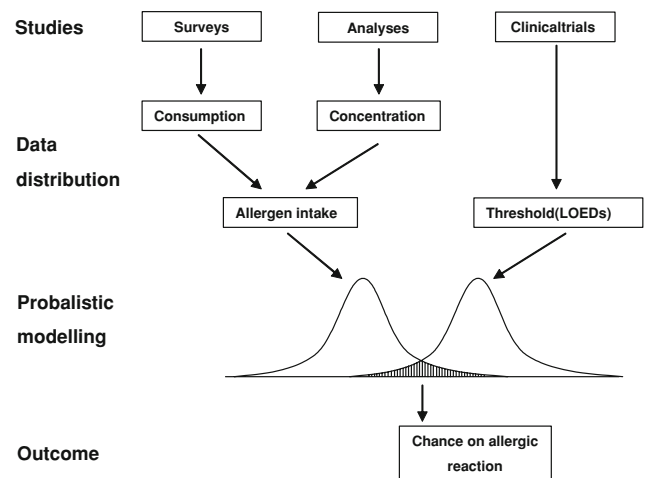


Fig. 1 Schematic presentation of the probabilistic approach in food allergen risk assessment [6]

Regulations and threshold levels

Legislative authorities worldwide ordered that producers declare possible allergens, deliberately added to food according to a recipe. Within the European Union, the legislation on allergen labelling is based on the Directive 2000/13/EC as amended by Directives 2003/89/EC and 2007/68/EC. These directives established a list of 14 food ingredients (Annex IIIa), which must be labelled on food-stuffs, as they are likely to cause adverse reactions in susceptible individuals. Within the USA, the Food Allergen Labelling and Consumer Protection Act (Title II of public law No. 108-282) also provides a list of nine major

Table 2 Legislation in allergen and gluten declaration within the European Union, Canada, Japan, Australia/New Zealand and the USA

European Union ^a	Canada	USA	Australia/NZ	Japan
Egg	Egg	Egg	Egg	Egg
Milk	Milk	Milk	Milk	Milk
Peanuts	Peanuts	Peanuts	Peanuts	Peanuts
9 Nuts ^b	9 Nuts ^b	Tree nuts	Tree nuts	Wheat
Fish	Fish	Fish	Fish	Buckwheat
Soybeans	Soybeans	Soybeans	Soybeans	Recommended: abalone, squid
Crustaceans	Crustaceans	Crustaceans	Crustaceans	shrimp/prawn, crab salmon roe,
Molluscs	Shellfish	Shellfish	Sesame seeds	salmon mackerel, chicken,
Sesame seeds	Sesame seeds	Wheat	Sulphur dioxide	pork, beef, peaches, oranges,
Sulphur dioxide	Sulphur dioxide			apples, kiwifruit, tree nuts,
Mustard	Wheat ^c			soybeans, mushrooms,
Celery				yams, gelatine
Lupine				
Cereals containing gluten	Cereals containing gluten		Cereals containing gluten	
Gluten declaration with thresholds				

^a Exceptions are given in the Directive 2007/68/EC

^b Almonds, Brazil nuts, cashews, hazelnuts, macadamia nuts, pecans, pistachios, walnuts, pine nuts (Canada only), Queensland nuts (EU only)

^c Including kamut, spelt and triticale

allergens. Labelling requirements for foods in Canada are described in an official regulation (vLex 41976834), which contains a list of 12 allergens. Within Australia and New Zealand the Standard 1.2.3. (Australia New Zealand Food Standards Code) contains a comparable list. The food sanitation law in Japan differentiates between 5 mandatory and 19 recommended allergens to be labelled. Table 2 gives an overview on the food ingredients regulated in the different countries.

In contrast, small traces of an allergen at the mg/kg-level are often the result of a contamination during food processing and are not addressed by the legislation. Because they are not apparent to consumers (e.g. peanut in cocoa), this lack of guidance is a genuine risk for consumers. Immune-mediated reactions do not discriminate between regulated and non-regulated allergens.

Threshold levels have been discussed [7–9], but no levels have been given in the above-mentioned regulations so far (except for sulphite with a limit of 10 mg/kg). As a result, the food industries have been subjected to increased scrutiny of allergen controls and are required to measure to as sensitive levels as possible.

Within Australia and New Zealand, a new approach, called the VITAL concept (voluntary incidental trace allergen labelling) is voluntarily used by some food producers [10]. This concept includes two transition points, which are based on the lowest observed adverse effect levels (LOAEL) from literature (Table 3). Action level 1

means that no precautionary labelling is required, whilst level 2 requires a statement (“may be present: xxx”). At level 3, the allergen needs to be listed as an ingredient. This is only valid if the allergen is dispersed within the food. In contrast, if the allergen could be present in a particulate form, the “may be present” statement irrespective of the amount should be used. The only problem using this approach is the determination of the total protein of the allergenic ingredient, since no method is given and analytical testing should be used for validation purposes only.

In the case of coeliac disease, threshold levels for gluten are proposed and now accepted by the Codex Alimentarius Standard 118-1979 [11] and are adopted within the European Community by the Directive 2009/41/EC. Up to 20 mg/kg could be labelled as “gluten-free”, whereas levels below 100 mg/kg may bear the term “very low gluten”. Within the USA, an FDA Working Group published several approaches and is seeking public comments on the paperwork and regulatory burdens of an “experimental study”, which will collect information from consumers who have and do not have coeliac disease [8, 9].

Food matrices and processing

Determination of allergens in food involves testing a wide range of highly complex matrices even for one allergen, e.g. the food industry asks for casein measurement in

Table 3 Transition points (expressed as mass of total protein) used in the determination of the VITAL action levels

Allergen	Transition point 1 (action level 1–2) Total protein (mg)	Transition point 2 (action level 2–3) Total protein (mg)
Milk	0.25	2.5
Egg	0.1	1
Soy	0.5	5
Fish	1	10
Peanuts	0.1	1
Sesame seeds	0.1	1
Tree nuts	0.1	1
Crustacea	0.1	1

bakery goods, chocolate, sausages, ice cream and wine. The concentration of the allergenic part ranges from trace levels (mg/kg) up to main ingredient levels (g/kg or more). In contrast to chemical substances, food allergens are in most cases a mixture of proteins, which can vary in amount and composition due to the region of origin, variety, growth, harvesting, transport, storage and processing during industrial or home preparation. For example, apple cultivars show a great variety in their allergenic reaction in DBPCFC studies [12], with a 30-fold difference between the varieties Golden Delicious and Santana. Whilst celery contains the heat-sensitive allergenic protein Api g1 and a heat-stable profilin protein, carrots for e.g. will be no more allergenic (IgE-mediated) after heat treatment [13]. The allergenic potential of peanuts is only slightly influenced or even more pronounced by roasting [14], and the coeliac toxic potential of wheat is not decreased by hydrolysis of the proteins to small peptides [15]. Mills and Mackie [16] hypothesized that the matrix itself and processing procedures may be responsible for the apparent lability differences when comparing homologues from the Bet v 1 superfamily (e.g. apple and celery).

At every point of the food chain, contamination with allergens is possible, e.g. traces of wheat in oats can be caused by crop rotation carried out every year in a particular area or peanut contamination in chocolate can occur due to difficult or incomplete cleaning procedures in the line of production.

Methods for allergen determination

When the legislative directives recommended labelling the presence of allergens in food, methods to control the content of allergens were under progress and many systems were already in place. All such methods are mainly based on the detection of specific markers that allow extrapolation for the

presence of the allergenic food of interest [17]. The methods can be divided into three groups based on their different measurement principles.

Chromatography

Chromatographic methods are based on a physical separation step of different proteins based on their size, charge and hydrophobicity. Depending on these properties, columns with ion-exchange, size-exclusion or (in most cases) reversed-phase (RP) materials are used. After separation, proteins are detected by their UV absorption or in a mass-specific detector (mass spectrometer or matrix-assisted laser desorption time of flight device, MALDI-TOF). A well-characterized and simple system is the RP-HPLC determination of lysozyme [18], where only one protein needs to be separated from interfering substances and the calibrant is easy to prepare. This situation is quite unique for allergens, because normally an allergenic protein mixture will be separated into single proteins and several peaks occur in the chromatogram. Further peaks will appear, if modified proteins (by food processing or extraction prior to analysis) are sufficiently distinct to be separated. Therefore, as for all methods discussed in this study, the standardization of the calibrants requires special emphasis.

Even if set up properly, chromatographic methods are time-consuming, expensive, less sensitive and should only be used by experts. Nevertheless, chromatographic methods combined with a mass-spectrometric detection seem to be perfectly suitable for confirmation, since they are also able to detect the allergenic part of the protein.

DNA-based methods

Real-time polymerase chain reaction (PCR) is based on the amplification and on-line detection of specific DNA segments using specific DNA primers and a heat-stable DNA polymerase. Since it is an exponential process, it takes some cycles until a signal is detected, which means that the final result is dependent on the amount of DNA at the beginning. Unfortunately, the DNA content is the variable when allergens are determined by PCR in a quantitative way and the extraction from food is highly dependent on the food matrix. Therefore, attempts were undertaken to standardize the real-time PCR using a competitive principle by insertion of a competitive DNA calibrant before DNA extraction. Using sequence-specific fluorescence probes, it is possible to detect and measure peanut in chocolate at amounts of 2 mg/kg (Holzhauser, personal communication). The fundamental problem in using the PCR in a quantitative approach is that the DNA is measured and not the allergenic protein. Since DNA is much more stable than a protein and there is no steady ratio

between DNA and protein content, the information from PCR methodology needs confirmation of the protein content. Furthermore, the DNA extraction process cannot be checked for recovery (except for the laborious competitive proposal), since it is nearly impossible to spike food with DNA. In conclusion, DNA-based methods are suitable for confirmation, but without further standardization not suitable for quantitative approaches.

Antibody-based methods

For antibody-based methods, suitable extraction methods are necessary in addition to well-characterized antibodies/antisera and calibrants.

Raising antibodies for use in antibody-based systems requires a suitably defined immunogen. It is possible to immunize using the whole protein isolated from the allergenic food or with a specific protein or a peptide sequence out of this protein. Depending on the later application, the immunogen can be used to inoculate rabbits, goats or sheep to produce polyclonal antisera or in mice to produce monoclonal antibodies (mAb). The latter has the advantage of permanent availability in (nearly) unlimited amounts, but development is tedious and more or less directed against one epitope. From a practical point of view, this means that a minimum of one mAb is produced for one protein. In case of egg, patients could be allergic to the egg white proteins, ovalbumin, ovomucoid, ovotransferrin and lysozyme, all of which show different stabilities against heat treatment [19]. Since legislative authorities require manufacturers to declare that a product contains “egg”, it is difficult to set up a method based only on a single mAb. On the other hand, mAbs are superior when only a single reactivity is sufficient, as in the case of coeliac disease, where the Codex Alimentarius [11] agreed that the mAb “R5” directed against the potentially toxic peptide sequence QQPFP and related structures [20, 21] should be used. This R5-ELISA is one of the rare examples where the antibody recognizes the potentially toxic (“related-to-causes”) epitope. In most cases, ELISAs are developed to more or less stable proteins and only some of them lead to reactions with IgE of sensitized patients. Overall, ELISAs based on mAbs are more susceptible to matrix effects because of the lower complexity in protein-specific recognition sites. False-negative results occur if the target protein or target epitope is affected by food processing or because other proteins that are not detected are important allergens for a subgroup of patients [17]. Irrespective of whether monoclonal or polyclonal, the antibodies used in an ELISA are (for practical reasons) not raised against all possible variants of an allergen, so that various antibodies used in non-commercial or commercial test kits give varying immune responses.

As mentioned above, analysts are confronted with a tremendous number of all kinds of matrices in the liquid, paste, solid or powdered form. Furthermore, recent food production processes utilize physical processes such as heating, high pressure, microparticulation, ultrafiltration and irradiation, as well as chemical processes such as proteolysis, fermentation and refining by extraction. In summary, these facts influence the success of an allergen extraction prior to determination. It is quite simple to solubilize and therefore extract proteins from nearly every matrix by harsh methods, for example, by using 6 mol/L urea or under reducing condition using a high salt content, but these extraction buffers will degrade protein structures and therefore influence the antigen–antibody binding. Depending on the analyte, an extraction could be very specific as in the case of gliadin. These prolamins are soluble in 60% of aqueous ethanol (volume fraction) and a special extraction solvent such as the “cocktail solution” introduced by Garcia et al. [22] or the comparable cross-validated “extraction solution” (R 7099, R-Biopharm AG) are able to extract prolamins from heat-treated samples in a quantitative manner without disturbing the ELISA results.

Until now, researchers or test kit manufacturers have prepared their own standards, which results in increased variability. Commercial test kit suppliers will always try to minimize the effects of different calibration standard lots by using characterized samples to check the quality for every lot and, therefore, to assure the quality over a long period. The greatest problem in each case is the kind of material used for standardization. Depending on the antibody, one manufacturer, e.g. uses an extract of roasted peanuts for calibration. This means that the amount of roasted peanut protein in a sample will be detected correctly, but detection of raw peanut protein may be incorrect. Customers must therefore be provided with a description of the antibody, the calibrant supplied in the kit and a detailed validation report. In this way, they can compare tests from different manufacturers and decide on the test that is best for their purposes.

As a consequence of inadequate information, customers often carry out internal validations and try to evaluate test kits using their own spiking procedures. Within the scientific community, there are two main opinions on how to spike a sample. One group prefers to imitate a contaminated food as realistically as possible and perform the spiking experiment during the production of the food under controlled (allergen-free) conditions. Since the spike passes through the whole processing stage, a change of allergenicity and antigenicity may occur. For example, a preparation of egg-containing noodles will start with the addition of raw whole egg to wheat and other ingredients. During processing, the proteins from egg white will denature for an unknown part and the result of the spiking

experiment seems to be a “loss” of the spike. The apparent “bad” performance of the test kit is only observed when a comparison of added raw whole egg and processed measured egg in the noodles is undertaken. The other group prefers to spike a processed food sample with a characterized spiking solution before extraction. In this case, it is clear that the spike is not modified and the influence of matrix and extraction procedure on the recovery can be determined. Additionally, it is possible to spike with modified allergens prepared under defined conditions. Different spike solutions (e.g. cooked egg white extract, diluted raw egg white or single egg white proteins) can be investigated in a short time to characterize the limits of the system.

Nevertheless, antibody-based ELISA systems are routinely used and seem to be the most robust assays at the moment. As with all quantitative analytical systems, an ELISA should be extensively characterized using a number of validated parameters such as specificity, precision, recovery, robustness and sensitivity. The suitability of several commercial ELISA systems has been demonstrated in collaborative studies and the boundaries for each system defined.

Whereas an ELISA gives a quantitative result, immunochromatographic methods such as lateral-flow devices (LFD) lead to qualitative “yes or no” results with a certain cut-off value of the method. In brief, extracted allergens will react at the start point of the strip with, e.g. a specific gold-labelled antibody. A pad on the top of the strip will produce a permanent flow trough the strip. In the reaction zone, another specific antibody is immobilized on the surface and “captures” the allergen–antibody (gold) conjugate. Above a specific cut-off level, a coloured line will occur, if there was an allergen in the sample. The run time is only a few minutes and the technique is perfectly suited for use in the production line and for hygiene control. If set up properly, the system is excellent but often needs some additional internal validation. The matrix and the buffer used can have a high influence on the extraction, which can lead to false-negative results; furthermore, a high dose effect can occur, which leads to false-negative results at high concentrations.

Surface plasmon resonance (SPR) technology uses ultrathin gold layers on a chip to which antibodies are linked covalently via thiolate bonds. During the real-time studies, analytes (proteins) are dissolved in buffer and flow over the surface, whilst SPR spectroscopy is used to measure the changes in refractive indices occurring at the metal surface on interaction between the two specific partners. The SPR phenomenon occurs when a beam of polarized light strikes the surface at a given angle. The intensity of the reflected light is reduced if a change of “mass” at the surface occurs [23]. First attempts on allergen measurement were described [24], but the devices are still expensive.

Examples for the current status concerning standardization

Egg

Legislation all over the world demands the labelling of egg in food. The egg consists of white and yolk, and the most allergenic proteins are included in the egg white, which contains about 10% of protein (mass fraction). Table 4 shows a characterization of the main allergenic proteins in egg white [25]. The food industry uses fresh whole liquid egg, liquid egg white, whole egg powder or egg white powder as ingredients. In most cases, the resulting products are heat-treated and therefore undergoes an unknown shift in their allergenic potential. It is therefore necessary that a method is able to quantify the heat-stable ovomucoid, since it is the only heat-stable protein (Table 4). The main question is which kind of standard should be used. At the moment, commercial test kit suppliers developed their own standard preparations, which are different from each other. Collaborative studies lead to different results depending on the antibody and standards used for calibration. At the moment, two whole egg powder NIST reference materials are or were available (Table 5). RM 8415 (no longer available) was intended for measurement of trace elements and fatty acids. It contains mass fractions of 37.8% protein, 2%

Table 4 Characterization of egg white proteins [25]

		Part of egg white protein ^a (%)	Thermal stability	Sensitization ^b (range) (%)
Ovomucoid	Gal d 1	11	High	70 (40–95)
Ovalbumin	Gal d 2	54	Low	60 (35–90)
Ovotransferrin	Gal d 3	12	Null	30 (20–45)
Lysozyme	Gal d 4	3.5	Null	10 (4–18)
Ovomucin		1.5–3.5		2

^a Mass fraction

^b Percentage of occurrence in patients allergic to egg

Table 5 Overview on possible test materials for standardization of ELISA systems

Allergen	Material	Description	Purpose ^b	Comments
Egg	RM 8415 ^a	Whole egg powder	FA, MMTE, vitamins	γ -radiated, silica aluminates
	RM 8445	Whole egg powder	Allergens	
Milk	RM 8435 ^a	Whole milk powder	FA, MMTE, vitamins	γ -radiated
	BCR-380R	Whole milk powder	CP, fat, ash, lactose	
	BCR-685	Skim milk powder	CP, fat	
	SRM 2387	Peanut butter	FA, aflatoxins, vitamins, elements	Sugar and vegetable oils added
Peanut				
var. Runners	IRMM-481a ^c	Whole peanut powder	Identity	Blanched, strong air-roasted
var. Common natal	IRMM-481b ^c	Whole peanut powder	Identity	Raw, mild air-roasted
var. Virginia	IRMM-481c ^c	Whole peanut powder	Identity	Blanched, strong oil-roasted
var. Virginia	IRMM-481d ^c	Whole peanut powder	Identity	Blanched, mild oil-roasted
var. Jumbo runners	IRMM-481e ^c	Whole peanut powder	Identity	Blanched only
	IRMM-481f ^c	Whole peanut powder	Identity	Mixture of 481a–481e
Prolamin/glutelin	PWG standard ^c	Prolamin fraction from different wheat varieties	Allergens	Accepted within the scientific community
	RM 8418 ^a	Wheat gluten	FA, MMTE	γ -radiated

^a No longer available^b FA fatty acids, CP crude protein, MMTE major, minor and trace elements^c Not a reference material

silica aluminate and is γ -radiated (^{60}Co). It is known from literature that γ -radiation leads to a substantial loss of 25% of tryptophan in lysozyme [26]. Therefore, it is not remarkable that this treatment led to a loss in antigenicity of factor 10 compared to another whole egg reference powder RM 8445 (Fig. 2). This is not an effect of denaturing processes, because heat-treated egg white powder reacts similar to RM 8445 (Fig. 2). The difference between RM 8415 and 8445 is partly based on the addition of silica aluminates, because there is a clear difference of more than factor 2 between a “stirred” and a “non-stirred” standard solution when diluting these solutions before measurement. It could be speculated that silica aluminates adsorb proteins (Fig. 3) and “release” them at higher dilutions. Nevertheless, RM 8415 was used to prepare reference materials incurred (RMI) for food allergens initiated by the Working Group Food Allergens of the EU-funded project Monitoring and Quality Assurance in the Food Supply Chain [27].

RM 8445 is a spray-dried whole egg powder without additives containing 49% protein (mass fraction) and is intended for use in ELISA systems. This is the first opportunity to calibrate all methods with one characterized standard, but one should bear in mind that egg white powder gives other antigenic responses compared to whole egg powder and that the protein content of egg preparations used in the food industry can differ significantly. It is obvious that the measurement of “egg” in all possible variants is a complex intention and difficult to realize.

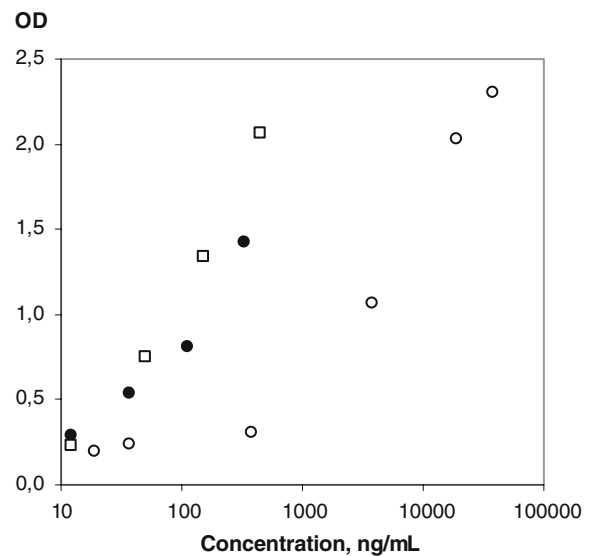


Fig. 2 Calibration curves using two different whole egg powders, RM 8415 (filled circle) and RM 8445 (open circle), in comparison to a heat-treated egg white powder (filled square). The total protein concentration of the standards is given as ng/mL. OD (optical density) was measured at 450 nm. The RIDASCREEN®FAST Ei/Egg system was used

Milk

Milk has to be declared as a food ingredient and consists of the whey fraction (containing β -lactoglobulin as the main allergic component) and the casein fraction (allergenic α , β , γ , κ -caseins). The food industry uses milk, whey,

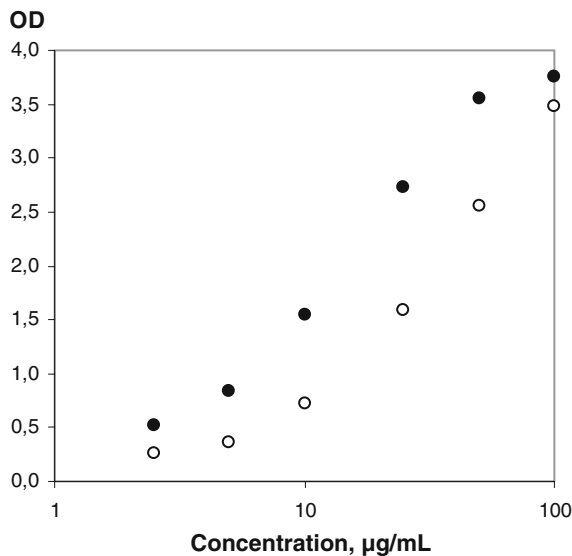


Fig. 3 Calibration curves prepared from γ -radiated RM 8415 (containing 2% silica aluminate) with (filled circle) or without (open circle) stirring the solution before dilution to the final concentrations. The total protein concentration of the standards is given as $\mu\text{g/mL}$. OD (optical density) was measured at 450 nm. The RIDA-SCREEN®FAST Ei/Egg system was used

hydrolysed whey, caseinates and hydrolysed caseinates. Most available ELISAs are directed against β -lactoglobulin or casein, whilst only one is suitable for the determination of total milk (containing β -lactoglobulin and casein antibodies, probably calibrated with a milk powder).

IRMM prepared a whole milk powder (BCR-380R) for determination of crude protein, fat, ash and lactose and a skim milk powder (BCR-685) for crude protein and fat (Table 5).

NIST provided a ^{60}Co -radiated whole milk powder (RM 8435; no longer available) for determination of trace elements, fatty acids and vitamins, which contains a mass fraction of 26% protein. Since γ -radiation is a general problem when using a protein as a standard in antibody-based methods, a better whole milk powder is necessary. This should be characterized for its protein content and composition (β -lactoglobulin and caseins) before it could be used as a standard material in ELISAs for determination of milk, β -lactoglobulin and casein.

Nuts

Tree nuts and peanuts are important allergens all over the world. The protein content of different varieties of hazelnuts can vary [28], whereas the protein content of different peanut varieties are quite similar [29]. Nearly all nuts are roasted at different temperatures and durations, which influence the protein structure and therefore the antigenicity. Proteins from peanuts and hazelnuts denature by

more than 30% after treatment at 140° for 40 min. At a higher temperature (160°C), proteins from peanut will be denatured by more than 80% within 30 min [28, 29]. Comparable results were shown for two different almond cultivars [30].

For this reason, often mixtures of different processed peanut materials and various kinds of peanuts are used (IRMM-481; peanut test material kit) or the assays are calibrated to an NIST peanut butter material (SRM 2387), which was created for the determination of vitamins, fatty acids, aflatoxins, acrylamide and elements (Table 5). It had been chosen as a calibrant within an AOAC Research Institute performance test method study in 2002.

Prolamins and glutelins

Official authorities recommend the labelling of gluten in food. Gluten consists of the alcohol-soluble prolamins and the insoluble glutelin fractions of wheat, rye and barley and it was assumed in the past that there was a fixed ratio of 1:1 between both fractions. The glutelins consist of a low-molecular weight (LMW) and a high-molecular weight (HMW) fraction, which are differently soluble in ethanol after thiol cleavage [31]. Historically, prolamins were seen as coeliac-active proteins. This led to the development of prolamins assays (detecting gliadins, hordeins and secalins) using the Skerit antibody (against ω -prolamin) or the antibody R5 as mentioned above. The Codex Alimentarius accepted the latter assay as a suitable method 1 in 2008, together with the use of the above-mentioned cocktail solution.

In parallel to the development of the R5-ELISA [32], it was recognized that standardization should be performed. Therefore, a mixture of the most frequently used European varieties of wheat was taken to prepare a calibrant consisting mainly of prolamins, which should be established as an official calibrant for antibody-based determinations. This so-called PWG standard was initiated by the WGPAT (Working Group on Prolamin Analysis and Toxicity) in 2000, but until now it has not been possible to establish it as an official material (Table 5). Nevertheless, the preparation is used in the scientific community and could be requested from Martin Stern (University of Tuebingen, Germany) in small amounts for scientific evaluations. This material contains mainly prolamins, and additionally albumins/globulins and few HMWs [33]. The R5 antibody recognizes the prolamins.

The NIST RM 8418 material is a whole wheat gluten standard, radiated with a radioactive source, made from a Canadian Western Spring wheat variety flour with a protein mass fraction of 76.7% (only valid until 31/08/08). The problem is in the selection of the calibrant to be used. Again, each kit supplier produces his own standard and there is no accepted international standard available until now.

In the recent past, it was described that not only the prolamin fraction, but also the glutelin fraction of wheat, rye and barley are toxic to patients with coeliac disease [34, 35]. This led to a discussion for the preparation of a total gluten standard consisting of prolamins and glutelins in their natural ratio [36] and the development of new antibodies since the R5 is not able to react with HMW glutelins (van Eckert, personal communication).

As a further complication, assays are needed for food containing hydrolysed gluten, e.g. beer and starch. The R5 antibody is suitable for the detection of peptides containing the potentially toxic sequence QQPFP and related sequences, but only in a competitive manner because the peptides are too small to have two epitopes required to build a sandwich ELISA. At the moment, the existing assay (Gliadin competitive, R-Biopharm AG) is calibrated using the QQPFP peptide. In the future, a hydrolysed calibrant of a mixture of wheat, rye and barley will be prepared, which will make it possible to recalculate the measured values to the gluten limit values defined in the Codex Alimentarius or the EU legislation (Geßendörfer and Wieser, personal communication).

Clinically validated reference samples (EuroPrevall)

Within the EU-funded project, EuroPrevall (<http://www.europrevall.org>), the recruitment of patients (so-called “cohorts”) is one fundamental issue. The cohorts range from newborns to adults and from regions spanning the various climates of Europe and some other parts of the world. A cornerstone is the preparation and testing of specially prepared meals containing either minute quantities of allergy causing food (peanut, hazelnut, celeriac, apple, peach, egg, milk and fish) or placebo meals without the problematic food in the above-mentioned double-blind placebo-controlled food challenges (DBPCFC). Common food matrices have been developed for diagnosis in older children and adults. These include a chocolate mousse desert formulated by Unilever and manufactured together with Kraft, plus a dark chocolate matrix prepared by Nestlé.

It was the idea to double check these materials in patients and in analytical (antibody-based) systems. Spikes (egg, milk) are therefore checked by commercial test kit suppliers for suitability in their assay systems. A collaborative study will follow and highly characterized reference materials will be available at the end of these trials.

Recommendations

The standardization of systems for allergen determination, especially ELISA systems, is a complex intention, which could be separated in the following single tasks:

- Legislatives should set thresholds for allergenic food ingredients and contaminants, maybe on the basis of the VITAL concept. It should be noted that allergenicity and antigenicity are not the same and that legislation requires the measurement of the food irrespective of its allergenic “status” or potential. This way, both industry and patients will have increased confidence and security.
- Introduction of characterized calibrants that all test kit manufacturers could relate to. Then the results of different ELISAs will be more comparable.
- Introduce analytically and clinically validated samples as are being developed within EuroPrevall; allergenicity and antigenicity would be characterized. Commercial test kit suppliers should publish and actualize validation reports with a special emphasis on the detection of processed food and easier interpretation of results by users would be possible.
- Commercial test kit suppliers should provide spiking protocols.
- In case of PCR, studies should be performed, which show the variability of the ratios between DNA and protein in different processed food, before performing more collaborative studies.
- Chromatographic methods should be encouraged by specialists to show the usability of routine screening of allergens in different processed foods.
- A working group for allergen standards and standardized validation reports should be initiated.

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