

Commercialized rapid immunoanalytical tests for determination of allergenic food proteins: an overview

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Abstract Food allergies have become an important health issue especially in industrialized countries. Undeclared allergenic ingredients or the presence of “hidden” allergens because of contamination during the food production process pose great health risks to sensitised individuals. The EU directive for food labelling lists allergenic foods that have to be declared on food products by the manufacturers. The list includes gluten-containing cereals, crustaceans, eggs, fish, peanuts, soybeans, milk, various nuts (e.g. almond, hazelnut, and walnut, etc.), celery, mustard, sesame seeds, lupin, and molluscs. Reliable methods for detection and quantification of food allergens are needed that can be applied in a fast and easy-to-use manner, are portable, and need only limited technical equipment. This review focuses on the latest developments in food allergen analysis with special emphasis on fast immunoanalytical methods such as rapid enzyme-linked immunosorbent assays (ELISA), lateral-flow immunochromatographic assays (LFA) and dipstick tests. Emerging technologies such as immunochemical microarrays and biosensors are also discussed and their application to food allergen analysis is reviewed. Finally, a comprehensive overview of rapid immunochemical test kits that are currently available commercially is given in tabular form.

Keywords Immunoassay · Strip tests · Microarray · Biosensors · Surface plasmon resonance · Resonance-enhanced absorption

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Introduction

Food allergies have become an important health problem all over the world. Adverse health effects due to allergic reactions to foods or food ingredients occur in about 1–3% of the population and about 4–6% of children (including food intolerances) [1]. Even the intake of minute amounts of food allergens can cause allergic reactions in sensitised individuals. A wide range of allergic symptoms are observed, for example digestive disorder, respiratory symptoms, circulatory symptoms, and skin irritations. However, for some allergic individuals contact with specific food allergens can lead to life-threatening situations (anaphylactic shock). It is important to distinguish between food intolerances and food allergies. Reactions due to food intolerances do not involve the immune system whereas allergies involve an abnormal immunological reaction to a food or certain food ingredient, which provokes the production of allergen-specific IgE antibodies (or IgA and T cells) against a food allergen. In this context, celiac disease, also known as gluten-sensitive enteropathy, is worth a special mention. Gluten (a specific protein fraction of, e.g., wheat and rye) is known to cause damage to the absorptive epithelial cells of the small intestine, leading to malabsorption of nutrients. Although celiac disease is not classified as an allergy, an immunological aspect does exist, which suggests that the disease is at least partly mediated by IgA and cytolytic T Cells [2].

A vast number of food materials have been identified as potentially allergenic; of these only eight account for more than 90% of all food allergies [3]. For an allergic consumer it is particularly important to know the exact composition of foodstuffs, especially regarding the presence of potentially allergenic ingredients or contamination. In order to protect consumers, the European Commission has issued Directive EC 2003/89 as an amendment to EC Directive 2000/13 for

ingredient declarations on foodstuffs [4, 5]. Annex IIIa of this guideline includes a list of food ingredients and products thereof classified as being possible allergens or leading to possible intolerance, e.g. cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk (including lactose), nuts (almond, hazelnut, walnut, cashew, pecan nut, Brazil nut, pistachio nut, macadamia nut, and Queensland nut), celery, mustard, sesame seeds, and sulfur dioxide and sulfites. Directive EC 2003/89 was issued on 11–25–2005 and requires that any of the twelve described potentially allergenic ingredients are labelled even if they make up less than 25% of the food product. In 2006, the list of allergens in Annex IIIa was expanded to include another two food ingredients, namely lupin and molluscs [6].

An additional problem is contamination of originally non-allergenic food products with traces of food allergens through the production process, so-called “hidden” food allergens. As a precaution, food manufacturers often label their food products with “may contain” a certain allergen. This drastically limits the variety of food products suitable for allergic consumers. It is not only in the interest of the allergic consumer, but also in the economic interest of food manufacturers to be able to guarantee the absence of allergens in food products. Therefore, analytical tools are necessary for reliable and accurate detection and quantification of allergens in foodstuffs.

The methods currently used for the detection of potential allergens in foods target either the allergen itself or a marker that indicates the presence of the offending food. Such food markers are usually proteins specific for the investigated allergenic food or characteristic DNA fragments. Poms et al. [3] gives a comprehensive overview of the methods currently used for allergen analysis in foods. Protein-based methods include immunoblotting, rocket immunoelectrophoresis (RIE), radio-allergosorbent test (RAST), enzyme allergosorbent test (EAST), radioimmuno-inhibition assay (RIA), enzyme-linked immunosorbent assay (ELISA), and liquid chromatography–tandem mass spectrometry (LC–MSⁿ). Immunoblotting and RIE only offer qualitative or semi-quantitative results whereas RAST, EAST, ELISA, and LC–MSⁿ are quantitative methods. DNA-based approaches involve the amplification of a specific DNA fragment of the allergen gene or food-specific protein gene through polymerase chain reaction (PCR). The amplified DNA-fragments are usually detected by gel electrophoresis or ELISA. Superior quantitative results can be achieved by employing real-time PCR or PCR–ELISA [3, 7].

The threshold dose above which sensitised individuals show allergic reactions varies widely among individuals and is, therefore, difficult to assess. There is, however, general agreement that the detection limits of analytical methods developed for different food products need to be somewhere between 1 and 100 mg of allergenic protein per

kg food (ppm) [3]. Most commercially available test kits for the detection and quantification of allergens in food products involve ELISA or PCR technology, which already meet this criterion and have limits of detection (LOD) in the range of 0.1 to 10 mg kg⁻¹ (ppm). The declaration of LODs, however, differs between the test kit suppliers and refers either to the allergic protein or to the amount of allergenic food in a certain food product.

Most of the methods developed for allergen analysis in food are time-consuming, labour-intensive, and mostly need expensive laboratory equipment (such as a micro-plate reader or thermocycler) and well-trained people to operate them. Therefore, great emphasis has been put in the development of analytical methods that can be applied in a fast and easy-to-use manner in order to enable legislation, and maybe also consumers themselves, to monitor food products for the presence of allergenic food ingredients or hidden allergens, and to offer manufacturers a tool for on-site check during food production, to avoid cross-contamination.

This review focuses on rapid immunochemical methods for the determination of allergens in food, for example rapid ELISAs using microwells, ELISAs in lateral flow (LF) device or dipstick formats, immunochemical microarrays, and biosensors. Recent developments in both academic and commercial research are highlighted. Special emphasis is given to the major food allergens that are listed in Annex III of the EU food labelling directive [4–6] and on commercial available rapid tests. Sulfur dioxide and sulfite are not included in the discussion, because they are not protein-based food allergens, and are only listed in the EU labelling directive because of their association with asthmatic reactions in sulfite-sensitive individuals.

Rapid ELISA

In the 1980s, ELISA technology had its breakthrough in biochemical and biomedical applications because of its high specificity, sensitivity, and simple sample handling. Its high potential for standardisation and automation enables high sample throughput which is essential for screening purposes. ELISA is currently the most widely used analytical technique for routine measurements and screening of allergens in food. Easy-to-use and more or less rapid test kits have become commercially available for a great variety of food allergens with LODs ranging from 0.05 to 10 mg kg⁻¹, depending of the allergen and the food matrix (Table 1). ELISAs test for the presence of allergens or specific food marker proteins by using specific antibodies that are usually raised in mammals such as mice, rabbits, goats, and sheep. However, antibodies generated in mammals are harvested from blood, which implies frequent bleeding or even killing of the immunised animal. Because

Table 1 Commercially available rapid immunochemical test (November 2008)

Allergenic food	Targets	Test format	LOD (LOQ)	Validation	Testing time	
Cereals with gluten	Gliadin, secalins, hordeins	ELISA	2 (5) ^f		30 min	RIDASCREEN FAST Gliadin (R-Biopharm,)
	Gliadin	LFA	2.5 (only qual.) ^f		5 min	RIDA QUICK Gliadin (R-Biopharm)
	Gluten	LFA	<20 ^{a,f}		< 10 min	RAPID 3-D Gluten test kit (Tepnel,)
	Gliadin	ELISA	<10 (only qual.) ^f		30 min	Alert for Gliadin Allergen (Neogen,)
	Gliadin	ELISA	n/s (5) ^f		30 min	Veratox for Gliadin Allergen (Neogen)
	Gliadin	ELISA	n/s (2.5) ^c		n/s	Gliadin assay (ELISA Systems,)
	Gluten	LFA	10 (only qual.)		10 min	EZ Gluten Test (ELISA Technologies,)
	Gluten	dipstick	10–20 (25) (semiquant.)		6–15 min	HAVen GLUTEN FlowThrough Test (Hallmark,)
Crustaceans	n/s	LFA	5 (only qual.) ^a		<10 min	RAPID 3-D Shellfish test kit (Tepnel)
	Tropomyosin	ELISA	n/s (0.05) ^c		60 min	Crustacean Residue ELISA (ELISA Systems)
Eggs	Egg-white protein (ovalbumin, ovomucoid, ovotransferrin, lysozyme)	ELISA	0.6 (1)		35 min	RIDASCREEN FAST Egg (R-Biopharm)
	Egg white protein	LFA	n/s ^a		<10 min	RAPID 3-D Egg test kit (Tepnel)
	Egg protein	ELISA	<5 (only qual.)		30 min	Alert for Egg Allergen (Neogen)
	Egg protein	ELISA	n/s (2.5)		30 min	Veratox for Egg Allergen (Neogen)
	Egg-white protein	ELISA	n/s (1) ^c		35 min	Egg Residue ELISA (ELISA Systems)
Peanuts	Ara h1	ELISA	0.15 (0.25)	AOAC-RI 2003	30 min	RIDASCREEN FAST Peanut (R-Biopharm)
	Ara h1	LFA	5 (only qual.)		10 min	RIDA QUICK Peanut (R-Biopharm)
	Peanut protein	LFA	1 (only qual.) ^a	JRC-IRMM 2006	<10 min	RAPID 3-D Peanut test kit (Tepnel)
	Peanut protein	LFA	<5 (only qual.)	JRC-IRMM 2006	10 min	Reveal for Peanut Allergen (Neogen)
	Peanut protein	ELISA	<5 (only qual.)		30 min	Alert for Peanut Allergen (Neogen)
	Peanut protein	ELISA	n/s (2.5)	AOAC-RI 2003	30 min	Veratox for Peanut Allergen (Neogen)
	Ara h1, Ara h2 and others	ELISA	n/s (1) ^c		35 min	Peanut Residue ELISA (ELISA Systems)
Soybeans	Soy protein	ELISA	<5 (only qual.)		30 min	Alert for Soy Flour Allergen (Neogen)
	Soy protein	ELISA	n/s (2.5)		30 min	Veratox for Soy Flour Allergen (Neogen)
Milk	Casein	ELISA	0.12 (0.5)		30 min	RIDASCREEN FAST Casein (R-Biopharm)
	Casein	LFA	n/s ^a		< 10 min	RAPID 3-D Casein test kit (Tepnel)
	Casein and whey	LFA	<5 (only qual.) ^d		5 min	Reveal for Total Milk Allergen (Neogen)
	Milk protein	ELISA	<5 (only qual.) ^e		30 min	Alert for Total Milk Allergen (Neogen)
	milk protein	ELISA	n/s (2.5) ^e		30 min	Veratox for Total Milk Allergen (Neogen)
	β-lactoglobulin	ELISA	n/s (0.1)		45 min	Beta Lactoglobulin Residue ELISA (ELISA Systems)
	Casein	ELISA	n/s (1) ^b		45 min	Casein Residue ELISA (ELISA Systems)
Nuts						
Almond	Almond protein	ELISA	0.17 (0.25)		30 min	RIDASCREEN FAST Almond (R-Biopharm)
	Almond protein	LFA	1 (only qual.)		<10 min	RAPID 3-D Almond test kit (Tepnel)
	Almond protein	ELISA	<5 (only qual.)		30 min	Alert for Almond Allergen (Neogen)

Table 1 (continued)

Allergenic food	Targets	Test format	LOD (LOQ)	Validation	Testing time	
	Almond protein	ELISA	n/s (2.5)		30 min	Veratox for Almond Allergen (Neogen)
	Almond protein	ELISA	n/s (0.5)		35 min	Almond Residue ELISA (ELISA Systems)
Hazelnut	Hazelnut protein	ELISA	0.15 (0.25)	BVL 2006	30 min	RIDASCREEN FAST Hazelnut (R-Biopharm)
	Hazelnut protein	LFA	5 (only qual.)		10 min	RIDA QUICK Hazelnut (R-Biopharm)
	Hazelnut protein	LFA	1 (only qual.) ^a		<10 min	RAPID 3-D Hazelnut test kit (Tepnel)
	Hazelnut protein	ELISA	n/s (2.5)		30 min	Veratox for Hazelnut Allergen (Neogen)
	Hazelnut protein	ELISA	n/s (0.5)		35 min	Hazelnut Residue ELISA (ELISA Systems)
Molluscs	n/s	LFA	5 (only qual.) ^a		<10 min	RAPID 3-D Shellfish test kit (Tepnel)

n/s, not specified

LOD/LOQ, limit of detection/quantification in mg kg⁻¹ (ppm) of allergenic food/target in food sample (corresponding to product name), in cases where no LOQ were reported, the lowest concentration level of the control samples supplied for quantification is given

LFD, lateral flow device AOAC-RI, Association of Analytical Chemist-Research Institute, validation according to the Performance Tested Method Program BVL, German Federal Office of Consumer Protection and Food Safety, validation according to the official collection of test methods pursuant to §35 Foods and Other Commodities Act (LMBG) [30]

JRC-IRMM, Interlaboratory study organised by the European Commission Joint Research Centre - Institute for Reference Materials and Measurements [31]

^a Matrix-dependent

^b ppm skim milk

^c ppm tropomyosin

^d ppm total milk

^e ppm non-fat dry milk

^f ppm gliadin/gluten

chickens accumulate antibodies in their eggs, and immunisation is usually better tolerated compared with mammals, the use of antibodies derived from egg yolk is an inexpensive, convenient, and animal-friendly alternative [8–13]. The detection step involves the binding of an enzyme-labelled antibody which subsequently reacts with a specific substrate forming a coloured product. The concentration of the antigen–antibody complex can be determined by measuring the absorbance of the coloured product.

Practically all ELISA formats have found their applications in food analysis. In general, two formats can be distinguished depending on whether the detection principle is based on sandwich or competitive assays [14]. The so-called sandwich ELISA (s-ELISA) involves a capture antibody specific to the protein of interest (allergen or food marker) which is immobilized on a solid phase (e.g. microtiter plate or multiple well strips). The analytes in the sample are captured by the immobilized antibody and detected by a second analyte-specific antibody, which is enzyme-labelled and binds to the protein of interest by forming a “sandwich”. If no second enzyme-labelled analyte-specific antibody exists, it is also possible to use the indirect detection procedure by also using a labelled

species-specific antibody, with the “disadvantage” that both analyte-specific antibodies must be derived from different species. The absorption of the coloured product formed after adding the substrate is directly proportional to the analyte concentration (Fig. 1). Because the antigen has to offer more than one epitope in this format, s-ELISAs are only applicable to large molecules such as proteins. Thus, the sandwich assay is the most common format for allergen detection in food.

The competitive format (c-ELISA) is applicable also to the detection of small analytes. There are more possibilities to run a competitive assay. On the one hand the analyte (antigen) is directly bound to the solid phase. Sera containing the specific enzyme-labelled antibody together with the appropriately diluted sample extract are pre-incubated before addition to the antigen-coated wells. Again if no labelled antibody is available, detection can be performed indirectly. The assay consists of bound analyte, sample in solution with analyte-specific antibody, and another additional incubation step has to follow with a labelled species-specific second antibody. On the other hand the antibody can be bound to the surface and the sample analyte competes with a tracer (= enzyme-labelled

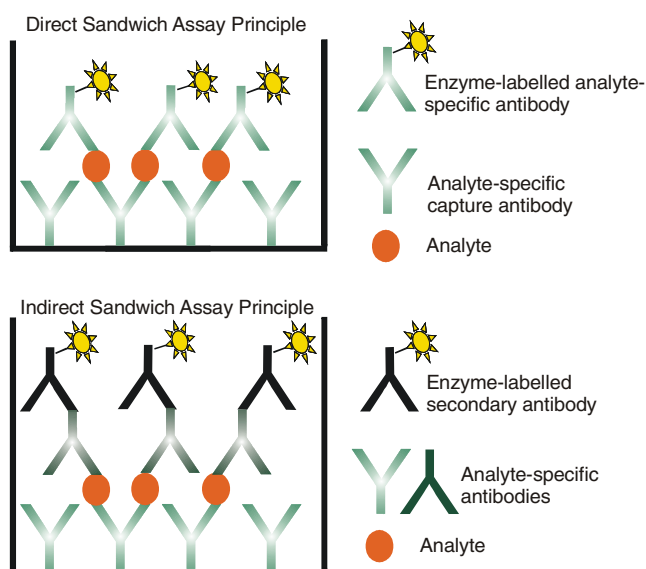


Fig. 1 Sandwich ELISA with direct detection by means of an enzyme-labelled analyte-specific antibody and the indirect sandwich assay with an enzyme-labelled species-specific secondary antibody for detection

analyte). In all three cases the antigen concentration present in the sample is indirectly proportional to the absorbance of the coloured substrate product (Fig. 2).

Rapid ELISA test kits, that produce qualitative and/or semi-quantitative results within 30 and 60 min have been developed and are commercially available for gluten, milk, soybean, peanut, hazelnut, almond, egg, and crustaceans (Table 1). With most of these ELISA kits, quantification is possible in conjunction with a microwell reader.

Lateral-flow assays (LFA) and dipstick tests

LFAs and dipstick tests for allergen detection in food are simplified versions of ELISAs with a membrane strip (usually poly(vinylidene difluoride), Nylon, or nitrocellulose), on which immunoreactants such as antibodies or antigens are applied. LFAs and dipstick tests are inexpensive, rapid, and portable, and do not require technical skill to perform. Both formats enable fast and cost-effective screening of foods because no specific instrumentation, for example microplate reader and washer, is necessary.

The technological basis for the development of immunochemical lateral-flow assays (LFA), also called strip tests, was founded in 1980, when Leuving et al. [15] reported the use of colloidal particles as labels for immunoassays. The first, and one of the most popular, applications for this test format was use of human chorionic gonadotropin (HCG) for the detection of pregnancy [16, 17]. In 1997, Mills et al. [18] developed one of the first dipstick immunoassays used in food allergen analysis for detection

of a peanut allergen (conarachin) in food. During the last decade, academic and industrial research has focused on the development of rapid allergen tests based on LFAs [19, 20] and dipstick tests [21, 22]. This has led to an explosion of rapid allergen test kits since 2003, when only two immunochromatographic allergen tests were commercially available [3]. Currently there are at least fourteen LFAs and dipstick test kits on the market, which enable the detection of the following food allergens with LODs between 1 and 25 mg allergen (allergenic food) per kg foodstuff: nuts (almond, hazelnut), crustaceans (shellfish), gluten (gliadin), peanut, milk (casein), soybean, and egg (Table 1).

LFAs are immunochromatographic tests with a mobile phase that moves the immunoreactants and/or the sample along a test strip. This type of assay satisfies the increasing demand for techniques for field and home use because they are portable, easy to operate, and rapidly produce reliable results within 3–15 min without expensive laboratory equipment and reagents. Although quantification is technically possible by using special strip test readers, commercially available LFAs are usually visually interpreted and give only qualitative or semiquantitative results, i.e. is an allergen present (yes or no) or is the allergen concentration

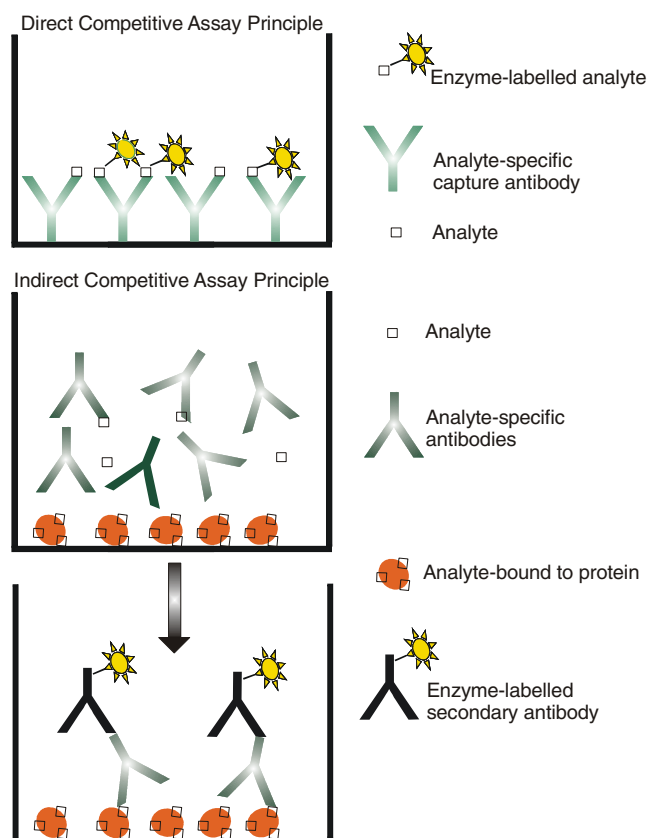


Fig. 2 Two examples of the competitive assay with direct detection by means of an enzyme-labelled tracer and with indirect detection by means of an enzyme-labelled secondary species-specific antibody

high or low. LFAs are commercially available for gliadin, egg, milk, peanut, almond, hazelnut, crustaceans, and molluscs (Table 1).

Similar to ELISA, two formats can be distinguished depending on whether the detection principle is based on sandwich or competitive format [14]. For sandwich assays, a binding agent specific for the analyte (usually, but not necessarily, an antibody) is immobilised on the membrane. The detection reagent, typically an antibody (either enzyme-labelled, or coupled to latex or colloidal metal) is deposited (but remains unbound) in the conjugate pad. When liquid sample (e.g. food extracts or liquid foods directly) is added to the conjugate pad, the detection reagent is solubilised and begins to move with the sample flow front up the membrane strip. The analyte present in the sample is bound by the antibody coupled to the detection reagent. As the sample passes over the zone to which the capture reagent has been immobilised, the analyte–detection reagent complex is trapped. Here, colour development is proportional to the amount of analyte present in the sample, which is only applicable for analytes with more than one epitope (high-molecular-mass analytes) (Fig. 3).

In competitive immunoassay procedures, the detection reagent can be the analyte bound to a protein or an antibody of the analyte bound to latex or a colloidal metal. As the analyte-containing sample and the detection reagent pass over the zone to which the capture reagent (antibody or analyte–protein conjugate) has been immobilised, some of the analyte and some of the detection reagent are bound and trapped. The more analyte present in the sample, the more effectively it will be able to compete with the immobilised analyte on the membrane for binding to the limited amount

of antibodies in the detection reagent. Hence an increase in the amount of analyte in such competitive immunoassays will result in a decrease in signal in the read-out zone.

The principle of dipstick tests is similar to that of LFAs but without a mobile phase moving up the test strip. A capture antibody is immobilized on the membrane which is located at the tip of the test strip. After contact with a liquid sample, the membrane is incubated before applying a solution containing the detection antibodies. The detection antibodies are usually labelled with an enzyme which subsequently reacts with a substrate to form a coloured product. In such “sandwich” assays, the absorbance of the coloured product is directly proportional to the concentration of the analyte present in the sample. Dipsticks applying competitive ELISA as detection principle have also been reported [23–25].

The analysis time for such dipstick tests ranges from 10 min up to 3 h and depends on the number of incubation cycles involved and the time needed for all incubations. Similar to LFAs, dipstick tests provide qualitative or semi-quantitative results.

As far as we are aware, there is currently only one, actually modified, dipstick assay commercially available, which is marketed as a flow-through test for semi-quantitative determination of gluten (Hallmark). This test works similar to a dipstick assay, but with the difference that sample and reactants are dropped on to a reaction field instead of dipping a coated membrane into a solution.

Microarrays

The development of microarrays was driven by the growing demand for high-throughput, multiplexed protein analyses in the microliter to nanoliter range, and miniaturization is becoming a key element enabling maximization of the biological information gained from small, precious sample volumes [26, 27]. Microarrays contain specific (bio) molecular recognition elements (e.g. antibodies specific to a certain allergen or food marker protein) that are immobilized by microprinting or microstructuring processes and form a patterned surface. A 2D read-out can be achieved by various techniques depending on the analytical principle used for the microarray, and data analysis is achieved with software for image processing. ELISA technology or other immunoassays can be implemented by using flow-through microarray platforms that contain additionally integrated instrumentation for such a multiplexed analytical application, similar to that of a flow-injection analysis (FIA) system. Flow-through microarray platforms consist of a fluidic system for sample introduction, a reagent supply, a flow cell, a microarray on a substrate, and a detection system.

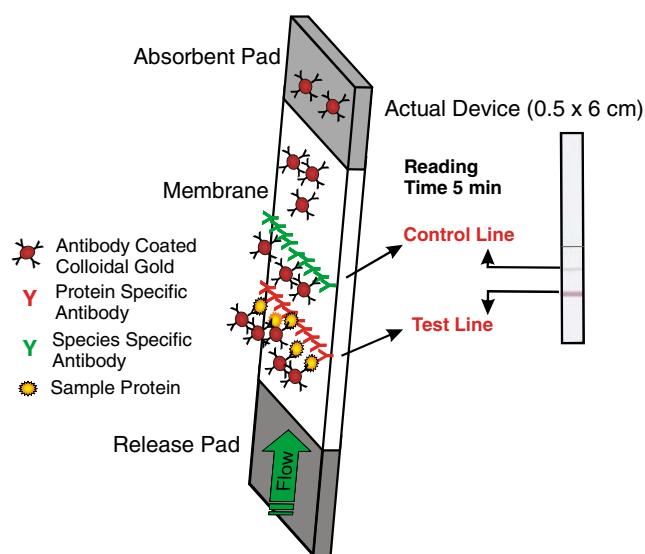


Fig. 3 Example of a lateral-flow assay based on the sandwich format

An automated and portable array biosensor developed by the Naval Research Laboratories (NRL) is capable of multiple target analysis and parallel sampling that also enables controls and standards to be analysed in parallel with unknowns [28]. The system is extremely versatile and existing immunoassay techniques can be easily implemented. Commercial versions of the portable NRL array biosensor are available (CT-ABS, Constellation Technologies; UltraRapid, Hanson Technologies), the latter also with food allergen applications.

Lateral-flow assays can also be implemented in microfluidic platforms that enable the miniaturization, integration, and automation of biochemical assays. Such microfluidic platforms can be combined with microarrays within a well defined and consistent fabrication technology to implement application-specific biochemical assays in an easy, flexible, and ideally monolithic way [29]. Miniaturized immunoassays with spotted antibodies as capture agents have been applied to the simultaneous screening of allergen-specific IgE in human serum [30]. Although only a very limited number of applications in the field of food allergen analysis have been published so far, such microarrays promise to be a powerful tool by enabling simultaneous detection of various allergens in food samples in a single step.

Biosensors

One of the emerging technologies in the past decade has been the immunochemical biosensor which enables measurement of a specific molecular interaction in real-time. Target molecules (e.g. proteins or single-stranded DNA fragments) are immobilized on a sensor chip surface and the binding activity between one or more molecules can be measured quantitatively. Immunochemical biosensors generally consist of three components: a biological receptor of appropriate specificity for the analyte, a transducer to convert the recognition event into a suitable signal, and a signal-processing system that detects, processes, and displays the analytical data. Detection and quantification is achieved by various physicochemical techniques, for example surface plasmon resonance (SPR), resonance-enhanced absorption (REA), or fluorescence evanescent-wave sensors, to name a few. In recent years, biosensors have also found increasing attention in food analysis and food allergen detection.

SPR immunoassay sensors, especially, have been frequently applied [31–34]. The increasing spread of this technology is because of the commercial availability of special SPR platforms and kits (e.g., Biacore Q, GE Healthcare and Spreeta, Texas Instruments). SPR biosensors monitor the interaction of a molecule with a prepared sensor surface in the presence of a target molecule in solution (solution inhibition) or excess analyte (surface

competition). The measurable output is either a resonance angle or refractive index value; thus, there is no more need to label molecules with fluorescent or radioactive tags. The Spreeta system is an experimental SPR evaluation kit which enables time-efficient and low-cost development of immunoassay biosensors. Its feasibility for food allergen analysis has been shown with the development of a peanut biosensor [31]. The Biacore Q system has been especially developed for food analysis applications and commercial kits are available for drug residue and vitamin analysis in food. A Biacore Q optical biosensor has been used to develop both direct and sandwich immunoassays for detection of proteins from milk, egg, hazelnut, peanut, shellfish, and sesame in food samples [32]. Furthermore, a localized surface plasmon resonance (LSPR) immunosensor based on a gold-capped nanoparticle substrate has been developed for detection of casein in milk [33]. The LODs achieved with such biosensors is comparable with those of traditional ELISAs and are in the range 1 to 10 mg kg⁻¹.

Optical biosensors based on resonance-enhanced absorption (REA) have been developed for milk allergens (β -lactoglobulin) [35] and egg-white allergens (ovalbumin and ovomucoid) [36]. REA, however, needs labelled detection reagents in contrast with label-free SPR technology. To achieve a strong REA signal, the detection antibodies are labelled with monodisperse colloidal gold clusters (or gold nanoparticles). The REA effect makes use of the optical near-field phenomenon and is observed when noble metal nanoclusters are deposited at a nanometric distances from the highly reflective mirror of an interferometric set-up.

Method validation

The market for commercial allergen test kits has rapidly developed in the past five years; however data on method validation is still poor. To ensure the quality (reliability and accuracy) of analytical data produced by these test kits, it is of utmost importance to perform validations or to participate in interlaboratory comparisons.

The Performance Tested Methods Program of the AOAC Research Institute (RI) provides an independent third-party review of test kit performance claims. Test kits found to conform with their claims are granted Performance Tested Methods status by the AOAC RI. Performance Tested Methods status assures the test kit user that an independent assessment has been conducted and the kit performs as claimed. A large number of commercial test kits have been approved by the AOAC RI including four food-allergen tests (three peanut tests and one test for gliadin) [37].

A commercial hazelnut ELISA (R-Biopharm) has been validated for dark chocolate by the German Federal Office

of Consumer Protection and Food Safety (BVL). The BVL offers an “Official collection of test methods pursuant to §35 Foods and Other Commodities Act (LMBG)” as an online database [38].

In 2006, an interlaboratory study was organised by the European Commission Joint Research Centre—Institute for Reference Materials and Measurements (JRC-IRMM) [39]. Two commercially available dipstick tests (lateral flow devices) designed for detection of peanut residues in food matrixes were evaluated in an interlaboratory validation exercise with 18 participating laboratories worldwide. The test samples used in the study were cookies containing peanuts at seven different concentrations in the range of 0–30 mg peanuts kg⁻¹ food matrix. However, some false-negative results were reported for all matrixes containing <21 mg peanuts kg⁻¹ cookie, partly because the test kits were challenged beyond their cut-off limits (~5 mg kg⁻¹, depending on the food matrix). One test kit showed fewer false-negative results, but it led to some false-positive results for the blank materials. The sensitivity of the dipstick tests approaches that achieved with enzyme-linked immunosorbent assays.

Beside the official validation and harmonization approaches much effort is being applied to proving test results by individual researchers. On the one hand commercially available tests are subjected to inter-laboratory validation [40–43] or the individually developed immunoassays pass through adequate in-house validation. Another very important up-coming effort is the development of reference methods other than immunoanalytical detection methods, e.g. LC–MSⁿ methods, for detection of food allergens [44–47]. These reference methods are at the beginning of their development and, hopefully, it will soon be possible to compare immunoanalytical results with those obtained by use of these alternative methods.

Rapid tests for selected allergens in food products

Cereals containing gluten and products thereof

Gluten is the dough-forming protein fraction of wheat flour. The use of wheat flour and gluten in bakery products and other foodstuffs is extremely common because of their heat stability and useful effects on, e.g., texture, moisture retention and flavour. The new Codex Alimentarius of 2008 defines a maximum content of 20 ppm gluten in naturally gluten-free products and 200 ppm gluten in products rendered gluten-free [48].

Rapid ELISAs in microwell format for the quantification of gluten/gliadin with incubation times of only 30 min are commercially available (Table 1).

The common format used in these test kits is a sandwich ELISA that provides high sensitivity and specificity and enables quantitative analysis of prolamins from wheat (gliadin), rye (secalin), and barley (hordein) in raw and processed foods down to 2.5 mg kg⁻¹ gliadin (which corresponds to 5 mg kg⁻¹ gluten). LODs of the ELISA test kits range from 2 to 10 mg kg⁻¹ gliadin.

There are also three commercial immunochromatographic tests (LFAs) and one commercial dipstick test (marketed as a flow-through test) for detection of gluten traces in food products. The times needed to obtain a result lies within 5 to 15 min. LODs down to 2.5 mg kg⁻¹ gliadin (which corresponds to 5 mg kg⁻¹ gluten) are reported (Table 1).

Crustaceans and products thereof

Shellfish and mussel are common foods in Europe. The major allergen of crustaceans is the abundant muscle protein tropomyosin and constitutes at least 20% of the soluble protein in shrimp. There is also strong immunological evidence that tropomyosin is a cross-reactive allergen among crustaceans and molluscs, in particular squid.

Two commercial test kits are available for the detection of soluble crustacean/tropomyosin levels (Table 1). A rapid ELISA test kit has been designed to provide a highly sensitive and convenient method for screening food samples for crustacean (tropomyosin) residues. The time required for testing an extracted sample is approximately 60 min. Quantification can be performed down to 0.05 mg kg⁻¹ tropomyosin by using a microwell reader.

A lateral flow test for crustaceans and molluscs (including crab, lobster, brown shrimp, tiger prawn, langoustine, crayfish, scallop, oyster, mussel, cockle, and squid) has been marketed with a testing time of less than 10 min and LODs around 5 mg kg⁻¹. Because the LOD is matrix dependent, validation is recommended for each sample type.

Eggs and products thereof

Hen's egg is one of the most frequent causes of adverse reactions to foods in children. Egg can be present as an ingredient or as a contaminant in raw and cooked products. Allergenic proteins are found in egg white and egg yolk. The allergenic potential of the proteins present in egg yolk is only moderate. Threshold doses are the lowest doses at which severely egg allergic patients reacted and lie in the microgram and low milligram range.

Four rapid ELISA microwell kits have been marketed for qualitative and/or quantitative analysis of egg protein residues in various food matrices. The tests produce results within 30 to 35 min and enable quantification down to

1 mg kg⁻¹ egg protein by using a microwell reader. The tests are characterised by high sensitivity (LODs down to 0.6 mg kg⁻¹) and high specificity. Sandwich ELISA formats are commonly used in these test kits, which usually apply polyclonal antibodies specific for one or more of the major egg-white proteins. A rapid immunochromatographic assay device which tests for the presence of egg proteins in various food matrix extracts within 10 min is also commercially available (see Table 1).

Baumgartner et al. [21] developed a dipstick assay for determination of egg proteins in food that has not yet reached the commercial market. The detection principle is based on a non-competitive ELISA format with direct spotting of the egg-white-specific capture antibody (raised in rabbit) on to a nitrocellulose membrane and detection with a peroxidase labelled antibody (also against egg white). The highly specific assay proved to be sensitive enough for detection of egg protein in the lower microgram per kilogram range and served well for obtaining qualitative information on the presence of egg protein in foods above 20 mg kg⁻¹. However, further research is needed to enable quantitative application of the developed dipstick assay. Another major aspect which must still be worked on is the detection of processed egg, which is highly dependent on the immunogen source the antibody was developed with. The ready-for-use dipsticks showed sufficient stability, which is essential for commercial application, and could be stored for 3 months at 6°C still with complete function.

A REA-based immuno chip sensor has been developed as a rapid method for allergen detection in complex food matrices, and its application for detection of the egg white allergens ovalbumin and ovomucoid was evaluated [36]. A colorimetric solid-phase immunoassay was realized on a planar chip in direct and sandwich assay formats using antibodies functionalised with gold nanoparticles. In the direct assay format, a coating time with allergen of only 5 min was sufficient for accurate reproducibility and sensitivity. A LOD of 1 ng mL⁻¹ has been achieved. The biosensor proved to be reproducible and selective in its performance and enables high-throughput screening.

Peanuts and products thereof

Peanut (*Arachis hypogaeae*) is a very nutritious fruit among the legume family. Unfortunately peanuts belong to the group of most allergenic foods. Even consumption of few milligrams of peanut can induce allergic reactions in highly sensitised individuals. Because peanut allergy is persistent during life and treatment of this allergy is not possible, avoiding peanuts is extremely important for these patients. Peanut can be present as an ingredient or as traces in contaminated raw and cooked products.

For protection of consumers against “hidden peanut allergens” and for hygiene monitoring during food manufacture, intense efforts have been made by academic and industrial laboratories to develop rapid immunochemical methods.

The semi-quantitative sandwich-ELISAs in dipstick format developed by Stephan et al. [22] for detection of peanut traces in food extracts, however, involves several incubation steps that result in a total testing time of 75 min. Peanut residues as low as 1 mg kg⁻¹ of peanut protein in food could be detected with this assay.

A competitive lateral-flow assay for the detection of the major peanut allergen Ara h1 has been developed on the basis of a direct competitive immunoassay using anti-Ara h1 polyclonal antibodies as capture reagents and Ara h1-tagged liposomes as detection reagents [19]. Its application was tested in chocolate and achieved results within 30 min and an LOD of 158 mg kg⁻¹ of peanut in food sample [20].

Various rapid immunochemical test kits for detection and quantification of peanut residues in foodstuffs are already commercially available (Table 1). Four rapid ELISAs in microwell format currently on the market produce qualitative and quantitative results within 30 to 35 min. Sandwich ELISA again is the format of choice, because of its high specificity and sensitivity. LODs between 0.15 and 5 mg kg⁻¹ have been reported. Two of the four rapid ELISAs have been approved by the Performance Tested Method Program of the AOAC Research Institute (RI) [37].

Three commercially available lateral-flow assays based on ELISA technology enable checking of the presence of peanut residues in various foodstuffs in 10 min or less with LODs between 1 to 5 mg kg⁻¹.

Soybeans and products thereof

For a long time, the allergenicity of soybeans has been underestimated. Soy is a common dietary protein and often introduced into the diet from an early age. Soy has been used as a standard milk formula in healthy children and as a substitute in children with suspected or proven cows' milk allergy. This practice, however, is now discouraged, because soy is regarded as an important food allergen, although the incidence of allergy to soybean proteins is quite low in comparison with that to other major food allergens.

Several immunochemical assays have been developed for detection of soy allergens in food products. However, they are usually time-consuming and labour-intensive. Most of these methods lack sufficient sensitivity for monitoring of soy traces in food product below the g kg⁻¹ range [3].

There are currently two rapid soy allergen test kits on the market that use a sandwich ELISA format and produce results within 30 min. The sensitivities of both assays are

satisfactory, with LODs $<5 \text{ mg kg}^{-1}$. In combination with a microwell reader, quantitative results can be obtained. Both test kits are provided by the same manufacturer (Table 1).

Milk and products thereof

Milk is a major allergenic food, especially in infancy. Cows' milk allergy must not be confused with intolerance of lactose, which is a milk sugar and does not contain protein allergens. Lactose intolerance is a consequence of lactose maldigestion which is because of genetic intestinal lactase deficiency occurring in large sections of the general population.

Several rapid immunoassay kits have been developed that enable checking for the presence of milk allergens in various foodstuffs (Table 1). Five rapid ELISA test kits are currently on the market and these deliver qualitative or quantitative results within 30 to 45 min. All these ELISA kits exhibit high sensitivity with LODs between 0.1 and 5 mg kg^{-1} . The kits either detect selected milk allergens like caseins or β -lactoglobulin, or check for the presence of total milk proteins. In products from which whey has been significantly removed, for example cheese, the estimated level of milk material, and thus also the level of β -lactoglobulin, will be lower. In these cases, a casein test kit might be more suitable to detect milk residues.

There are also two lateral-flow assays on the market that detect casein and whey residues in food products down to 0.12 mg kg^{-1} with a testing time of less than 10 min.

The applicability of an REA biosensor with a direct immunoassay on chip has been proven for rapid detection of β -lactoglobulin in food matrices [35]. A conventional ELISA for β -lactoglobulin was converted to a cluster-linked immunosorbent assay (CLISA) by labelling the read-out antibody with monodisperse colloidal gold clusters. For generation of a strong REA signal 30 min of coating of the target protein was sufficient.

Minh et al. [33] used a localized surface plasmon resonance (LSPR) immunosensor based on a gold-capped nanoparticle substrate on which anti-casein antibodies were immobilized for detection. The casein immunosensor achieved an LOD of 10 ng mL^{-1} . With the LSPR-based biosensor no time-consuming and labour-intensive labelling of detection agents is necessary and it has several advantages, for example easy fabrication, simple handling, low-cost, and high sensitivity.

Nuts and products thereof

Although nuts belong to several different botanical families, allergologically they can be grouped together. They have similarities in allergenic properties, way of consumption, clinical picture provoked, and protein pattern. Together

with peanuts, nuts (particularly Brazil nuts and hazelnuts) are the most powerful food allergens. They are able to provoke severe or even fatal anaphylactic reactions at very low threshold doses. A few milligrams of nut proteins can provoke allergic reactions in sensitised individuals [1]. Currently, rapid immunochemical test kits are only available for the detection of almond and hazelnut. Therefore, the following discussion is restricted to these two tree nuts.

Almond

Almond (*Amygdalus communis* L.) is one of the most popular tree nuts worldwide because of its aromatic taste. Thus, almonds are widely used as ingredients in food products such as snacks, breakfast cereals, and bakery products (e.g. cookies, cakes, confectionary products, etc.). Moreover, almonds are a source of gourmet edible oils that potentially contain residual protein. The protein content in almonds is very high (approx. 25%) and 95% of these proteins are water-soluble, which makes them easily accessible. Even consumption of few milligrams of almond can induce allergic reactions in highly sensitised individuals.

Rapid ELISA test kits in microwell format are commercially available that enable detection of almond proteins in various food matrices with LODs ranging from 0.15 to 5 mg kg^{-1} (Table 1). Sandwich ELISAs with high sensitivity and selectivity are commonly used. Qualitative or quantitative results are obtained within 30–35 min. Strong cross reaction is observed to apricot stone, which is closely related to almond. A lateral-flow assay has been marketed and detects almond traces down to 1 mg kg^{-1} in less than 10 min.

Hazelnut

The prevalence of hazelnut allergy in the European population is 0.1 to 0.5%. Hazelnut allergy is often associated with allergy to pollens from trees like birch. Hazelnut is used as a food ingredient in pastry, confectionary products, and ice cream, but it is also processed to oils. Undeclared hazelnut might be present as cross-contaminant in various food products, for example cookies, muesli bars, or nut-free chocolate products.

There are already three rapid ELISA test kits commercially available that detect hazelnut traces in foods such as cereals, baked goods, ice cream, and chocolate (Table 1). In conjunction with a microwell reader, they can also produce quantitative results in 30 to 35 min. LODs achieved with these test kits lie between 0.15 and 5 mg kg^{-1} and depend on the degree of roasting, e.g. highly roasted hazelnuts are recovered with approximately 80% efficiency. One of the three commercial ELISA test kits has been validated for

dark chocolate following the official collection of test methods pursuant to §35 Foods and Other Commodities Act (LMBG) by the German Federal Office of Consumer Protection and Food Safety (BVL) [38].

Stephan et al. [22] developed a semi-quantitative sandwich-ELISA in dipstick format for detection of hazelnut traces in processed foods. The dipstick test, however, has a very long testing time (3–4 h) because of several incubation steps which take up to 40 min each. The limit of detection (LOD) is approximately 1 mg kg⁻¹ of hazelnut protein in food.

The two LFAs on the market enable checking for the presence of hazelnut traces in various food products within 10 min. The two test kits have LODs of 1 and 5 mg kg⁻¹, respectively. High cross reactivity is observed for sesame and sunflower seeds.

Molluscs and products thereof

Although molluscs (gastropods, bivalves or cephalopods), are most often consumed in their natural form, they are also used as ingredients, after processing, in a number of preparations and in products such as surimi. The main allergenic protein in molluscs is tropomyosin, which is the same as that in crustaceans, and cases of cross-allergies between molluscs and crustaceans occur frequently. In 2006, molluscs were included in the list of allergens of the EU food labelling directive [6].

There is currently one commercial lateral flow test available that detects residues of molluscs and crustaceans down to 5 mg kg⁻¹ with a total testing time of less than 10 min. LODs are matrix-specific and validation is recommended for each sample type.

Others

Lupin has been recently added to the EU list of allergens that must be labelled on food product in all circumstances [6]. Lupin flour is used in food as additive to wheat flour and in gluten-free bakery products and food for celiac disease patients. The frequency of cross-allergy between peanut and lupin is high. Recently, methods for detection of lupin in food products have been developed that are based on either ELISA technology or real-time PCR. One commercial kit based on real-time PCR for qualitative detection of lupin with an LOD of 10 mg kg⁻¹ is already on the market (R-Biopharm). Cross-reactions with alfalfa (48%) and pea (0.001%) have been observed. However, no rapid immunochemical test methods that enable detection within 1 h have yet been reported.

Although fish, celery, mustard, and sesame seeds were added in 2003 to the list of allergenic foods that need to be labelled on food products [4, 5], there has been a lack of

analytical methods for these four food allergens. Only recently, ELISAs [49–51, 57] and real-time PCR methods [52–55] have been developed for the determination of fish [57], celery [52, 55] mustard [49–52], and sesame [52–54] in foods. Commercial ELISA test kits are available for sesame (Tepnel, R-Biopharm, ElisaSystems) and mustard (ElisaSystems). Test kits based on real-time PCR can be obtained from R-Biopharm for all four food allergens; these achieve LODs of 5 to 10 mg kg⁻¹, depending on the allergen and food matrix. A surface plasmon resonance (SPR) biosensor has been developed for detection of the major fish allergen, parvalbumin. The SPR biosensor enables detection of parvalbumin within 5 min with LODs below 1 mg kg⁻¹ and promises to be a rapid and powerful tool for allergen detection and quantification. However, for the other three allergenic foods no rapid immunochemical test with testing time of 60 min or less has yet been developed.

Buckwheat (*Fagopyrum esculentum*) has been recognized as a common food allergen in Asian countries. There is a growing interest in buckwheat products as a health food and as a substitute for wheat flour for gluten-allergic persons in Western countries. Buckwheat is not included in the food allergen list of the EU food labelling directive. It is however mentioned here in this context because a rapid ELISA test kit (ElisaSystems, Windsor) is commercially available that enables detection of buckwheat protein residues in various food matrices in approx. 45 min.

Conclusion

The demand for rapid and reliable analytical tools for the detection of food allergens in food products has increased since the implementation of the EU food labelling directive [4–6] into the legislation of many countries. In recent years many rapid test kits that check for the presence of food allergen residues within 60 min or less have become commercially available. In particular, numbers of fast ELISA test kits (testing times 30 to 35 min) and immunochromatographic tests (LFAs and dipsticks with testing times of 10 min or less) have significantly increased. Not only has analysis time been shortened, but the sensitivity and selectivity of the methods have also improved. LODs of less than 10 mg kg⁻¹ of allergenic protein in a food sample have become standard and cross-reactivities that lead to false-positive results have been reduced. A lack of rapid analytical methods has been identified for several allergenic foods including celery, mustard, sesame seeds, fish, and lupin. All of these allergenic foods are listed in the EU directive for food allergen labelling and their presence in food products must be declared on food packaging.

Although much research effort has been applied to the development of optical biosensors [56] (with more than 1000 publications per year) and microarray techniques [26, 27] in the past decade, the application of these novel techniques to food allergen analysis is still limited. However, great potential has been recognised, especially for the development of portable optical biosensors, where experimental development kits are already available on the market (Spreeta and Biacore Q). Microarrays in combination with immunochemical analytical approaches offer the technical basis for simultaneous multiple-allergen detection and parallel analysis of multiple samples. With this technology, large numbers of samples can be analyzed within a relatively short time, which is essential in food screening and monitoring programmes.

The availability of rapid immunochemical allergen tests makes the task of monitoring foods for allergen contamination readily achievable, providing the industry with an important tool for quality control of raw materials, processes, and products. Increased testing will give food allergic consumers increased confidence in the safety of the food they eat, because food labelling will become more accurate and vague declarations on food labels such as “may contain traces of a specific allergen” may be widely avoided.

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