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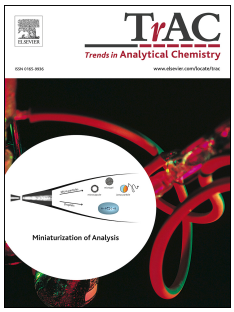

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Recent advances and challenges in food-borne allergen detection

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Highlights

- Review of recent approaches and commercial kits for food-borne allergen detection
- The applicability of allergen detection methods to food samples is stressed
- The advantages and limitations of current methods are discussed
- Future perspectives for reliable on-site food-borne allergen quantification

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Abstract

Food allergy is reported as the commonest adverse reaction to food components, whose prevalence has increased in recent years. As food avoidance is mainly in practice the only way to prevent hypersensitive consumers from ingesting allergenic substances, it is imperative to provide complete and accurate information on food ingredients. In this scenario, there is a need for precise, fast and cost-effective methods for the high-throughput screening of specific allergen content in food products. This work reviews recent approaches, existing kits for food-borne allergen detection and cutting-edge applications by focusing on the sensitivity, selectivity and applicability of current methods in food samples. In addition, the advantages, benefits and limitations of each approach are discussed to establish the most suitable methods and which challenges are to be addressed in forthcoming years from an analytical viewpoint.

Keywords

Food-borne allergens, bioanalytical methods, immunoassay, nucleic-acid detection, biosensors

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1. Introduction: food allergy and food intolerances. Legal status

Anomalous reactions that derive from food ingestion are often defined as “adverse reactions to food”. They are classified by the European Academy of Allergology and Clinical Immunology based on the responsive pathogenic mechanism as toxic and non-toxic reactions.[1] Toxic reactins result from a primary harmful effect that food has on all the individuals who intake it. Non-toxic reactions depend on individual susceptibility, are not commonly dose-related, and are subdivided into immunological (food allergy) and non-immunological (food intolerance).[2],[3]

Food allergy is an adverse immune-mediated response that occurs reproducibly upon exposure to a given food, component or ingredient. The immune response is classified as IgE-mediated, non-IgE-mediated, or a mixture of both. IgE-mediated food allergy is based on the interaction of allergenic proteins with specific IgEs linked with mast cells/basophils present in the gut. Conversely, non-IgE-mediated food allergy is governed mainly by T-cell-mediated processes and antibody isotopes that differ from IgE, i.e. IgG, IgM and IgA.[4] Food intolerances are adverse reactions to food that do not involve the immune system. They are often related to enzymatic defects, such as lactose intolerance due to β-galactosidase deficiency, or to the presence of vaso-active pharmacological substances, such as histamine that provokes similar symptoms to those of an allergic reaction.[5]

When an adverse reaction to food is suspected, a clinical history must be taken to establish the pathogenic nature of the disease. Only having ruled out food toxicity and intolerance does the patient undergo specific allergology tests, of which Skin Prick tests (SPT) are the most widely used as a cost-effective method that provides immediate results. However, they are usually related to false-positive results due to cross-reactivity.[3],[6] Hence the Double Blind Placebo Controlled Food Challenge (DBPCFC) appears a more accurate alternative, which involves the prolonged ingestion of increasing amounts of either the suspected allergen or a placebo while monitoring symptoms.[7] Besides, *in vitro* tests for the specific detection of IgE levels have gained more attention in the last few years because they non-invasively provide sensitive results to complement the clinical information that *in vivo* tests provide.[8]

While awaiting a remedy or effective treatment for food allergy, sensitive consumers must rely on allergen-suspicious food avoidance, which makes complete and accurate information of ingredients on food labels imperative. Indeed countries and international bodies are collaborating to enact laws, regulations and standards for food allergen labelling, and have implemented requirements to identify the offending allergen in packaged food products. To

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date, over 200 foods have been proven allergenic. Hence, governments and regulatory agencies have recognised the need to focus allergen-labelling regulations on a limited set of "priority allergens". Some differences in regulations in the number of foods designated as allergens appear worldwide. In Europe, labelling regulation Directive 2000/13/EC and further amendments (in 2007/68/EC) include the major eight allergens originally recommended by Codex Alimentarius, namely: milk, egg, peanut, tree nuts, soya bean, cereal-containing gluten, fish and crustacean shellfish, as well as celery, mustard, sesame seed, lupine and molluscs.[9] Currently, there are 10 regions in the world that share between five and fourteen allergens on their regulation lists (Table 1).[10],[11]

Besides, no regulatory threshold exists for allergenic contents in food samples. This is partially explained by the limited availability of clinical data from DBPCFC tests and because standardised protocols for diagnostic purposes are lacking.[9] For instance, the International Codex Alimentarius Standard states that food samples with gluten levels below 20 mg/kg, and from 20 to 100 mg/kg, should be labelled as "gluten-free" and "very low gluten", respectively.[12] Japan is the only jurisdiction to have adopted regulatory thresholds of 10 mg/kg (ppm) of soluble protein. In Australia, the Allergen Bureau (a food industry initiative) has proposed a standardised allergen risk assessment tool (Voluntary Incidental Trace Allergen Labelling), but it unfortunately lacks legal relevance.[9] Therefore, organisations like the WHO, FDA and CFIA are collaborating in the standardisation of allergen detection methods, threshold values and food-labelling requirements.

By taking into account the exposed clinical and legal scenario, it is intuitive that precise, cost-effective and fast analytical methods are required for the high-throughput screening of specific allergen contents in commercial food products.

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Table 1. Food allergens: Immune response, symptoms and regional regulations

Food	Major allergenic protein	Type of Immune response	Symptoms	Labelling	Ref.
Cow's Milk	Bos d 4; Bos d 5; Bos d 8	Mixed and non-IgE mediated	Hives; wheezing; itching; abdominal pain; diarrhoea; vomiting	EU, CH, USA, CAN, AUS	**[13]**
Egg	Gal d 1; Gal d 2; Gal d 3; Gal d 4; Gal d 5	Mixed and non-IgE mediated	Hives; nasal congestion; coughing; vomiting; abdominal pain; wheezing	EU, CH, USA, CAN, AUS	**[14]**
Crustacean	Pen a 1	IgE-mediated	Hives; itching; swelling; nasal congestion; vomiting; abdominal pain; diarrhoea, dizziness	EU, CH, USA, CAN, AUS	**[15]**
Fish	Lep w 1; Pon 1 4; Pon 1 7; Seb m 1; Xip g 1	IgE-mediated	Hives; swelling of lips; itching; throat tightening; vomiting; abdominal pain, diarrhoea	EU, CH, USA, CAN, AUS	**[16]**
Peanut	Ara h1; Ara h2; Ara h3; Ara h 4-9	IgE-mediated	Hives; itching; swelling; Vomiting; abdominal pain; diarrhoea; nausea	EU, CH, USA, CAN, AUS	**[17]**
Tree nuts		IgE-mediated	Abdominal pain; difficulty swallowing; itching, diarrhoea; nasal congestion	EU, CH, USA, CAN, AUS	**[18]**
Hazelnut	Cor a 1; Cor a 2; Cor a 8; Cor a 9; Cor a 11; Cor a 12; Cor a 13; Cor a 14				
Brazil nut	Ber e 1; Ber e 2				
Cashew	Ana o 1; Ana o 2; Ana o 3				
Almond	Pru du 3; Pru du 4; Pru du 5; Pru du 6				
Walnut (Black)	Jug n 1; Jug n 2; Jug n 4				
Walnut (English)	Jug r 1-6				
Pecan	Car i 1; Car i 2; Car i 4				
Pistachio	Pis v 1; Pis v 2; Pis v 3; Pis v 4; Pis v 5				
Soya bean	Gly m Bd 30K; Gly m Bd 60K;Gly m Bd	Mixed and non-IgE	Hives; abdominal pain; difficulty	EU, CH, USA, CAN,	**[19]**

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	28K	mediated	breathing; nausea; vomiting; diarrhoea	AUS	
Wheat	Tri a 12; Tri a 14; Tri a 18; Tri a 25	Mixed and non-IgE mediated	Swelling; itching; hives; wheezing; nasal congestion; nausea; vomiting; diarrhoea; anaphylaxis	EU, CH, USA, CAN, AUS	**[20]**
Gluten	Tri a 26 & Tri a 36; Tri a 19 & Tri a 20	IgE-mediated	Abdominal pain; diarrhoea; nausea; headache; brain fog	EU, CH, USA, CAN, AUS	**[20]**
Sesame	Ses i 3; Ses i 2	IgE-mediated	Urticaria; abdominal pain; Diarrhoea; vomiting	EU, CAN, AUS	**[21]**
Mustard	Sin a 1; Sin a 2; Sin a 3; Sin a 4	IgE-mediated	Urticaria; itching; swelling (face/throat); abdominal pain; nausea; vomiting; severe asthma	EU, CAN	**[22]**
Sulphites	E220 – E228	Mixed and non-IgE mediated	Dermatitis; urticaria; flushing; hypotension; abdominal pain; diarrhoea	EU, CAN, AUS	**[23]**
Lupin	Lup-1; Lup-2; Lupin PR-10 protein	IgE-mediated	Urticaria; itching; swelling (face/throat); abdominal pain; nausea; vomiting; severe asthma	EU, AUS	**[24]**
Celery	Api g 1; Api g 2; Api g 3; Api g 4; Api g 5	IgE-mediated		EU	**[25]**

EU: European Union; CH: China; USA; United States of America; CAN: Canada; AUS: Australia

The aim of this review is to critically analyse the most recent approaches developed for food-borne allergen detection purposes by considering immunoanalytical, mass-spectrometry, nucleic-acid-based methods and biosensors **(Figure 1)**. Special attention is paid to the achieved sensitivity and the practicability of tests when evaluating commercial food samples. In addition, a glance at the commercially available kits for food allergen detection is made to attain a complete evaluation of the technology status.

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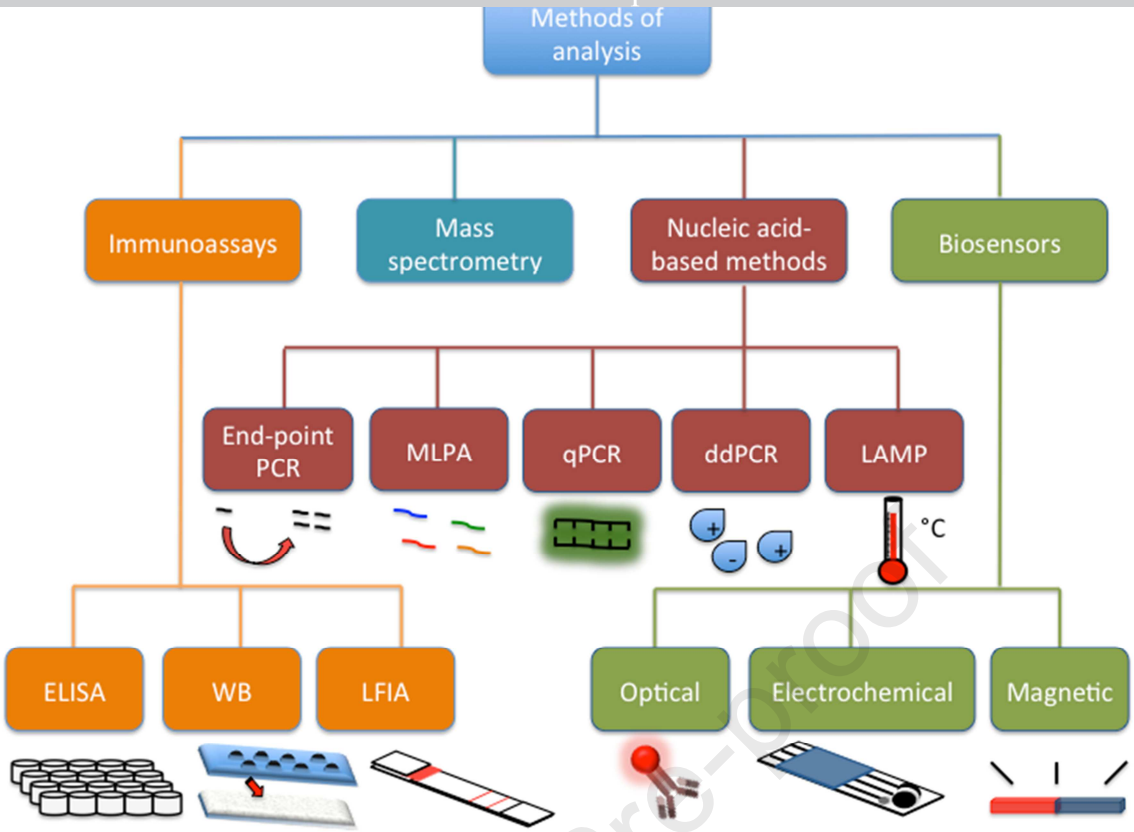

Figure 1. Outline of the current methods to analyse food-borne allergen detections. Immunoassays are subclassified into enzyme-linked immunosorbent assay (ELISA), Western blot (WB) and lateral flow assay (LFIA). Nucleic-acid based methods are subdivided into end-point PCR, multiplex ligation-dependent probe amplification (MLPA), quantitative PCR (qPCR), digital droplet PCR (ddPCR) and loop-mediated isothermal amplification (LAMP). Biosensors are classified according to the signal transduction mode into optical, electrochemical and magnetic.

2. Methods of food analysis

2.1. Immunoanalytical methods

Immunoanalytical methods rely on using specific and high-affinity antibodies for the detection of protein/peptide biomarkers that indicate the presence of allergenic ingredients in food samples. Antibodies can be either polyclonal or monoclonal, depending on their ability to bind more than one epitope. The former are cheaper and faster to produce, but are more prone to batch-to-batch variability. The latter provide higher consistency among experiments, but take longer to develop. The selection of one or other type very much depends on the desired assay format, and monoclonal antibodies are often used in competitive assays, with a combination of polyclonal and monoclonal antibodies in sandwich-based assays.[26],[27]

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Alternatively, the new generation of immune-based bioreceptors has been recently applied to food-borne allergen detection, such as single-domain antibodies, and provide analytical methods with improved properties. They are also reviewed in this section.

The most frequently used techniques to detect protein allergens are enzyme-linked immunosorbent assay (ELISA), which is the traditional method, Western blot and lateral flow immunoassay (LFIA). In this section, the most recent approaches for food-borne allergen detection in each method are discussed.

2.1.1. ELISA

2.1.1.1. Sandwich ELISA

Of today's detection methods, ELISA is the most widely used platform and standard method[28] for the detection of allergenic ingredients in different sample matrices in both research and food manufacturing. The reason behind this is its outstanding analytical sensitivity and simplicity. The assay is based on the functionalization of a well plate with capture reagents and the use of enzyme-labelled antibodies as detector reagents. The most widely used enzymes in ELISA are horseradish peroxidase (HRP) and alkaline phosphatase (ALP). ELISA's operation is based on detecting colour change when a dedicated substrate is added that is catalysed by the enzyme.[29–31] Hence, colour intensity can be easily related to the concentration of the target analyte, which is usually quantified by a spectrophotometer developed to directly read the plate containing the assays developed in wells. Depending on the properties of the target analyte, the food matrix and the selected antibody, ELISA is performed by the competitive or sandwich format.[32]

In the sandwich ELISA, colour, fluorescence or luminescence intensity are directly related to the amount of target allergen present in the extracted food sample. This method has been applied to detect many different food-borne protein allergens in recent last years. For instance, Peng *et al.* developed a highly sensitive sandwich ELISA to detect ovalbumin (OVA) related to the egg allergen. Specificity studies were performed with 17 monoclonal antibodies produced in different murine hybridomas. The combination of anti-OVA mAb17 as a capture antibody and anti-OVA mAb15-HRP as a detector antibody was the most sensitive as it gave a limit of detection (LOD) of 0.51 ng/mL in the egg matrix. This approach offered good

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precision, accuracy and repeatability when evaluating commercially processed foods, including egg protein.[33]

Costa and co-workers developed an indirect sandwich ELISA to detect hazelnut traces in chocolate. Rabbit polyclonal antibodies and mouse monoclonal antibodies were raised against the Cor a 9 hazelnut allergen, and were used as a capture antibody and a primary antibody, respectively. The evaluation of antibodies' specificity was made by Western blot and LC-MS/MS to confirm that the produced antibodies did not show any cross-reactivity with the other components present in the sample. In order to overcome the matrix effect and to avoid non-specific interactions, samples were diluted 10-fold in blank sample and plates were blocked with 2% milk powder. The system gave a LOD and a limit of quantitation (LOQ) of 1 mg/kg and 50 mg/kg in chocolate samples, respectively, which well agreed with other reported ELISA assays for hazelnut allergen detection purposes.[34]

Kiyota and colleagues proposed combining monoclonal antibodies (as capture reagents) and polyclonal antibodies (as detector reagents) as a sensitive strategy to detect the profilin (Cit s 2) allergen in Navel oranges and other citrus fruit. It is noteworthy that the antibodies were not raised against Cit s 2, but against its homologue rBet v 2, which shares 75% similarity in the amino acid sequence of Cit s 2. The reason for this was the instability of Cit s 2 during the antibodies' purification process. Besides, the antibodies raised against rBet v 2 showed strong immunoreactivity with Cit s 2, which enabled high sensitivity (LOD of 1.81 μg/g) for detecting Cit s 2 in Navel oranges and other citrus fruit. These authors also confirmed 1.5-fold higher concentrations of the allergen in pulp than in peel, which is relevant in allergy prevention terms.[35]

Sandwich ELISA has also been applied for multiplexing purposes, e.g. by Schocker *et al.*, who designed an assay for the Ara h2 and Ara h6 peanut allergens in human breast milk. Both proteins belong to 2S albumins, have a 59% sequence homology and are relevant marker allergens of peanut. The ELISA operation was based on using mAbs and biotinylated pAbs as the capture reagent and the detector reagent, respectively. Signal amplification was achieved by employing the poly HRP-streptavidin conjugate, which binds to biotinylated pAbs. This strategy gave a LOD of 1.3 ng/mL and 0.7 ng/mL for Ara h 2 and Ara h 6, respectively, and proved more sensitive than other reported approaches. In addition, the developed assay showed no cross-reactivity when evaluating a complex breast milk matrix, and proved to be a reliable method for determining peanut allergen in real samples.[36]

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He and co-workers developed a fluorescent-based sandwich ELISA method as a sensitivity enhancement strategy for detecting bovine β-lactoglobulin in hydrolysed infant formulas. mAbs and biotinylated pAbs were used as the capture antibody and the detection antibody, respectively. The detection mode was based on the fluorescence quenching of thiolated CdTe QDs by hydrogen peroxide (H_2O_2). With this approach, the authors accomplished a 16-fold higher sensitivity compared to the conventional sandwich ELISA based on HRP (0.49 ng/mL and 7.81 ng/mL, respectively). In addition, a 102.47% signal recovery was achieved when challenging the assay with hydrolysed infant formula samples, which revealed a good correlation with the results obtained by the HRP-based conventional sELISA and a commercial sELISA kit.[37]

2.1.1.2. Competitive ELISA

Contrary to sandwich ELISA, the signal intensity generated in the competitive format is inversely related to the amount of target allergen present in the sample. In recent years, this assay format has been preferred for detecting several food allergens. For instance, Castillo *et al.* developed an indirect competitive ELISA (icELISA) to quantify traces of β-casein milk allergen in raw and processed food. Two highly specific monoclonal antibodies (1H3 and 6A12) were raised against β-casein, from which 1H3 was selected as the most sensitive, and a LOD of 0.29 µg/mL was reported in raw and processed foods, with the most specific showing no-cross-reactivity with the other proteins present in the food matrix.[38]

Xi and colleagues developed an icELISA kit to detect the Gly m Bd 28K protein which, together with Gly m Bd 30K and Gly m Bd 60K, is the most frequent soya bean allergen in infants and adults. A specific monoclonal antibody was raised against the recombinant Gly m Bd 28K protein, which gave a high-affinity constant between 10^7 and 10^{12} L/mol. Interestingly, despite using the *E. coli*-expressed recombinant Gly m Bd 28K protein to perform the assay, the kit gave high sensitivity and selectivity when detecting the native Gly m Bd 28K content in several soya bean products: soya bean seeds, soya bean protein isolate, soya bean meal, tofu, soya milk, soya sauce, natto, sufu and lobster sauce. In fact, the obtained LOD (0.235 µg/L) was lower than others reported to date, which do not fall within the low ppb range.[39,40] Of all the tested soya bean products, soya milk, soya sauce, natto and lobster sauce had unexpected lower allergen levels, mainly due to protein denaturation

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during food processing. The developed kit also proved stable and remained so for more than 6 months when stored at 4ºC.[41]

Karina and co-workers designed a competitive ELISA for the detection of soya traces in meat products. In this case, the antigen was coated on the plate, a soya protein (SP)-specific rabbit polyclonal antiserum was used as the primary antibody and alkaline phosphatase-conjugated anti-IgG as the developing antibody. The obtained LOD and LOQ were 9.0 and 18.0 µg/mL, respectively, when evaluating real meat products, which is higher than those obtained with a commercial ELISA kit. Signal recoveries were lower than expected when analysing model systems of raw meat, which suggests that the assay was affected by the matrix effect. Despite these issues, the developed kit's low price compared to the commercial one, 0.6 dollars and 13.00 dollars, respectively, allows it to be used as a preliminary screening method.[42]

To date, we have reviewed the latest allergen detection approaches based on both competitive and sandwich ELISAs. It is well-known that the assay format has a dramatic effect on the detection method's analytical capabilities, which means that it is imperative to evaluate both formats in the assay development phase and to select the optimum one. Accordingly, Segura-Gil *et al.* reported a comparative study between an indirect competitive and sandwich ELISA for β-conglycinin soya allergen detection in processed food. The sandwich ELISA proved more sensitive (LOD: 0.90 ng/mL; LOQ: 2.1 ng/mL) than the indirect competitive format (LOD: 30 ng/mL; LOQ: 70 ng/mL). The authors suggest that the higher analytical capabilities of sandwich ELISA might be attributed to the use of two primary antibodies with specificity to different β-conglycinin epitopes.[43]

2.1.1.3. Commercial ELISA Kits

If we leave research approaches to one side, several commercial ELISA kits used for food allergen detection purposes have been launched in the last decade. R-Biopharm developed ELISA kits for almost all commonly labelled food allergens.[44] Of these, *RIDASCREEN®FAST Crustacean* and *RIDASCREEN®FAST Peanut* should be highlighted. The former is a sandwich ELISA kit for tropomyosin allergen detection in raw or cooked food that employs an extraction buffer. This kit provides a LOD and a LOQ within the low ppm range (2 and 20 mg/kg, respectively), but shows cross-reactivity with mustard, curcuma, beans, mussels and arthropods.[45] The latter is approved by the AOAC Performance Tested

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Methods Program, which certifies that the test method's performance fulfils an appropriate standard for its intended use.[46] The kit enables the detection of peanut allergen Ara h 1 and Ara h 2 at a concentration as low as 0.03 mg/kg in peanut butter. It also shows cross-reactivity to green pea, lentils, wheat semolina and fenugreek.[47]

The Morinaga Institute of Biological Science [48] offers ELISA kits for several food allergens, which enable effective protein solubilisation and extraction in processed and unprocessed foods by using an innovative non-toxic extraction buffer. The extraction solution enables higher recovery rates that permit the use of small sample volumes (0.1 mL). Interestingly, the assay format is based on sandwich ELISA and employs polyclonal antibodies. Besides, this configuration enables LODs of: 0.31 µg/mL for egg, β-lactoglobulin, casein, wheat, buckwheat, peanut, soya and crustacean; 0.26 µg/mL for gluten; 0.16 µg/mL for hazelnut. This kit's lot-to-lot reproducibility is noteworthy (C.V.≤ 10%).

Eurofins Technologies also developed ELISA kits for all common food allergens, with proven outstanding sensitivities for β-lactoglobulin (LOD 1.5 ng/mL), tropomyosin (LOD 1.7 ng/mL) and ovalbumin (LOD 4 ng/mL), which are not so good for gluten (LOD 3 µg/mL), hazelnut (LOD 0.3 µg/mL) and almond (LOD 0.2 µg/mL). Conversely to the kits developed by other companies, Eurofins only declares cross-reactivity to Ewe's (sheep's) milk (< 0.2%), Goat's milk (< 0.002%) and Casein (< 0.02%) in the bovine β-lactoglobulin kit.[49]

Currently, there are several more commercially available ELISA kits available to determine food allergens that offer similar analytical performance in terms of sensitivity, selectivity, reproducibility, total assay time, etc., such as those marketed by Neogen, Zeulab and CristalChem.[50–52]

2.1.2. Western blot

Western blot is an analytical method that combines SDS-PAGE electrophoresis for protein allergen separation (based on molecular mass), followed by immunoassay on a membrane support for allergen detection. Despite its low sensitivity compared to ELISA, Western blot is interesting for food allergen detection because it gains insight into the protein/peptide profile of differently processed foods. This is useful, on the one hand, for designing optimum antibodies based on the antigenic fragments present after food processing and, on the other hand, for distinguishing the allergen of interest from protein inhibitors that might lead to false-positive results in ELISA assays. Panda and colleagues developed a Western blot assay

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for gluten detection purposes using HRP-conjugated gluten-specific antibodies (G12, K5, 2D4, MIoBS, and Skerritt) from nine commercial gluten ELISA test kits. These authors analysed 59 fermented-hydrolysed foods from four food groups (beer, soya-based sauces, vinegar, and sourdough bread) and classified them into clusters based on differences in proteolytic fermentation processes. The assay proved highly specific, but unsuitable for allergen quantification. So this analysis method should be combined with ELISA for proper allergen quantification when following appropriate calibration standards.[53]

2.1.3. Lateral Flow immunoassay

As globally standardized allergen labelling legislation is lacking, faster and simpler detection methods are required for improved cost-effectiveness, portability and ease-of-use.[54] Accordingly, lateral flow immunoassay (LFIA) is a simple and cost-effective platform that has been applied in the past decade for the on-site determination of food allergens.[55],[56] Most LFIA approaches for food allergen detection have been based on using gold nanoparticles (AuNPs) for their simple synthesis and straightforward bioconjugation. For instance, Masiri *et al.* developed a panel of AuNPs-based LFIA tests to detect allergens in almond milk, cashew milk, coconut milk, hazelnut milk and soya milk. Both sandwich and competitive formats were evaluated, and the competitive one was more sensitive. However, the authors suggested that the sandwich assay could be useful for analyzing samples with high concentrations of the target analyte because they produce false-negative results in the competitive assay. Semi-quantification can be performed when pairing strips with a dedicated lateral flow reader. Besides, the assay was able to detect as low as 1 µg/mL of protein allergen in 25 minutes.[57]

Anfossi and co-workers took advantage of colorimetric nanoparticles with different surface plasmon resonance peaks to develop a multiplex LFIA for casein, ovalbumin and hazelnut allergenic proteins in commercial biscuits. The multiplexing strategy was based on using three test lines, each one representative of one allergen, and by using AgNPs, spherical and desert-rose AuNPs, which provided a yellow magenta cyan (YMC) colour code **(Fig. 2 A)**. The visual LOD was estimated to be 0.1 µg/mL, which is comparable to that reported by other LFIAs for detecting single allergens.[58]

In recent years, the use of novel nanomaterials to improve signal transduction has enabled sensitivity enhancement in LFIA together with the possibility of performing simple multiplexing.[59–61] For instance, quantum dots (QDs) provide outstanding fluorescence

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signal emissions within a wide wavelength range. Wang and colleagues designed a competitive QDs-based LFIA to detect major crustacean allergen tropomyosin (TM) in real food samples. CdSe/ZnS core-shell QDs were conjugated to TM-specific polyclonal antibodies and used as detector reagents **(Fig. 2 B)**. Moreover, assay optimisations, such as the TM concentration in test lines or the addition of tween-20 to test lines in order to avoid false-positive results proved effective to obtain high sensitivities. LODs were 0.5 µg/mL for visual detection and 0.05 µg/mL for the instrument analysis, and showed consistency with ELISA. The developed LFIA evidenced applicability when evaluating commercial food samples and showed consistency with ingredient lists, except for shrimp sauce, for which the unexpected negative result could be related to TM denaturation during food processing.[62]

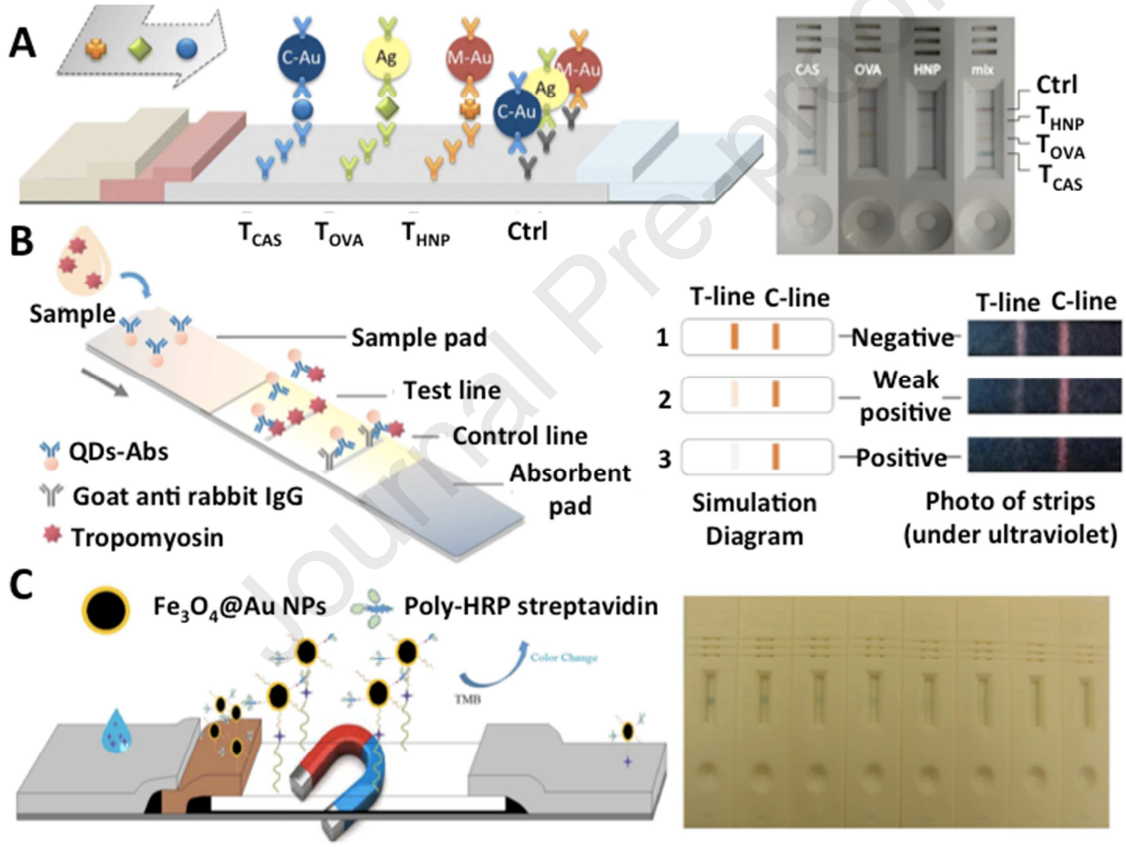

Figure 2. Lateral flow immunoassay approaches for food-borne allergen detection. (A) Multiplex LFIA based on AgNPs, spherical and desert-rose AuNPs for the simultaneous detection of casein, ovalbumin and hazelnut allergenic proteins in commercial biscuits. **(B)** Fluorescent LFIA based on CdSe/ZnS core-shell QDs for the detection of tropomyosin allergen in fish-containing food samples. Adapted with the permission of ref. 48, Copyright 2019 Elsevier. **(C)** Sensitivity enhancement approach in LFIA based on enzymatic signal amplification and magnetic focusing to detect β-conglutin in lupin-containing food samples. Adapted with the permission of ref. 49, Copyright 2018 Elsevier.

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Alternatively, Wu *et al.* developed an ultrasensitive sandwich-based LFIA for β-conglutin detection in lupin-containing food samples by combining three strategies. Firstly, they used highly specific aptamers instead of antibodies because they lack immunogenicity and are stabler. Secondly, they performed enzyme-based signal amplification by loading Fe_3O_4@Au core-shell nanoparticles with HRP. Thirdly, they applied a magnetic concentration to test lines using an external magnetic field to enhance the interaction between β-conglutin and capture aptamers **(Fig. 2 C)**. The combination of these three strategies gave an outstanding LOD of 8 fM when a smartphone camera was used coupled to image analysis software. Magnetic focusing had the strongest impact on assay sensitivity, as the achieved LOD was 1,000-fold lower than that obtained by similar assays that did not use the magnetic concentration. Aptamers were also specific for β-conglutin, but not for its structural analogues (α-, γ-, δ-conglutins).[63]

2.1.3.1. Commercial LFIA kits

Similarly to commercial ELISA kits, companies are offering LFIA kits for all common food-borne allergens. For instance, R-Biopharm developed colorimetric-based LFIA kits for the qualitative determination of soya (Rida®Quick Soya) and gliadin (Rida®Quick Gliadin), and the latter is an AACC- and AOAC-approved method. Both kits are based on the sandwich format, but have some interesting differences. The lateral flow strip of Rida®Quick Soya does not contain a conjugate pad. Otherwise the assay procedure includes a step in which the sample is mixed with the conjugate solution beforehand. Besides, Rida®Quick Gliadin has the conjugate pad integrated into the lateral flow strip, but two coloured labels are used; blue for the control line and red for the test line. Both assays take some 25 minutes (it can take up to 2 h depending on the extraction method) and are evaluated by the naked eye when one line or two are present. The test line only appears if allergen content surpasses threshold values, which are 10 mg/kg and 6.3 mg/kg for the soya- and gluten-containing processed food samples, respectively.[64],[65]

Romerlabs developed AuNPs-based LFIA kits to detect all allergens that require labelling. These kits enable the qualitative determination of allergens in swab and processed foods in no more than 11 minutes, with LODs falling within the low ppm range. As the assay format is non-competitive, the appearance of two lines indicates the presence of allergens above the

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threshold value. These kits include the extraction buffer, which enables allergen extraction in 1 minute.[66]

Morinaga [67] also offered AuNPs-based LFIA kits (Rapid Test Pro II) for egg, milk, wheat, buckwheat and peanut detection in food-processed samples. The assay takes 15 minutes, without including the extraction procedure, which is the same as Food Allergen ELISA Kits II. It is a sandwich assay in which red appears in TL if more than 5 µg/mL of allergen are present in food samples. Similarly, Zeulab [68] developed a colorimetric-based LFIA for the qualitative determination of milk, egg, gluten and soya allergens in just 10 minutes, with LODs within the low ppm range. Neogen [69] offers qualitative LFIA tests using coloured particles and the non-competitive assay format to detect the commonest food allergens in 5-10 minutes, with LODs within the low ppm range.

2.1.4. Nanobodies applied for allergen detection purposes

Nanobodies (also known as heavy chain-only antibodies) have attracted much interest in the biomedical field since they were discovered in 1989. Regarding their use as bioreceptors in diagnostics, nanobodies offer advantages over conventional antibodies, such as smaller size, higher stability, bigger and cheaper production yields.[70] Chen and colleagues developed nanobodies for peanut allergen detection purposes for the first time. They constructed a phage-displayed library by randomising the antigen-binding region of a highly stable VHH backbone. The candidates against Ara h 3 allergen were isolated, and Nb16 provided the highest affinity (Kd of 400 nM). Work currently underway is to improve Nb16 affinity and to evaluate its applicability for peanut allergen detection purpose in food samples.[71]

Similarly, Garcia-Garcia *et al.* ran a phage display strategy to isolate single-domain antibody fragments (dAbs) against gluten. The ability of dAbs to detect gluten in wheat, barley, rye and triticale samples was assessed by indirect phage ELISA, where clone dAb8E-phage was the most specific. However, the LOD achieved (20 mg/kg) was not better than that obtained by other immunoassays, which limited its applicability when evaluating commercial gluten-containing products. Besides, the affinity of isolated clones can be further enhanced by genetic engineering by means of phage display technology.[72]

2.2. Mass spectrometry

Mass spectrometry (MS) has become an alternative to immunoassays for food-borne allergen detection in the last decade for offering interesting advantages from the analytical viewpoint. For instance, immunoassays show little sensitivity when food allergens undergo harsh processing or transformation (fermentation, acidification, precipitation, etc.), which also imply false-negative results. They also tend to provide false-positive results due to antibody cross-reactivity with homologous proteins. It is noteworthy that minor differences in sample preparation or antibody composition can dramatically affect assay repeatability, and immunoassays rely on complex strategies for multiplexing purposes, although this aspect has greatly improved in recent years. Indeed MS has overcome these limitations because it does not require antibodies, offers simple multiplexing and is indifferent to denatured proteins, although modified proteins do not often show allergic effects.[73] However, MS relies on expensive instrumentation and trained personnel. Therefore, unlike LFIA that is performed on-site, performing MS is restricted to specialised laboratories.

The MS technique consists in four stages; peptide target selection, peptide specificity verification, running the targeted method and food allergen quantification. The last step includes several steps, such as the enzymatic digestion of proteins, followed by HPLC separation and MS analyses. Coupling MS with liquid chromatography has been the most widely used method for food allergen detection in the last decade, which is applied to detect fish allergens,[74][75] sesame,[76] barley, corn, oats, rice, rye and wheat.[77] Then, the triple quadrupole (QQQ) and quadrupole ion trap (Q-IT) systems have drawn attention because they enable food allergen quantification. Recently, several excellent manuscripts that review the latest MS-based approaches for food-borne allergen detection have been published.[78–81]

2.3. Nucleic acid-based methods

These analytical methods rely on the detection of allergen-coding genes. Despite being an indirect detection method, it presents several excellent advantages over the detection of the allergen itself. On the first hand, DNA is much stabler than proteins, which are commonly denatured if extreme conditions (temperature, acidity, etc.) are applied during food processing. The consequent conformational change of protein epitopes aborts antibodies' targeting, which leads to false-negative results. On the second hand, DNA is also less affected

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by allergen extraction methods, which are typically based on using acidic solutions and mechanical forces. Therefore, nucleic acid-based detection methods are compatible with a wide range of allergen extraction protocols, and are typically related to higher recovery efficacies when challenged with commercial food products.[82],[83]

2.3.1. End-point PCR

End-point PCR is the simplest and oldest way to analyse PCR products, which are usually visualised by gel electrophoresis to determine their size and relative quantity.[84] This technique is often applied for cloning, sequencing, genotyping and sequence detection. In the past few years, end-point PCR has been applied mainly for multiplexing purposes to detect food allergens. Multiplex PCR assay is based on using several primer pairs that are specific to multiple targets, and to enable their amplification in a single reaction, which thus lowers assay costs and time. However, differences in each target's amplification efficiency and issues related to primers competition mean that optimising PCR conditions an essential requirement.

Suh *et al.* developed a PCR assay for the simultaneous detection of tomato, apple, peach and kiwi allergen-coding genes. Primers were designed to amplify products with no more than 200 bp as this enables assay applicability in processed foods where DNA is commonly degraded to small fragments.[85] Moreover, the annealing conditions gave optimum specificity and sensitivity when performed at 62ºC for 20s. Amplicons were analysed by gel electrophoresis. The specificity assay evidenced that primer pairs exhibited no cross-reactivity when evaluating 23 plant species. The sensitivity assay revealed that multiplex PCR provides a 10-fold lower LOD compared to single-analyte PCR. Moreover, the LOD achieved when testing DNA mixtures (0.08 ng) enables multiplex PCR to be applied to evaluate commercial food samples.[86]

Alternatively, the same author proposed employing capillary electrophoresis instead of gel electrophoresis for evaluating amplicons after multiplex PCR assays. The reason for this is that capillary electrophoresis provides a higher resolution of separated DNA fragments and is able to represent DNA concentrations in complex mixtures. Along these lines, the authors applied this approach to simultaneously detect tropomyosin allergens from oyster, mussel, abalone and clam mollusc species. Specific primers were designed to amplify tropomyosin-coding genes and the 18s rRNA gene, which is universal of eukaryote species and serves as a

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positive control. In order to minimise non-specific interactions, several parameters were optimised, such as Taq DNA polymerase concentrations and PCR buffer. The multiplex PCR assay proved highly specific because no false-positive amplicons were produced when evaluating non-target species. Besides, the achieved sensitivity (0.016 ng) is comparable to that reported for similar detection methods. Interestingly, these authors performed two sample pretreatments to enhance DNA recovery efficiency when evaluating 19 commercially available processed seafood products. Firstly, samples were washed with water prior to DNA extraction to remove any potential PCR inhibitors. Secondly, a double amount of lysis buffer was used for target DNA extraction. The tropomyosin allergen was successfully detected in seafood products, which proved the practicability of the developed multiplex PCR assay for the simultaneous detection of allergenic mollusk species.[87]

To date, eight was the maximum number of allergen-coding genes detected in a single reaction.[39],[88] Cheng and co-workers recently developed a decaplex PCR assay, combined with capillary electrophoresis, for the simultaneous detection of 10 common food allergen-coding genes from hazelnut (*Cor a 1*), pistachio (*2S albumin*), oat (*Avenin*), sesame (*2S albumin*), peanut (*Ara h 2*), cashew (*Ana o3*), barley (*B1 hordein*), wheat (*Gliadin*), soya bean (*Gly m Bd28K*) and pecan (*11S-1*) **(Figure 3 A)**. The designed PCR primers had similar Tm values and produced amplicons with lengths shorter than 200 bp, which is recommended when evaluating processed foods.[85] Interestingly, the optimal annealing temperature for the primers in the multiplex assay was several °C lower than in simplex assays. This suggests that not all assay optimisations must be performed individually for each set of primers as optimum PCR conditions vary when performing multiplex detection. The decaplex assay proved able to maintain specificity for the target allergen's detection, despite the presence of the other nine primers. The achieved LOD (0.005% w/w) was lower than those reported for other multiplex PCR approaches. This assay proved also reliable as similar results were attained in three different laboratories.[89]

2.3.2. Multiplex ligation-dependent probe amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) appeared in 2002 as an alternative to multiplex PCR because it offers advantages in terms of cost-effectiveness (probes are less expensive), flexibility to increase the multiplexing level, is easy to use, as well as higher

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reproducibility and throughput.[90] Recently MLPA has been applied for the simultaneous detection of five food allergens: sunflower, poppy, flaxseed, sesame, soya. Specific ligation probes were designed to amplify the nuclear ITS1 region of ribosomal DNA, which is species-specific. Another set of probes was also designed to target 18S rRNA in order to serve the assay a positive control. Amplicons were analysed by capillary electrophoresis **(Fig. 3 B)**. The specificity of the designed probes was the first thing to be assessed by evaluating DNA extracts from 46 plant species and four animal species. Non-specific peaks were not observed in capillary electrophoresis, so it was concluded that MLPA meets specificity requirements. Besides, the sensitivity assay revealed that the lowest concentration to be detected was 10 mg/kg, which was 10-fold higher than that achieved with TaqMan RT-PCR. Hence non-declared food was positively detected by TaqMan RT-PCR, but not by MLPA.[91]

2.3.3. RT-PCR

Real-time PCR-based methods have been established and accepted in food analyses for many years now, and represent an indirect approach for food allergen analyses by measuring allergen-coding genes, in which the analytical target is not the allergenic protein itself, but gene sequence encoding. This technology, however, enables a very specific, highly sensitive and quantitative detection of food allergens. As DNA is a very stable molecule, PCR-based real-time methods can also be applied in highly processed food matrices, but this requires sample preparation for specific DNA extraction purpose. The detection of allergenic food components can be easily integrated into existing PCR routines and delivers consistent results.

Real-Time PCR (RT-PCR), or quantitative PCR (qPCR), has been the most extensively applied DNA amplification tool since Prof. Higuchi introduced it in 1992. Its success lie in its ability to monitor the amplification of a target sequence in real-time using fluorescent labels.[84] It also enables the precise quantification of nucleic acids, even when the starting material is used at very low concentrations. RT-PCR has been employed in recent years as the reference method for the identification and quantification of allergen-coding genes in food samples.

Costa *et al.* developed a RT-PCR method coupled with a fluorescent hydrolysis probe to detect soya bean allergen in processed meat products. Primers were synthesised by Eurofins for the specific detection of the soya bean lectin gene, while DNA was extracted by the

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Wizard method. The assay gave a LOD of 10 mg/kg of soya bean in pork meat in both raw and thermally processed samples. The approach also displayed excellent applicability when evaluating 25 commercial meat samples, of which 90% showed a good agreement with the soya bean-labelled information.[92]

As an alternative to detect nuclear genes, Puente-Lelievre *et al.* developed a RT-PCR assay for detecting allergenic peanut using chloroplast genes as markers. The reason for this approach appears to be the large amount of copy numbers of chloroplast genome present in the plant cell, which confers the assay higher sensitivity and more robustness. With this strategy, the authors performed the simultaneous detection of the *matK*, *rpl16* and *trnH-psbA* target genes in a single reaction, with a LoD of 1.0 µg/mL in tomato-based sauces, chocolate and baked goods matrixes. The assay's sensitivity was at least 10-fold higher than that reported for the detection of *Arah* gene in food samples.[93] Similarly, Garino *et al.* developed a TaqMan RT-PCR based on the amplification of a chloroplast gene (tRNA-Leu) to detect pine nut traces in thermally and non-thermally treated foods. This assay proved to be highly specific and sensitive, with a LOD of 0.1 µg/mL when detecting the gene in spiked pesto sauce. Yet despite current regulations not including pine nuts on the list of allergenic nuts, the developed assay can be applied to detect pine nuts as a "hidden ingredient" in food.[94]

Xiao *et al.* created an assay based on RT-PCR to detect cow's milk α-lactalbumin gene. In order to confer the assay high specificity, the authors used the TaqMan Minor Groove Binder (MGB) probe instead of SYBR Green **(Fig. 3 C)**. The former's shorter length provides higher sequence specificity, while the latter favours the formation of primer dimers, which are often related to false-negative signals. MGB was also employed as a T_m enhancer, which is usually recommended when employing short probes. Applying higher melting temperatures provides hybridisation with enhanced stability. By this approach, the authors obtained a LOD of 0.05 ng of DNA, which enables only 2.5 mg of input material being used. No interference signals were recorded when challenging the assay with totally differently processed food from sweets to soft drinks. Hence the assay's versatility was demonstrated.[95] However when comparing different RT-PCR approaches, it should be noted firstly that MGB is expensive and, secondly, designing probes based on temperature melting predictions is extremely challenging.

2.3.3.1. High-Resolution Melting analysis (HRM)

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High-Resolution Melting analysis (HRM) has played an increasing role in food safety control and food adulteration detection since it was first introduced in 2002. This analysis technique is applied and combined with end-point and RT-PCR to identify and differentiate varieties and closely related species. Briefly, HRM is based on measuring the dissociation rate of double stranded to single stranded DNA via small increments in temperature. The operation can be summarised in three simple steps: PCR is firstly performed using specific primers; then amplicons are incubated with highly fluorescent intercalating dyes, which only emit fluorescence when bound to dsDNA; finally, temperature is slowly increased to force gradual dsDNA denaturation and to, thus, release the fluorescent dye. Sequences with minor nucleotide variations have a different melting temperature (*Tm*), which enables the generation of DNA melt curve profiles. HRM resolution allows the discrimination of two fragments that differ in a single nucleotide substitution.[96],[97]

In recent years, HRM has been applied mainly for authentication purposes, as in walnut in milk beverages,[98] gadoid fish species in fish-containing foods,[99] and PDO-certified olive oil and wine.[100] HRM has also been applied in food allergen detection as an approach to discriminate gluten-containing cereals. Tri a 18 is a wheat allergen whose encoding gene was selected as a target to identify wheat species (by RT-PCR) and its discrimination from other gluten-containing cereals (by HRM). HRM analysis was able to display wheat, rye, barley and oat in four clusters based on minor differences in their melting temperatures.[101]

2.3.4. Droplet Digital-PCR

Droplet digital PCR (dd-PCR) is also an interesting DNA-based method for determining allergenic ingredients in food. Its operation is based on partitioning target molecules into several thousands or millions of individual droplets in a water-oil emulsion. According to Poisson distribution, some droplets will contain target molecules, while others will contain no target molecules. After PCR cycling, counting the positive and negative droplet enables the absolute quantification of the target molecule. So unlike RT-PCR, standard calibration curves are not required in dd-PCR to perform the quantification of the target molecule, which makes the assay more straightforward and accurate.[102],[103] However, this detection method has one main limitation, the detection system's high price. The most widespread instrument for dd-PCR is QX200 AutoDG (Bio-Rad) which costs around $15000.

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In recent years, dd-PCR has gained popularity for detecting food allergens as it provides much higher analytical sensitivity than RT-PCR when using low concentrations of target DNA. Temisak *et al.* developed a dd-PCR assay to detect the *Arachis hypogaea allergen II* gene, which is a biomarker of peanut. DNA extraction was performed with a commercial kit and enabled peanut DNA isolation from PCR inhibitors (usually fat and oils), which are present at high levels in the peanut matrix. Besides, the optimisation of primer and probe concentrations and annealing temperature (set at 60ºC) enabled 103% PCR efficiency. Under these conditions, a LOD and LOQ of 0.015 ng/μL and 0.03 ng/μL were, respectively, achieved. The LOD was calculated as the lowest concentration detected a the 95% confidence level, while the LOQ was calculated as the lowest concentration detected with acceptable expanded measurement uncertainty below 25%. However according to Poisson distribution, the theoretical LOQ can vary depending on the number of analysed droplets.[104] In this case, other authors recommend determining the LOQ as the lowest concentration to give a %CV lower than 25%.[105] The dd-PCR assay gave an excellent correlation (R^2=0.9998) with Nanodrop instruments in terms of measured DNA copies and expected concentrations.[106] Thus dd-PCR is reliable and accurate quantification method for Peanut allergens.

Daga *et al.* developed a dd-PCR assay for detecting fish allergen by targeting 18S rDNA. The Bioedit software was used to identify highly conserved common rDNA regions of the fish species often employed in fish food. Hence the aim of the assay was to determine fish presence in food without considering any specific species. The designed primers were labelled with FAM fluorophore at the 5'-terminal and with a quencher at the 3'-terminal. The assay enabled the quantification of 0.18 pg of fish DNA when evaluating samples related to three fish species: *Gadus morhua, Salmo salar* and *Scomber scombrus*. However, in order to obtain the measured results in mass units, (which are more informative for costumers than DNA copies per microlitre, the authors were required to perform the assay under identical conditions using a reference material with a known mass concentration. In addition, the authors observed a population of droplets that were at an intermediate point between true positive and negative droplets. This phenomenon is commonly known as droplet rain and is caused by sequence variance occurring between species or non-specific amplifications.[107] In order to reduce droplet rain generation, gradient temperature annealing and changes in PCR cycling were performed.[108]

In order to obtain lower LODs and LOQs, Mayer *et al.* developed a dd-PCR assay in which they used chloroplast DNA instead of nuclear DNA for the quantification of the *Glycine Max*

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soya allergen **(Fig. 3 D)**. As discussed in the previous section, chloroplast DNA is present in number copies in plant cells, which usually contain around 40 chloroplast organelles, and each one has many DNA copies, which enables higher analytical sensitivity. In particular, the assay was designed to detect the ndhH gene, which is a subunit of the NAD(P)H dehydrogenase complex. Signal transduction was achieved by labelling primers with a TaqMan probe. Similarly to Daga's approach, a reference material containing the allergen at 40 mg/kg was used to convert the measured results into units of mass fraction. The assay enabled the detection of the DNA soya allergen at a concentration as low as 0.16 mg/kg, while the LOQ was 0.63 mg/kg. These values agree with the LODs required by experts, who claim that LODs should fall within the low ppm range when detecting food allergens. Assay specificity was also tested with DNA isolates from 72 plants, and it was proven that none yielded more DNA copies than the cut-off value. Selectivity was also evaluated by performing the assay with four different types of matrix to conclude that no matrix-specific effects were observed.[109]

Unlike previous approaches that require running n extra assay with reference material to determine the detected allergen concentration in mass fraction units, Köppel *et al.* developed an interesting strategy based on devising a conversion factor. This conversion factor considers the target DNA allergen concentration and the total DNA content (in cp/μL) in order to determine the %ratio of the target DNA allergen in w/w. This approach has been validated for determining apricot adulteration in marzipan samples, which should contain almond instead of apricot. Indeed duplex dd-PCR has been used to measure two fluorescence signals at once. The specific primers for apricot DNA and for prunus species were labelled with fluorophores FAM and HEX, respectively. DNA extraction was performed with a column-based DNA purification kit. Assay specificity was tested using the samples that contained animal and plants DNA, but none showed non-specific amplification. The LOD was estimated at 1%, as this was the lowest concentration to provide measurement uncertainty below 25%. This value correlated very well with the proficiency test performed with three other dd-PCR platforms in another laboratory.[110]

2.3.5. Loop-mediated isothermal amplification (LAMP)

The LAMP assay is based on the amplification of target DNA under isothermal conditions, combined with the visual detection of amplicons by the naked eye. The former is achieved by *Bacillus stearothermophilus* (*Bst*) DNA polymerase's strand displacement ability, which

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enables amplifications to be carried out at constant temperature (60-65 C). The latter is accomplished by the turbidity-based detection of insoluble by-products (magnesium pyrophosphate) or the fluorescent-based detection of DNA-intercalating dye molecules (SYTO 9 or SYBR Green). Both of them confer LAMP with real-time quantification capabilities when using dedicated readers. The possibility of carrying out the assay at constant temperature and making naked eye evaluations enables LAMP to be applied at the point-of-care because no expensive laboratory equipment (thermocycler or electrophoresis equipment) is required. This is probably the best advantage of LAMP over other DNA-based detection methods.[111–113]

However, the LAMP assay still lacks specific optimisation guidelines, which partly contributes to make an inappropriate comparison of its analytical capabilities with those obtained by other DNA-based detection methods. For instance, sensitivity in LAMP is often evaluated using 10-fold serial template dilutions, and the results are directly compared to the sensitivities of other PCR methods.[114] In order to deal withe this issue, Garrido-Maestu *et al.* compared the selectivity and sensitivity of qPCR and LAMP for detectiong the gluten *α2-gliadin* gene in cereals. In the interest of achieving the most realistic comparison of both detection methods, qPCR and LAMP were performed in a regular RT-PCR thermocycler. In addition, as the results in LAMP are expressed as Time-to-threshold (Tt) rather than cycle of quantification (Cq), the authors developed a mathematical model to enable the more accurate calculation of Tt values. They concluded that LAMP was 60 minutes faster in its detection, and its selectivity was similar to that of qPCR. Besides, qPCR showed a 10-fold lower LOQ in wheat and corn flour samples.[115]

LAMP has also been applied to detect peanut allergens in processed food. In this case, the above authors used the corresponding heating block, which kept a constant temperature (55-64ºC) for the 60 minutes that amplification lasted. LAMP primers were designed to amplify the *Ara h1* sequence and the internal transcribed spacer (ITS) of ribosomal DNA. Interestingly, the authors opted for gel electrophoresis to perform the detection of LAMP products rather than using other simpler, faster and cheaper detection methods. Moreover, the use of ethidium bromide for DNA staining is not longer the best option given its potential health risk. The specific and sensitive detections of peanut allergen (LOD of 1 pg and 100 pg for ITS and *Ara h1* detection, respectively) was achieved when evaluating 13 commercial foods. So the assay proved valid for precise peanut allergen identification.[116]

The same authors developed a LAMP assay for detecting mango in processed food. Evidence showing that proteins in mango are responsible for causing allergy is insufficient. Therefore,

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Shed and colleagues designed primers to amplify ITS of ribosomal DNA, which is the most popular target chosen for mango identification purposes. Similarly to the previous approach, these authors performed amplification at a temperature within the 55-64ºC range for 60 minutes. The assay was not affected by temperature oscillations as all the temperatures within that range enabled the generation of LAMP products at similar concentrations. Moreover when evaluating different heat-processed mango samples, the boiling process (up to 120ºC) did not influence mango authentication by LAMP and PCR. Conversely to LAMP, PCR was strongly affected by steam autoclaved mango samples.[117] LAMP showed equal sensitivity to PCR (LOD of 1 ng), but can be further improved by using loop primers, which are reported to be sensitivity enhancers.[118]

Mao et al. have reported using LAMP for pistachio Pis V 1 allergens in food samples. Highly specific LAMP primers were designed to detect the Pis V 1 coding gene, which enabled the gene to be detected at concentrations as low as 10 mg/kg in pistachio-containing wheat flour. This method proved to be 10-fold more sensitive than conventional PCR. Test adaptability in real scenarios was evaluated by analysing 92 commercial food products with no pistachio labelling. Of these, Pis V 1 was detected in 11 commercial foods, which means that the developed approach offered high applicability for the on-site detection of pistachio in foodstuff.[119]

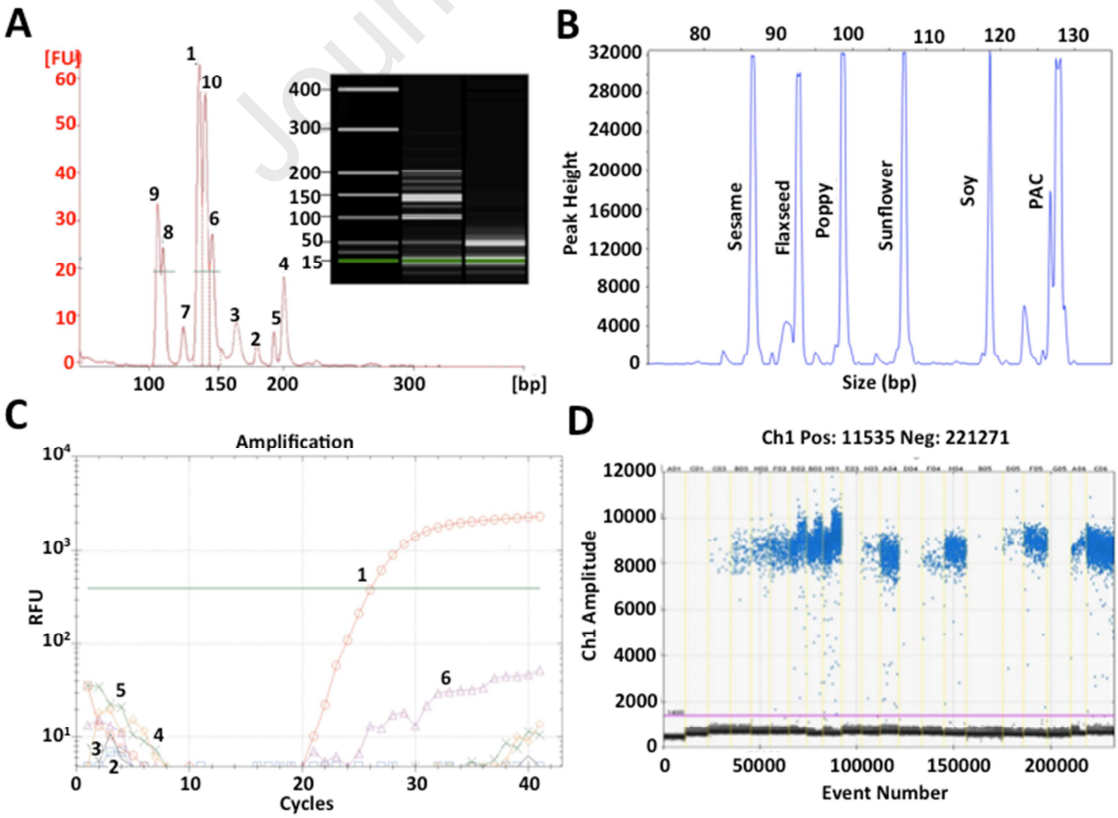

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Figure 3. Nucleic acid-based foodborne allergen detection methods. (A) End-point PCR for the simultaneous detection of 10 food allergens. Evaluation by gel electrophoresis. Peak 1: Hazelnut; Peak 2: Pistachio; Peak 3: Oat; Peak 4: Sesame; Peak 5: Peanut; Peak 6: Cashew; Peak 7: Barley; Peak 8: Wheat; Peak 9: Soya bean; Peak 10: Pecan. Adapted with permission from ref. 71, Copyright 2016 Elsevier. **(B)** MLPA combined with capillary electrophoresis for the simultaneous detection of sunflower, poppy, flaxseed, sesame and soya allergens, plus the positive amplification control. Adapted with permission from ref. 73, Copyright 2017 Elsevier. **(C)** RT-PCR method using the TaqMan minor groove binder to detect α-lactalbumin in cow's milk (1), goat's milk (2), soya bean milk (3), peanut (4), hazelnut (5) and Atlantic salmon (6). Adapted with permission from ref. 77, Copyright 2016 Elsevier. **(D)** dd-PCR for the detection of the glycine max soya allergen in food samples. Positive droplets (blue) and negative droplets (black) are clearly seen in two clusters.

2.3.6. Commercial nucleic-acid based kits

Similarly to immunoanalytical methods, several companies offer commercial kits for the detection of food-borne allergen-coding genes. Interestingly, they are all based on RT-PCR. For instance, R-Biopharm has launched RT-PCR kits for the determination of crustacean, fish, molluscs, celery, lupin, mustard, oak and buckwheat allergen-coding genes. It is noteworthy that this firm has also developed two quadruplex kits for the simultaneous detection and differentiation of wheat, barley and rye DNA sequences on the one hand, and macadamia, Brazil, pecan nuts and internal amplification control on the other hand. The former uses five fluorescent probes (FAM, VIC, HEX, ROX, and Cy5) and provides a LOD of 1 mg/kg when using its DNA extraction kit. The latter employs the same fluorescent probes and allows a LOD of 0.4 mg/kg with its DNA extraction kit. Besides, it shows cross-reactivity with DNA extracts from shagbark hickory.[120]

Biotecon Diagnostics has also developed RT-PCR kits for celery, gluten, hazelnut, peanut and soya allergen-coding genes. All these kits use hydrolysis probes and enable LODs of 0.1 µg/mL, 0.1 µg/mL, 1.0 µg/mL, 1.0 µg/mL and 0.1 µg/mL, respectively. Quantification can be performed when an allergen reference material is employed. The assay's repeatability in relation to the standard deviation is lower than 30% and 60% when detecting high (800 µg/mL) and low concentrations (1 µg/mL) of allergens, respectively. In addition, the specificity assays done with several plant and animal species, as well as commercial food products, have no cross-reactivity with other food ingredients.[121]

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Kogene Biotech offers both End-point PCR and RT-PCR tests for the detection of peanut, shrimp, pork, egg, soya bean and milk allergen-coding genes. The former kit is delivered with a positive control and loading dyes for electrophoresis, while the latter comes with an internal amplification control, a positive control and two fluorophore probes: FAM and VIC. This company does not provide any information on the analytical performance of its detection kits.[122]

3. Biosensors for food-borne allergen detection

Biosensors have been applied to a wide range of fields thanks to their excellent versatility in bioreceptors, materials and transduction modes terms. In the food processing industry, biosensors have emerged as an alternative to the laborious, expensive and time-consuming spectroscopy and chromatography assays used for food authentication and safety monitorin purposes. These include the detection of pathogens, pesticides, additives and allergens in food.[123–125] Biosensors can be classified according to the signal transduction mode, which can be optical, electrochemical, mass, magnetic, calorimetric or micromechanical, and the first two are the most frequently used. In this section, we review the most relevant biosensors developed for food allergen detection purposes in the last few years.

3.1. Optical biosensors

During optical detection, the transduced light signal can be generated directly by either the interaction of the target analyte with the transducer (label-free biosensors) or by the use of labels coupled to the bioreceptor (label-based biosensors).[126] There is a group of various optical phenomena useful for detection in biosensors. The most widely used can be classified upon the signal response, such as: absorption, reflection, refraction, dispersion, colorimetry, fluorescence, chemiluminiscence, phosphorescence, interferometry, surface-enhanced Raman scattering.[127]

3.1.1. Colorimetric biosensors

The easy operation and rapid reading of colorimetric biosensors make them particularly appealing. The transduction method is based on colour change related to the biorecognition of

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the target analyte. This can be visualised directly by either the naked eye or dedicated readout systems.

AuNPs are the most investigated colorimetric labels for their low cost, fast synthesis and simple functionalisation with either protein or oligonucleotide bioreceptors.[128] Yuan *et al.* developed a biosensor based on the hybridisation chain reaction coupled with AuNPs for peanut, sesame and soya bean DNA detection in commercial food samples. The principle of the biosensor is based on the ability of ssDNA to attach to the surface of AuNPs, while dsDNA are unable to do so. Two hairpin probes were designed to specifically target the genes of the three allergens. In the absence of the target allergens, hairpins are able to attach to the surface of AuNPs and avoid their aggregation upon the addition of NaCl (60 mM). Conversely in the presence of the target allergens, hairpin probes hybridise with them. Consequently AuNPs aggregate, which leads to a colour change from red to light purple due to the red shift in the maximum absorbance peak. The biosensor has a proven sensitivity as low as 0.5 nM **(Fig. 4 A)**.[129]

Alternatively, Yuan and colleagues designed a colorimetric biosensor based on integrating LAMP into a microfluidic chip for the simultaneous detection of peanut, sesame and soya bean allergen DNA in commercial food products. The use of NueRed dye, which is a common pH indicator, served as a reporter of allergen detection. In the absence of allergens, the pH of the solution was 8.8 and was light brown in colour. Besides, the detection of the target allergens by specific primers induced a LAMP reaction and the consequent production of hydrogen ions, which gradually increased pH. This was monitored by the colour change of the solution, which went from light brown to pink **(Fig. 4 B)**. The LOD was 0.4 ng/µl, which means it is comparable to the typical Taq-man RT-PCR. The biosensor enabled the accurate detection of the three allergens in 60 minutes.[130] Maquiera´s group developed a DNA microarray approach for the simultaneous detection of traces of hazelnut (*Corylus avellana L.*), peanut (*Arachis hypogaea*), and soya bean (*Glycine max*) in food. After DNA extraction, multiplex PCR was set up using 5′-labelled specific primers for the Cor a 1, Ara h 2, and Le genes, respectively. Digoxin-labelled PCR products were detected by hybridisation with 5′-biotinylated probes immobilised on a streptavidin-modified DVD surface.[131]

Badran and co-workers developed a multiplexed competitive immunoassay with a DVD microarray format for the simultaneous determination of gliadin, casein, β-lactoglobulin and ovalbumin in spiked baby foods, juice and beer. AuNPs were used as labels and the signal

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was amplified by the silver enhancement method. The biosensor gave LODs of 0.04, 0.40, 0.08 and 0.16 mg/L for gliadin, casein, β-lactoglobulin and ovalbumin, respectively.[132]

3.1.2. Fluorescence-based biosensors

Fluorescence-based biosensors consist in coupling a fluorescent responsive molecule to the bioreceptor. Fluorescence is based on photon emission after light absorption. Therefore, a short wavelength light source is required to initiate electronic transition in the fluorescent molecule.[127] Zhang *et al.* developed a robust platform based on the ability of graphene oxide (GO) to naturally adsorb and desorb unfolded and folded ssDNA, respectively. The system was validated for the detection of tropomyosin in buffer solution using highly specific aptamers. The operation mode is simple; GO can adsorb tropomyosin and aptamers, but not the tropomyosin-aptamer complex. Therefore, the concentration of tropomyosin in solution can be quantified upon the addition of Oligreen ssDNA reagent, which can emit only fluorescence after interacting with desorb aptamers. The biosensor achieved a 4.2 nM LOD with a working range from 0.5 to 50 µg/mL of tropomyosin.[133] Alternatively, Fu and co-workers designed a fast universal biosensor based on the Förster Resonance Energy Transfer (FRET) mechanism to detect ovalbumin (OVA). Antibody-modified carbon dots doped with nitrogen, oxygen and phosphor were used as energy donors, while GO was employed as an energy acceptor. The biosensor displayed a linear response to OVA from 0.5 to 15 µg/mL with a LOD of 153 ng/mL. It was successfully applied to determine the allergen in egg white powder, with recoveries ranging from 99.25 to 118.90%.[134]

Weng *et al.* also took advantages of the FRET mechanism by using aptamer-conjugated CdSe@ZnS QDs and GO as the energy donor and acceptor for the detection of the Ara h1 peanut allergen. GO was adsorbed on a microfluidic chip, which promoted homogeneous sample distribution. In the presence of Ara h1, the aptamer changed in conformation and desorbed from GO surface, which led to fluorescence recovery **(Fig. 4 C)**. The biosensor proved that it could detect Ara h1 at levels as low as 56 ng/mL in just 10 minutes.[135] Jiang *et al.* developed a fluorescent biosensor based on mast cells as the sensing probe to detect the major fish allergen paralbumin (PV). Mast cells provide stable and accurate antigen recognition through the abundant highly-affinity surface receptors that mimic physiological conditions. Interestingly, the interaction between cell receptors and IgEs promotes the release of intracellular inflammatory mediators, which enables the detection of PV at trace levels.

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However, it should be noted that mast cells respond to PV upon sensitisation with a specific IgE antibody. Biosensors gave a LOD of 0.35 ng/mL in buffer medium and a proven consistency with other reported methods for detections in a complex food matrix.[136]

Jauset-Rubio developed an innovative biosensor to detect β-conglutin in buffer medium using highly specific aptamers. Interestingly, these authors exploited the nucleic acid nature of aptamers by performing isothermal amplification as a sensitivity enhancement strategy. Eva green fluorescent DNA binding dye was used as signal reporter during RPA. This biosensor achieve a LOD of 1.8 x 10^{-11} M, which was 3,000-fold lower than that achieved with a conventional enzyme linked oligonucleotide assay (ELONA). These authors also proved that the incorporation of a magnetic focusing step could reduce the assay time from 100 min to 25 min.[137]

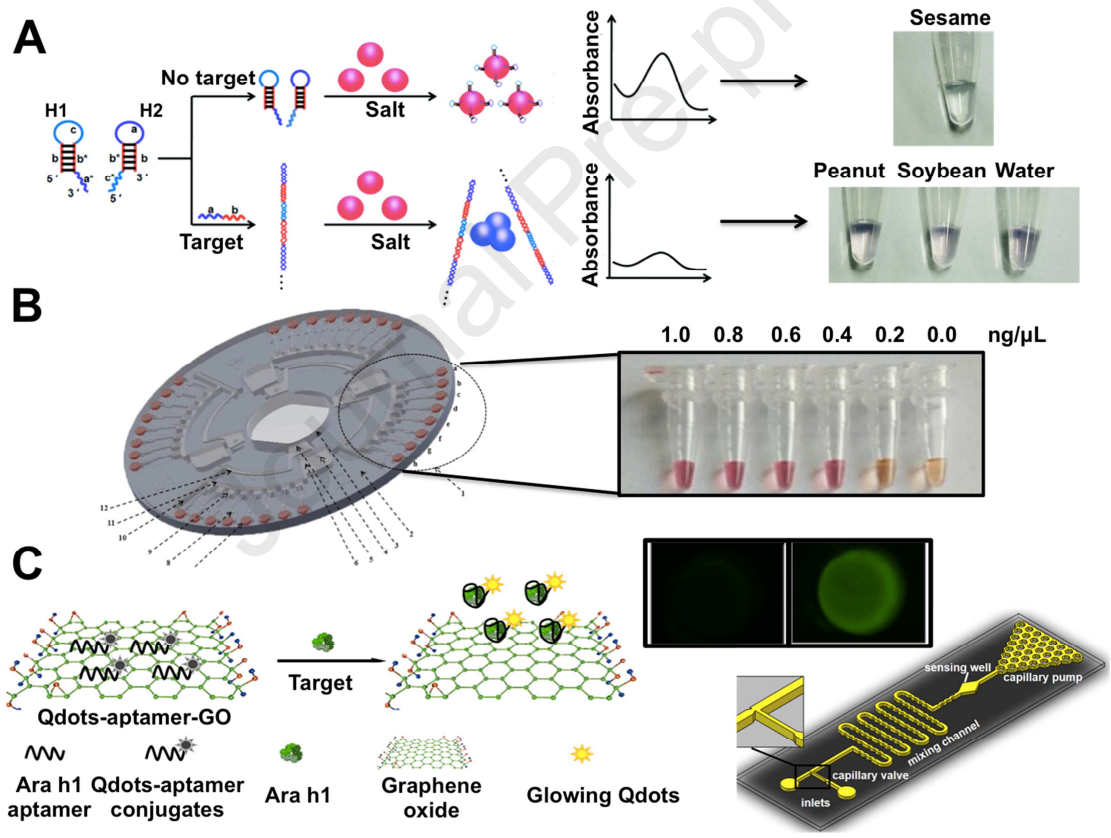

Figure 4. Optical-based biosensors for food-borne allergens detection. (A) A hybridisation chain reaction coupled with AuNPs for the detection of peanut, sesame and soya bean DNA in commercial food samples. Adapted with permission from ref. 115, Copyright 2019 Royal Society of Chemistry. **(B)** LAMP-integrated microfluidic chip for the simultaneous detection of peanut, sesame and soya bean allergens DNA in commercial food products. Adapted with permission from ref. 116, Copyright 2018 Springer Nature. **(C)**

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Microfluidic biosensor based on the FRET mechanism using CdSe QDs-functionalised aptamers and graphene oxide for detecting the Ara h1 peanut allergen. Adapted with permission from ref. 119, Copyright 2016 Elsevier.

3.1.3. Surface plasmon resonance

Surface plasmon resonance (SPR) is very interesting in the biosensing field for its distinct advantages, such as no labelling requirements, assay flexibility and versatility, together with rapid and fully automated real-time analysis. SPR has gained considerable attention in a wide variety of applications in recent years, including food allergen detection. For instance, Ashley *et al.* developed an SPR sensor for detecting β-lactoglobulin (BLG) in buffer medium. The format was based on a direct assay with a BLG polyclonal antibody-functionalised gold chip. The developed sensor showed appropriate sensitivity (LOD 0.164 μg/ml), which exceeded the required allergen detection levels for BLG (2 μg/ml).[138] Besides, these authors challenged the developed sensor with food samples to evaluate its practicability in real scenarios. Ashley and colleagues also developed an SPR sensor for α-casein detection in cleaning-in-place (CIP) final rinse wash samples. The sensor included a direct binding assay format and gave a LOD of 58 ng/ml, which is lower than the required detection levels for casein allergen in cow's milk (2 μg/ml).[139] The same authors developed an SPR sensor based on molecular imprinted polymers (MIPs) for determining α-casein levels in CIP cold wash samples collected from an ice cream pilot plant. MIPs offered a high affinity for α-casein (Kd ≈10×10^{-9} M) and selectivity. The achieved LOD (0.127 μg/mL) was superior to that of commercial ELISA kits, while the recoveries from CIP samples (87-120%) fell within an acceptable range.[140] Besides, SPR has also been applied to detect and quantify tropomyosin (TM) allergen in shellfish-containing food samples. In this case, the SPR platform was designed with a Kretschmann configuration, in which a 50-nm flat gold layer was functionalised with highly specific mAbs. The sensor was able to detect TM within 3 minutes, and its LOD and LOQ were 1 μg/mL and 2.5 μg/mL, respectively.[141] One major limitation of SPR is its difficulty to perform multiplexing. However, this has been bypassed after developing the SPR micro-matrix.

3.2. Electrochemical biosensors

Electrochemical biosensors use an electrochemical transducer to generate a signal in relation to a biorecognition event. The principle of this detection technique is the production or

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consumption of ions or electrons when the target analyte is recognised by immobilised bioreceptors. Consequently, a change in the current, potential, conductivity or other electrical parameter is recorded.[142]

In recent years, two electrochemical-based biosensors have been developed for food-borne allergen detection purposes. On the one hand, Pereira-Barros *et al.* designed a disposable amperometric biosensor for the detection of the Sola I 7 tomato allergen-coding gene in real food samples. This biosensor consisted in a screen-printed carbon electrode (SPCE) functionalised with a magnetic bead-conjugated specific RNA capture probe (RNACp). RNA detector probes and specific DNA/RNA primary antibodies were employed to detect a fragment of the Sola I 7 allergen-coding sequence, while anti-IgG-HRP was used as secondary antibodies. The biosensor principle was based on the magnetic focusing of the sample on the SPCE working electrode, followed by amperometry detection by a HQ/HRP/H_2O_2 system **(Fig. 5 A)**. Interestingly, the developed biosensor gave a LOD of 0.2 pM and its assay time lasted 90 minutes without having to perform PCR.[143] On the other hand, Angulo-Ibáñez *et al.* developed a similar strategy based on an immunoassay for shrimp tropomyosin (TPM) determination in food samples using an inexpensive disposable SPCE. Specific antibodies against TPM were conjugated to magnetic particles, which were used for the sample pre-concentration, while a secondary antibody was conjugated with HRP and enabled the amperometric detection of TPM by the H_2O_2/HQ system. The immunosensor achieved a LOD of 0.047 µg/L and proben excellent applicability in raw and cooked marketed food samples.[144] Similarly, Ruiz-Valdepeñas *et al.* developed an immunosensor for Ara h 2 peanut allergen by combining SPCE technology with the magnetic pre-concentration of the sample and the amperometric transduction mode based on HQ/HRP/H_2O_2. The immunosensor showed a wide working range of 10^4 orders of magnitude and an interesting LOD of 0.026 µg/L, which enabled trace amounts of the allergen to be detected in wheat flour samples.[145] Lin *et al.* developed a magneto-chemical biosensor with integrated antigen extraction, coupled with an electronic key-chain reader (iEAT). This assay firstly comprised an allergen extraction step using a disposable kit based on immunomagnetic enrichment and, secondly, a detection step that employed specific antibodies labelled with HRP. The oxidation of TMB and the reduction of H_2O_2 generated an electrical current, which was measured by SPCE **(Fig. 5 B)**. The biosensor was validated for the detection of peanut (Ara h 1), hazelnut (Cor a 1), wheat (Gliadin), milk (Casein) and egg (Ovalbumin) allergens, and gave a LOD of 0.1 mg/kg in just 10 minutes.[146]

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Alves *et al.* designed electrochemical biosensors based on the voltammetry signal transduction mode to detect the Ara h 1 peanut allergen. These authors generated gold nanoparticles directly on the surface of the working electrode of a SPCE and detected the allergen by employing specific antibodies conjugated with alkaline phosphatase, which catalyse a metal precipitation reaction than can be measured by anodic voltammetric potential scanning. The biosensor achieved an LOD of 3.8 μg/L and was able to detect Ara h 1 in food samples containing 1% peanut.[147]

Interestingly, Jiang and colleagues developed a portable microfluidic biochip to mimic the allergen detection mechanism of the human intestine. Briefly, RBL-2H3 mast cells and ANA-1 macrophages were co-cultured on a PDMS chip containing four groups of gold electrodes. Cell-secreted inflammatory cytokines were measured by cell impedance changes upon the addition of the allergen stimulus. The biosensor was validated to detect the mice dinitrophenyl allergen as a proof-of-concept. It gave a LOD of 10^{-1} μg/L and correlated well with the ELISA assay. The developed platform is interesting for real-time food allergen research.[148] Donglei Jiang *et al.* also developed a novel rat basophilic leukaemia cell (RBL-2H3) biosensor based on the electrochemical transduction mode. Cells were transfected with cationic fluorescent magnetic beads, which enabled cells to be isolated from the sample medium upon magnetic focusing on the working electrode. Anti-Pen a1 IgE and Anti-PV IgE-activated cells were employed to quantify both shrimp allergen tropomyosin (Pen a 1) and fish allergen parvalbumin (PV) with a LOD of 0.03 μg/mL (Pen a 1) and 0.16 ng/mL (PV), respectively. The strategy is convenient for removing electrodes and has proven applicability with real food samples.[149]

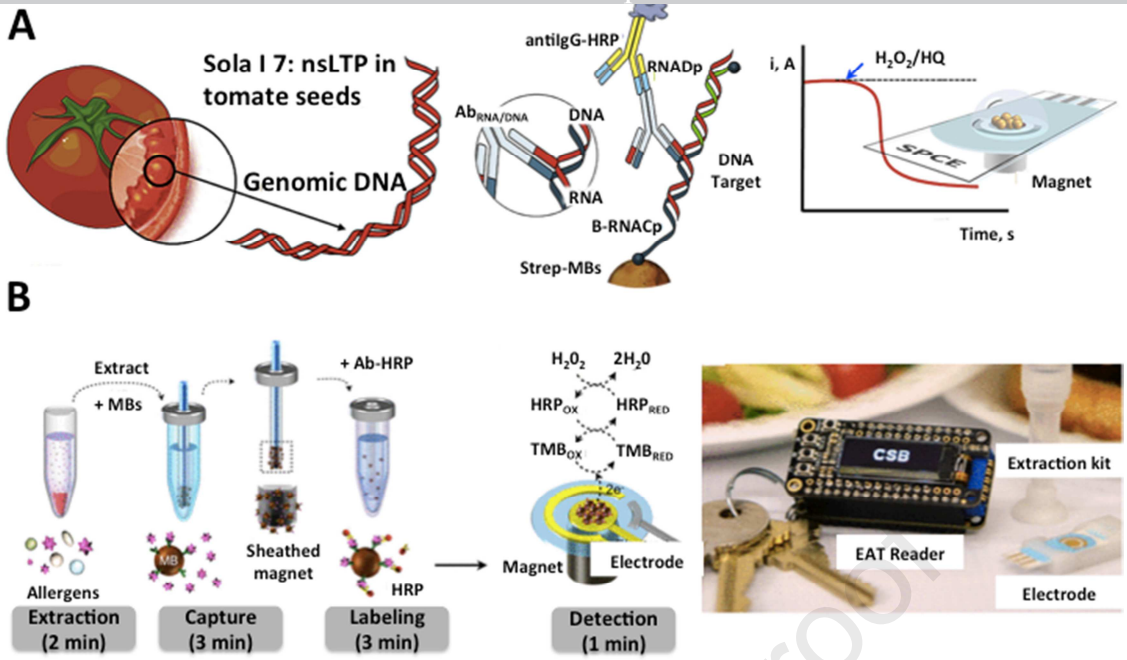

Figure 5. Electrochemical-based biosensors for food-borne allergens detection. (A) An amperometric biosensor based on DNA/RNA sandwich hybridisation for Sola I 7 tomato allergen detection. Adapted with permission from ref. 122, Copyright 2019 Elsevier. **(B)** An integrated exogenous antigen testing (iEAT) biosensor with integrated allergen extraction and a key chain reader for the simultaneous detection of peanuts, hazelnuts, wheat, milk and egg allergens in commercial food samples. Adapted with permission from ref. 123, Copyright 2017 American Chemical Society.

3.3. Biosensors based on alternative signal transducers

Apart from optical and electrochemical classic biosensing detectors, other types of signal transduction modes have been recently applied for food-borne allergen analyses. For instance, Ng and co-workers developed a giant magnetoresistive (GMR) biosensor for the simultaneous detection of major peanut allergens Ara h1 and Ara h2, and wheat allergen Gliadin. The operation mode was based on the functionalisation of the GMR biosensor with capture antibodies, which recognised the target allergens that formed a sandwich with detector antibodies. The latter were conjugated with magnetic nanoparticles to generate a localised magnetic field, and consequently led to a change in the sensor's resistance **(Fig. 6 A)**. They gave LODs of 7.0 ng/mL, 0.2 ng/mL and 1.5 ng/mL for Ara h1, Ara h2 and Gliadin, respectively, which were one order of magnitude lower than those of ELISA assays.[150]

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Angelopoulou *et al.* developed an optoelectronic platform for the simultaneous determination of bovine milk protein, peanut, soya and gliadin allergens in rinse water samples from a cleaning-in-place system (CIP). The platform consisted in an array of 10 Mach-Zehnder Interferometers (MZIs) and LEDs integrated into a silicon chip. Besides, continuous MZI transmission spectra were measured with a miniaturised spectrophotometer. In addition, the chip was combined with a microfluidic module to enable uniform delivery or reagents and, thus, the repeatability of the results improved **(Fig. 6 B)**. Highly specific antibodies were raised against allergens. The biosensor gave LODs of 1.0 µg/mL, 0.04 µg/mL, 0.8 µg/mL and 0.1 µg/mL, for peanut protein, bovine k-casein, soya protein and gliadin, respectively. These were lower than, or at least comparable to, those achieved with commercial ELISA and LFA kits. Interestingly, the assay time was only 6.5 min and the immunosensor can be reused for at least 10 times, which provides an assay cost of $0.16 per analysis.[151]

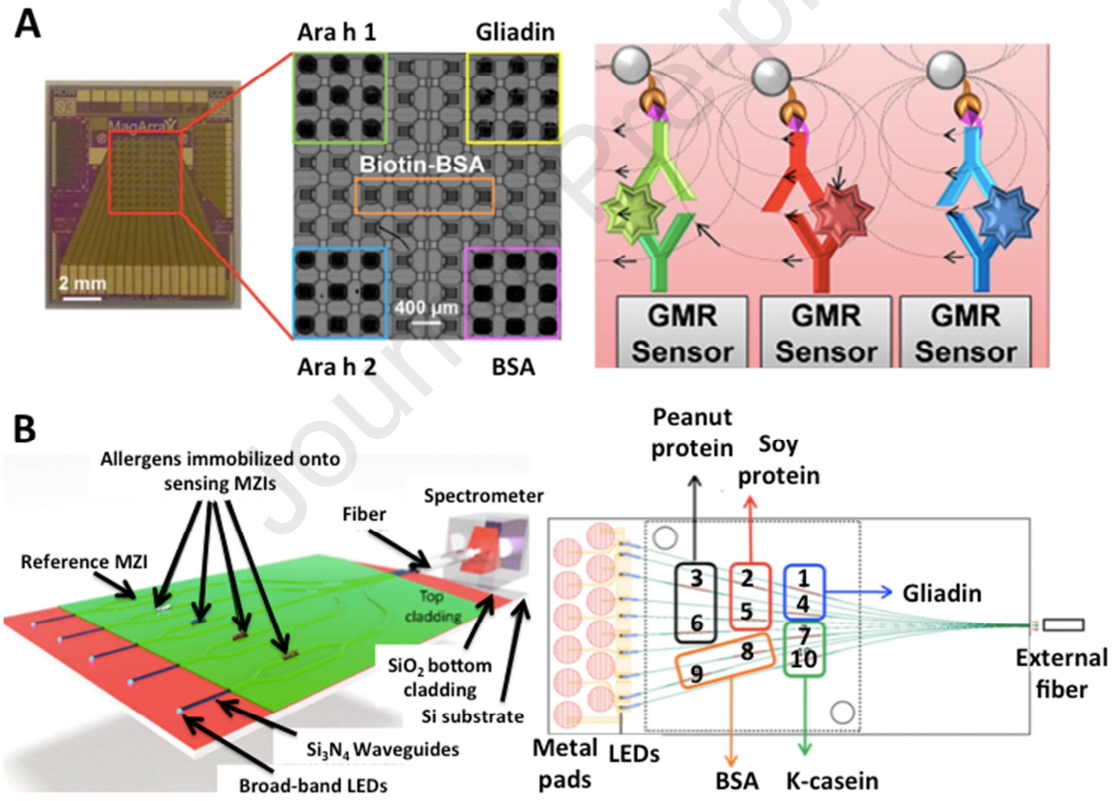

Figure 6. Biosensors based on alternative signal transducers for food-borne allergens detection. (A) A giant magnetoresistive biosensor for the simultaneous detection of major peanut allergens Ara h1 and Ara h2, and wheat allergen Gliadin. Adapted with permission from ref. 124, Copyright 2016 Elsevier. **(B)** An interferometric biosensor coupled with a microfluidic platform and a miniaturised spectrometer for the simultaneous detection of bovine milk protein, peanut, soya and gliadin allergens in rinse water samples from a cleaning

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-in-place system (CIP). Adapted with permission from ref. 123, Copyright 2018 American Chemical Society.

4. Challenges to be addressed

Challenges and barriers in developing food allergen detection methods are priority areas that need further motivation to improve the detection process. Improving detection methods would be a major advance for food allergic patients who must eat diets without allergens. The current food-borne allergen detection methods and biosensors are subject to several limitations, which somewhat hinder their applicability in real scenarios. For instance, this review discusses the analytical capabilities of the developed approaches, and leaves aside one of the most important steps of the analytical method: the sample extraction procedure. Despite seeming separate from the assay detection step, allergen extraction considerably influences the detection method's ability to succeed and should, thus, also be carefully optimised while the assay is underway.[152] The allergen extraction process usually consists of mechanical food trituration, guided by allergen solubilisation using an appropriate extraction solution, and finally a purification step based on centrifugation and/or filtration. The extraction solution must be selected according to the allergen's biological nature, with aqueous buffers, saline buffers and alcoholic solutions for the solubilisation of albumins, globulins and prolamines, respectively.

However, a challenge emerges when the simultaneous detection of several allergens is the aim. In this case, a "universal" extraction buffer should be used, in which pH and ionic strength are compatible with all allergens to ensure the quantitative co-extraction of different allergens from the matrix.[153] As allergens are structurally different proteins, distinct extraction procedures are required. If testing for multiple allergens, the extraction buffer should allow a compromise between quantitative extraction and the capability to co-extract several allergens form the food matrix. In fact, the standardisation of allergen extraction protocols is of key importance for food-allergen detection in a multiplex detection format. It is also noteworthy that a biosensor's analytical performance is usually worse when performing multiplexing for two main factors: firstly, the combination of bioreceptors with different optimal assay requirements, such as working buffer, biorecognition time or detection working ranges; secondly, undesired competition between bio-reagents, which might interact in-specifically and promote signal reduction and reproducibility issues. Hence, the selection of highly specific bioreceptors, such as nucleic acids probes, is completely recommended so

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as to avoid cross-reactivity. Moreover, the selection of bioreceptors with similar binding affinities is most interesting to achieve comparable assay conditions that can favour the simultaneous detection of several analytes in a single step.

Food processing is another issue related with food-borne allergen detection methods with a dramatic effect on both allergen extraction efficiency and allergen detection. Although it is true that most of the reported nucleic-acid based approaches present in this review are challenging, their detection methods with ultra-processed food samples, their proven excellent applicability and immunoanalytical methods still offer low recoveries with such food samples. Food processing causes the denaturation of allergens, which alters their detection with bioreceptors by modifying assay results. However, there are reports that food processing can also reduce the allergenic potential of protein, and consequently lower the incidence of sensitisation and allergic diseases. Therefore, would it be at all interesting to detect an uncertain allergenic compound that no longer poses a health risk? The answer is yes. Processing may influence, but not abolish, the allergenic potential of proteins. According to the food processing type, protein allergenicity can be modified to a greater or lesser extent, while microbial fermentation, and enzymatic and acid hydrolysis reduce protein allergenic integrity the most [154] Despite nucleic acid-based methods being less susceptible to food processing-related issues (due to the high stability of nucleic acids), it is noteworthy that immunoanalytical approaches are still the only methods capable of informing about the presence of allergens. Conversely, nucleic acid-based techniques report[155] the presence of an allergen-codifying gene without providing direct information about food's allergenic capacity.

Another major issue for detecting allergens in food products is the analytical method's robustness. Method validation is essential for providing reliable results, which can be comparable among different laboratories. To this end, the development of certified reference materials (CRMs) in different matrices is highly desirable. However, lack of CRMs for most target allergens in particular matrices makes it very difficult to standardise analytical protocols. Today different standards are used for the semi-quantitative and quantitative determinations of distinct allergens. Their use is fundamental for calibration, validating methods, proficiency testing and quality control. This highlights the need for reference materials that should be made available to develop standards. Quality of standards must be assured to compare the results of analyses. This is why the MoniQA association is leading the task to address certification issues. In addition, in 2017 it launched together with R-biopharm the first validated reference material to be developed for milk allergen determinations.[156]

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While there are currently several commercially available standards for gluten (PWG-Gliadin) detection, differences in their protein content have been found, which lead to wide intra- and inter- assay variability when validating analytical methods.[157]

The sensitivity of existing methods is another challenge for detecting increasingly smaller amounts of food allergens. Likewise, it would be desirable to increase the reproducibility of the extraction and the subsequent detection of allergens. To do so, reference materials or standards are essential for achieving homogeneity in analytical results. Therefore, the development of certified reference materials for other food allergens is envisioned in forthcoming years. This can be achieved by producing recombinant versions of allergens using molecular biology techniques, which would allow the research community to standardise semi-quantitative and quantitative methods, and would provide key best practices for allergen management and labelling guidance for the food industry.

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Table 2. Comparison of immunoanalytical-based methods for food-borne allergens detection.

Format	Allergen	LOD (µg/L)	Sample	Comment	Ref.
Non-competitive	Ovalbumin	0.51	Egg	Enhanced selectivity due to the use of specific antibodies	**[19]**
Non-competitive	Hazelnut	1,000	Chocolate	Use of milk powder and diluted samples to reduce the matrix effect	**[20]**
Non-competitive	Orangeprofiling	1.82	Navel oranges	Enhanced selectivity due to the use of specific antibody	**[21]**
Non-competitive	Ara h2, Ara h6	1.3, 0.7	Milk	Signal amplification via the biotin/strep. detection system	**[22]**
Non-competitive	β-lactoglobulin	0.49	Hydrolysed infant formulas	Fluorescence detection using H_2O_2-sensitive CdTe QDs	**[23]**
Competitive	β-casein	290	Raw and processed foods	Detection based on an indirect competitive immunoassay	**[24]**
Competitive	Gly m Bd 28K	0.235	Soya bean seeds, soya bean protein isolate, soya bean meal, tofu, soya milk, soya sauce, natto, sufu and lobster sauce	Indirect competitive assay coated with Gly m Bd 28K and blocked with GaMIgG-HRP	**[27]**
Non-Competitive	Soya	9,000	Meat products	Costless enzyme immunoassay	**[28]**
Competitive	β-conglycinin	0.90	Spiked and incurred model foods	Non-competitive more sensitive than competitive	**[29]**
Non-competitive	Tropomyosin	2,000	Raw and cooked food	Sandwich ELISA kit Cross-reactivity	**[31]**
Non-competitive	Ara h 1, Ara h 2	30	Peanut butter	Cross-reactivity	**[33]**
Non-competitive	Hazelnut	160	Not specified	-	**[34]**
Not specified	β-lactoglobulin, tropomyosin, ovalbumin, gluten, hazelnut, and almond	1.5, 1.7, 4, 3, 300 and 200	Not specified	Lower cross-reactivity	**[35]**
Not specified	Gluten	-	Beer, soya-based sauces, vinegar and sourdough bread	Classification of allergens based on proteolytic fermentation processes	**[39]**

Table 2. Cont. Comparison of immunoanalytical-based methods for food-borne allergens detection.

Format	Allergen	LOD (µg/L)	Sample	Comment	Ref.
Non-competitive and competitive	Modified proteins	1,000	almond milk, cashew milk, coconut milk, hazelnut milk and soya milk	Colorimetric detection using AuNPs	**[43]**
Non-competitive	Casein, ovalbumin, hazelnut	100	Commercial biscuits	Colorimetric detection using AgNPs, spherical and desert-rose AuNPs	**[44]**
Competitive	Tropomyosin	50	Crustacean	Fluorescent detection using QDs	**[48]**
Competitive	β–conglutin	8 fM	Foodstuffs	Catalytic signal amplification	**[49]**
Non-competitive	Soya protein	10,000	Soya processed food	No conjugate pad. Detection by the naked eye	**[50]**
Non-competitive	Gliadin	6,300	Gluten-containing processed food	Two coloured labels allow detection by the naked eye	**[51]**
Non-competitive	Not specified	< mg/L range	Swab Processed food	Rapid allergen extraction. Detection based on AuNPs	**[52]**
Non-competitive	Egg, milk, wheat, buckwheat, and peanut	5,000	Processed food	Detection based on AuNPs	**[53]**
Indirect phage	Gluten	20,000	Wheat, Barley, Rye, Triticale	Enhanced specificity using nanobodies	**[56]**

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Table 3. Comparison of nucleic acid-based methods for food-borne allergens detection.

Format	Allergen	LOD (µg/L)	Sample	Comment	Ref.
End-point PCR	Tomato, apple, peach and kiwi coding genes	0.08 ng	Processed food	Using gel electrophoresis	**[68]**
End-point PCR	Tropomyosin	0.016 ng	Oyster, mussel, abalone and clam mollusc species	Capillary electrophoresis-based method	**[69]**
10-plex End-point PCR	Cor a 1, 2S albumin, Avenin, Ara h 2, Ana o3, B1 hordein, Gliadin, Gly, m Bd28K, 11S-1	50,000	Hazelnut, pistachio, oat, sesame, peanut, cashew, barley, wheat, soya bean and pecan	Simultaneous detection of 10 allergen-coding genes with high specificity	**[71]**
MLPA	Sunflower, Poppy Flaxseed, Sesame, Soya	10,000	Not specified	Amplified nuclear ITS1 region by specific ligation probes	**[73]**
RT-PCR	Soya bean	10,000	Pork meat sample	Method coupled with a fluorescent hydrolysis probe	**[74]**
RT-PCR	matK, rpl16 and trnH-psbA	1,000	Tomato-based sauces, chocolate and baked foods	Detection using chloroplast genes as markers	**[75]**
RT-PCR	Pine nut traces	100	Spiked pesto sauce	Amplification of a chloroplast gene	**[76]**
RT-PCR	β-lactoalbumin gene	0.05 ng	Cow's milk	Enhanced specificity using MGB	**[77]**
dd-PCR	Arachis hypogaea allergen II	0.015	Peanut	103% PCR efficiency	**[88]**
dd-PCR	Fish allergen	0.18 pg	Gadus morhua, Salmo salar and Scomber scombrus	Labelled primers using FAM fluorophore at the 5'-terminal and a quencher at the 3'-terminal	**[89]**
dd-PCR	Glycine Max soya allergen	160	Not specified	Enhanced sensitivity using chloroplast DNA	**[91]**
Duplex dd-PCR	Apricot	10,000	Marzipan samples	Fluorescent detection using FAM and HEX fluorophores	**[92]**
LAMP	Gluten *α2-gliadin* gene	-	Cereals	60 min. faster than qPCR	**[97]**
LAMP	Ara h 1	1 pg	Processed food	Detection using gel electroph.	**[98]**
LAMP	ITS of ribosomal DNA	1 ng	Processed food	Not affected by temperature oscillations	**[99]**
LAMP	Pis V 1	10,000	Wheat flour with pistachio	10-fold more sensitive than End-point PCR	**[101]**
RT-PCR	Macadamia, Brazil, pecan nuts coding genes	400	-	R-Biopharm kit. Kits for other allergens.	**[102]**
RT-PCR	Celery, soya and gluten allergen coding gene	100	-	Biotecon Diagnostics. Includes other allergens with lower sensitivity	**[103]**

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Table 4. Comparison of biosensing-based methods for food-borne allergens detection.

Detection	Allergen	LOD (µg/L)	Sample	Comment	Ref.
Colorimetric	Peanut, sesame and soya bean	0.5 nM	Commercial food samples	AuNPs aggregation	**[111]**
Colorimetric	Peanut, sesame and soya bean	400	Commercial food products	LAMP-based microfluidic chip using NueRed dye	**[112]**
Colorimetric	Cor a 1, Ara h 2, and Le	1	Commercial food products	Multiplexed nucleic acid-based assay with a DVD microarray format	**[113]**
Colorimetric	Gliadin, casein, β-lactoglobulin and ovalbumin	40, 400, 80, and 160	Spiked baby foods, juice and beer	Multiplexed competitive immunoassay with a DVD microarray format	**[114]**
Fluorescent	Tropomyosin	4.2 nM	Buffer medium	Aptamer assay using Oligreen ssDNA reagent and GO as a fluorescence quencher	**[115]**
Fluorescent	Ovalbumin	153	Egg white powder sample	Immunoassay based on the FRET mechanism using carbon dots doped with nitrogen, oxygen and phosphor as energy donors and GO as the energy acceptor	**[116]**
Fluorescent	Ara h1	56	Peanut	Microfluidic chip based on the FRET mechanism using QDs and GO	**[117]**
Fluorescent	Parvalbumin	0.35	Buffer medium	Mast cells used as bioreceptors	**[118]**
Fluorescent	β-conglutin	0.18 pM	Buffer medium	Isothermal amplification	**[119]**
SPR	β-lactoglobulin	164	Buffer medium	BLG polyclonal antibody-functionalised gold chip	**[120]**
SPR	α-casein	58	CIP wash samples	Direct surface plasmon resonance format	**[121]**
SPR	α-casein	127	CIP cold wash samples	Using molecular imprinted polymers	**[122]**
SPR	Tropomyosin	1,000	Shellfish-containing food samples	Kretshmann SPR configuration	**[123]**
Electrochemical	Sola I 7	0.2 pM	Tomato	SPCE functionalised with a magnetic bead-conjugated specific RNA capture probe	**[125]**

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Table 4. Cont. Comparison of biosensing-based methods for food-borne allergens detection.

Detection	Allergen	LOD (µg/L)	Sample	Comment	Ref.
Electrochemical	Tropomyosin	0.047	Commercial food samples	SPCE-based immunoassay combining magnetic sample pre-concentration and amperometric signal transduction	**[126]**
Electrochemical	Ara h 2	0.026	Wheat flour	SPCE-based immunoassay combining magnetic sample pre-concentration and amperometric signal transduction	**[127]**
Electrochemical	Ara h 1, Cor a 1, Gliadin, Casein, Ovalbumin	100	White rice	Integrated antigen extraction	**[128]**
Electrochemical	Ara h 1	3.8	Commercial food samples	AuNPs-functionalised SPCE. Enzyme catalysis of metal precipitation monitored by anodic voltammetry.	**[129]**
Electrochemical	Dinitrophenyl	10^{-1}	Buffer medium	Mast cells-based portable microfluidic biochip that mimics the allergen detection mechanism of the human intestine	**[130]**
Electrochemical	Tropomyosin and Parvalbumin	30 and 0.16	Buffer medium	Mast-cells transfected with fluorescent magnetic beads	**[131]**
Magneto-resistance	Ara h1, Ara h2, and Gliadin	7.0, 0.2, and 1.5	Peanut, wheat	Localised proximity magnetic sensing	**[132]**
Interferometry	Peanut, bovine, K-casein, soya protein, gliadin	1,000, 40, 800, and 100	Rinse water samples	MZIs and LEDs integrated into a silicon chip	**[133]**

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5. Conclusions and future trends

The development of food-borne allergen detection methods is very important for the scientific community, especially as food allergy prevalence increases on a daily basis there is still no effective treatment for allergens. Thus, detection methods must be sensitive, specific, robust and reproducible. Besides, there is a need to develop cost-effective and simpler methods that can also enable the multiplexing of food-borne allergens. Of today's available current detection methods, we discuss which approaches provide the desired characteristics and, thus, offer more probabilities to succeed as commercial products.

Throughout this review, we pay special attention to the sensitivity and practicability of different detection methods. It is important to point out the factors that influence both of them. Regarding the former, the bioreceptor's binding affinity for the analyte is the main factor to determine an assay's sensitivity, while there are other aspects that might further improve analytical sensitivity, such as assay format (sandwich format is usually more sensitive that the competitive one), using signal transducers with signal amplification capabilities (chemiluminescence, fluorescence or alternative signal transducers) or assay conditions (longer incubation times, optimal pH and ionic strength of working buffers, etc.).

Several factors can generally enhance the practicability of analytical methods and biosensors for reliable on-site allergen quantification in real food samples. These mainly include using highly specific bioreceptors that overcome false-negative results and optimised assay conditions (washing buffers/steps, blocking reagents) that avoid false-positive results. Other factors exist and are related to assay ease-of-use and simplicity which can also increase the test's practicability by improving assay reproducibility and by maintaining intra- and inter-assay variability below 15%. For instance, developing single-step procedures with all the operations integrated into the device (LFA). Finally, assay stability is one of the major factors to affect a test's practicability. Bioreceptors are usually the fastest elements applied in assays to expire (with protein-based bioreceptors). Thus accelerated ageing tests must be performed to determine the test's expiry date and appropriate storage conditions should be implemented to ensure that assay stability lasts at least 1 year.

Most of the immunoanalytical methods developed in recent years are based on ELISA or LFIA. So, it is not unusual to find that they are the preferred formats when developing commercial kits. The former generally provides sensitivities within the ppb (µg/L) range, where the the non-competitive format is more sensitive than the competitive one. Contrarily, the latter offer sensitivities within the low ppm (mg/L) range, although this has not been an impediment when evaluating its applicability with commercial food products. Despite its lower sensitivity (mainly because internal incubation times are lacking), LFIA has the advantage of being cheaper and simpler than ELISA, and its operation mode is usually reduced to a single step. Besides, allergens should be present at high concentrations (ppm range) to induce an allergenic reaction. Current regulations do not include threshold values for most of the 14 food allergens, apart from 20 µg/mL in gluten-free food samples. For this reason, LFIA is expected to become the reference immunoanalytical method in forthcoming years. We can anticipate that improvements in LFIA will be related to enhance sensitivity towards ppb levels and to increase multiplexing capabilities to be able to simultaneously detect the 14 regulated food-borne allergens. We also expect the application of nanobodies or aptamers to overcome the cross-reactivity issues currently observed in commercial kits. Indeed test procedures that can be performed in short times are preferred. For this reason, ELISAs, lateral flow tests and dipsticks are very popular tests for obtaining initial information about the presence of allergic substances.

A marked trend has also been observed in recent years in relation to developing nucleic acid-based methods for food-borne allergen detection purposes. Despite being an indirect technique, it is less prone than immunoassays to false-negative results as it can successfully overcome food processing-related protein denaturation issues. So it is interesting to observe that more sophisticated PCR methods are applied for food allergen detection alternatively to end-point PCR, which is not quantitative and relies on a lengthy procedure. For instance, MLPA is a simpler method for performing multiplexing, but has no proven better sensitivity and multiplexing capabilities than end-point PCR. I would also appear that RT-PCR continues as the reference method because it is still the only one used in commercial kits. Besides, increasing developments have been made with ddPCR and LAMP methods. While the former provides the highest sensitivities, the price of the instrument is prohibitive. The latter avoids using bulky and expensive thermocyclers and can, thus, be easily applied to the

point-of-user. Therefore, we expect LAMP to gain more attention in the next few years. The improvements made to this method will focus on enhancing sensitivity and achieving a more agreed protocol. Apart from this, we will very likely evidence the first commercial kits based on LAMP in a few years time.

Furthermore, biosensors based on different signal transduction modes have also been developed for food-borne allergen detection. Indeed optical biosensors are the most widely reported, although the approach based on magneto-resistance detection has proven sensitivities within the low ppb range. Moreover, it is good to observe that improvements have been made to simplify the assay's procedure, as in the case of the iEAT device, where the allergen extraction step has been integrated. Microfluidics has been introduced in other approaches to achieve more homogeneous sample distribution, which in turn, enables higher assay reproducibility. Finally, it is also noteworthy that researchers opt for the miniaturisation of biosensors, as in the case of the spectrometer in the GMR device or the key chain format designed for the iEAT device. This will doubtlessly help to transform these methods into successful commercial products.

We also conclude that sample treatment is no doubt the backbone of the food allergen analysis. Accordingly, sample preparation (dissolution, extraction, dilution, etc.) is still the bottleneck of the analytical procedure. Extracting allergens from processed foods can be very complex, and the matrix in which the corresponding allergen is found often complicates its extraction. In addition, some substances present in certain foods can interfere with the extraction process. During food preparation, the chemical modifications that derive from following thermal and enzymatic treatments hydrolyse and modify allergenic molecules, which also complicates the extraction process. From our point of view, we must focus on these aspects to simplify the analysis in order to boost the multiplex detection of allergens. To do so, the study of universal extraction approaches, among other issues, is very interesting to quantitatively co-extract the most allergens. In line with this, a universal extraction buffer (extraction cocktail) allows the quantitative extraction of different allergens simultaneously. This is very advantageous approach as co-extraction procedures simplify the analytical procedure. However, chemical properties, the origin of allergens (animal and plant) and chemical modifications make it very challenging to follow a unique or universal extraction procedure to develop quantitative food allergen extraction procedures. The high prevalence of allergic populations to more than one allergy, due to cross-reactivity with

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similarly structured allergens, makes testing more than one allergen at one time most appealing. Thus, the co-extraction of different allergens will be extremely important.

Method validation is the other critical issue for developing reliable food-allergen analytical-based methods. Currently due to the very few commercially available reference materials and the expensive preparation of the materials specifically intended for use in food allergen testing at different allergen levels, harmonising strategies to develop and validate reliable methods is necessary to determine food allergens.

All in all, much progress has been made in the food-borne allergen detection methods, which is expected to continue as long as food avoidance remains the only way to avoid food allergy. As previously discussed, the trend in forthcoming years will not focus so much on improving the sensitivity of methods, but on achieving cheaper and simpler approaches to enable the high-throughput screening of food-borne allergens by non-specialised personnel, such as point-of-care tests for biomedical applications.

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Recent advances and challenges in food-borne allergen detection

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Highlights

- Review of recent approaches and commercial kits for food-borne allergen detection
- The applicability of allergen detection methods to food samples is stressed
- The advantages and limitations of current methods are discussed
- Future perspectives for reliable on-site food-borne allergen quantification

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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