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To cite this article: Jilin Li, Huifen Wang & Jun-Hu Cheng (2021): DNA, protein and aptamer-based methods for seafood allergens detection: Principles, comparisons and updated applications, Critical Reviews in Food Science and Nutrition, DOI: [10.1080/10408398.2021.1944977](https://doi.org/10.1080/10408398.2021.1944977)

To link to this article: <https://doi.org/10.1080/10408398.2021.1944977>



Published online: 29 Jun 2021.



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REVIEW



DNA, protein and aptamer-based methods for seafood allergens detection: Principles, comparisons and updated applications

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ABSTRACT

The increasing number of people with seafood allergy has caused a series of problems for practitioners and consumers in the seafood industry year by year. Thereby, development of efficient, convenient and low-cost allergen detection methods is urgently needed. This review introduces three important existing seafood allergen detection methods associated with DNA-based, protein-based and aptamer-based. Their principles and biological characteristics are firstly presented. The core of these three methods are DNA amplification techniques, specific binding of antigens and antibodies, and specific binding of aptamers and ligands, respectively. Among them, DNA-based detection method is an indirect analysis, which takes the gene of allergen as the detection object and is characterized by good stability and high sensitivity. Protein-based and aptamer-based, methods employ indirect analysis for allergen detection. The difference is that the latter uses an easily synthesized and highly efficient aptamer as the detection probe, showing great promising potentials. The advantages and disadvantages of the three mentioned detection methods are also discussed. In the future, as more efficient and reliable detection methods for seafood allergens come into practice, the possibility of seafood allergy patients eating seafood products by mistake will be greatly reduced, which will ensure the food safety and the health of allergy patients.

KEYWORDS

allergy; aptamer-based methods; DNA-based methods; protein-based methods; Seafood

Introduction

Seafood mainly refers to several distinct groups of edible aquatic animals including fish (such as cod, salmon), crustacean (such as shrimp, crab), and mollusk (such as squid, scallop). Seafood is an excellent source of proteins, essential vitamins such as fat-soluble vitamins (A, D), water-soluble vitamins (B3, B6), minerals like potassium, iodine and essential fatty acid, like eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA) (Daczowska-Kozon and Pan 2011; McManus et al. 2014; Ruethers et al. 2018; Sprague et al. 2017). However, adverse reactions like allergies are often associated with seafood consumption. Seafood allergy is a major global public health issue in the field of food safety, which leads to a decline in the quality of life for affected individuals and even endangers the lives of patients in the worst case, with an estimated death of 150 annually. A reliable survey from the Food and Agriculture Organization of the United Nations (FAO) showed that wheat, peanuts, soybeans, nuts, milk, eggs, fish and crustaceans are included in the most common allergic foods (FAO 1995). Seafood allergy was more common in adults compared with children (2.8% vs 0.6%) and in women compared with men according to a survey made in USA (Sicherer, Muñoz-Furlong, and Sampson 2004). Symptoms of allergies

after ingestion and exposure can appear within minutes, and they include nausea, vomiting, urticaria, asthma exacerbation, and anaphylaxis (Davis et al. 2020). Even exposure to trace amounts of allergens could be fatal to some allergic patients (Moneret-Vautrin et al. 2005). Unfortunately, there is currently no complete and reliable cure for food allergies. The most effective way to curb seafood allergies is by avoiding the ingestion of foods, which contain allergens, sensitization, proper labeling, and identification (Matricardi et al. 2016). As a result, recent advances in food processing have focused on identifying allergens in food matrices with a significant effort to develop rapid, reliable, and user-friendly allergen detection methods to facilitate risk assessment through proper management and labeling of allergenic foods. These methods allow seafood manufacturers to quickly detect and label allergens, which enables consumers' health to be guaranteed. Depending on different detection principles, the current used detection methods include three categories, namely DNA-based, protein-based and aptamer-based detection methods (Faisal, Vasiljevic, and Donkor 2019; Fernandes et al. 2015).

In 2015, Fernandes et al. (2015) reviewed the allergens of the highly-consumption seafood products and exhibited a concise compendium of the most common detection methods based on proteins or DNA, while Carrera, Pazos, and

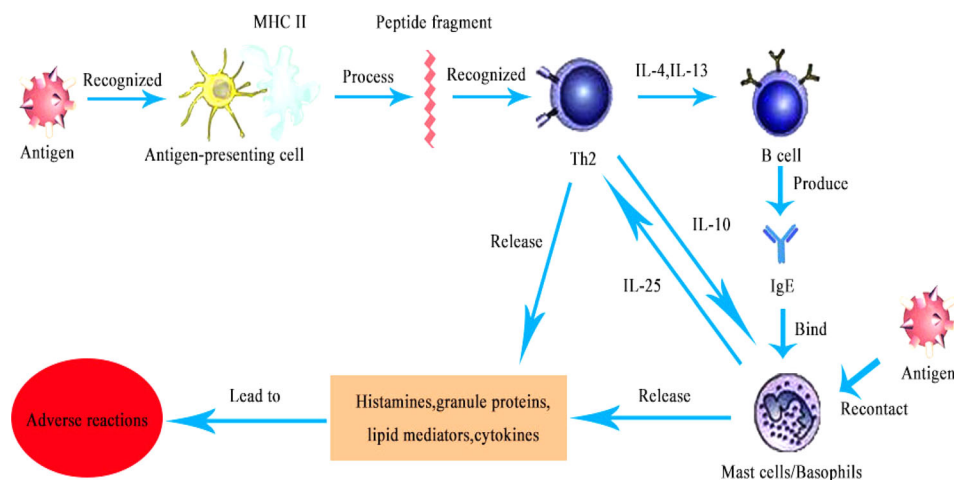


Fig. 1. Diagram of food allergy mechanism

Figure 1. Diagram of food allergy mechanism.

Gasset (2020) reviewed a protein-based detection using proteomics detection for seafood allergens with the mass spectrometry as the core. Compared with the above two reviews, seafood allergen detection methods are summarized in this review in a more comprehensive way. Therefore, the current review highlights the most important seafood allergens and interprets the mechanism of allergic attack and some adverse reactions. More importantly, DNA-based, protein-based and aptamer-based detection methods for seafood allergens are discussed in details and future developments are outlooked.

Seafood allergens and allergy

Ingesting, touching, or even inhaling marine life can cause some adverse reactions, which can be compartmentalized into immunological reactions (allergies, enterocolitis) and non-immunological reactions (biotoxins, parasites, food intolerances) by pathogenesis. Immunological and non-immunological reactions can only be distinguished by medical examination for patients because of similar clinical symptoms. However, this review focuses on the immunological reaction, namely, allergy.

Major seafood allergens and epitopes

Allergens is the substance that causes allergy and the reported seafood allergens usually consist of parvalbumin, enolases, aldolases, tropomyosin, arginine kinase, troponin C proteins, sarcoplasmic calcium-binding proteins, and myosin light chain proteins. Among these known allergens, parvalbumin is regarded as the main allergen in fish, and tropomyosin is considered as the main allergen in shellfish (Van Do et al. 2005; Kuehn et al., 2013; Faber et al. 2017; Shen et al. 2011; Kalyanasundaram and Santiago 2015; Wang et al. 2012; Ayuso et al. 2008; Aa; Aas and Jebsen 1967; Daul et al. 1994).

Epitopes are chemical groups that determine the allergenicity of the antigen. According to different structure,

conformational and linear epitopes are common categories of epitopes. Generally, a linear epitope is composed of consecutively arranged amino acid residues, and a conformational epitope consists of amino acids arranged discontinuously but with spatial interaction (Matsuo, Yokooji, and Taogoshi 2015). Since the epitopes are a direct cause of allergic reactions, inducing iterations in epitopes is a good way of inhibiting its potential IgE-binding capacity. In addition, allergen cross-reaction also poses a threat to consumers, which means that consumers may have allergic reactions to different species. For example, through IgE-mediated cross-reactivity studies, IgE from fish-allergic individuals could cross-react with extracts of other fish species (Bugajska-Schretter et al. 1998). However, the cross-reaction of protein is not limited to fish protein but exists in all kinds of protein. Homologue epitope is a main cause for cross-reactivity among homologue proteins, which often belong to the same families of proteins (Costa et al. 2021).

Seafood allergy mechanism

There are two stages involved in food allergy: IgE production stage and allergy symptom development stage (Bush 2001) and the specific mechanism is shown in Figure 1. In the former stage, seafood allergens are first processed into small peptide fragments and presented on the antigen-presenting cell (APC) surface through the major histocompatibility complex class II (MHC II). Subsequently, CD4 + helper T cells (Th2) recognize the allergen-MHC II complex by T cell receptor (TCR), while the secretion of IL-4 and IL-13 from Th2 promotes the differentiation from B cells into plasma cells. Finally, plasma cells release specific IgE. In the latter stage, IgE binds with IgE receptors located on the surface of mast cells or basophils. Once exposed to the same allergen again, the seafood allergens-IgE receptor binding behavior induces degranulation of mast cells or basophils (Rustemeyer et al. 2020). IL-12 secreted by Th2 and IL-25 secreted by mast cell/basophils play a feedback

Table 1. Adverse immunological reactions to seafood.

Categories	Disease	Symptoms
Gastrointestinal allergy	Physical discomfort	Nausea, vomiting, abdominal pain, diarrhea, poor appetite, intermittent abdominal pain, and malabsorption
	Oral allergy syndrome	Itchy or tingling tongue, swollen or numb lips
	Eosinophilic oesophagitis	Swallowing obstruction, food incarceration and reflux-like symptoms
	Gastroenteritis	Abdominal pain, bloating, diarrhea, constipation
Dermatological allergy	Urticaria	Edema, itching and erythema
	Protein contact dermatitis	Erythema, swelling, papules, blisters
	Eczematous contact dermatitis	Diffuse flushing, papules, blisters, erosion, exudate, scab
	Angioedema	Loose muscle tissue, red skin, and dizziness
Respiratory allergy	Asthma	Continuous wheezing, chest tightness, difficult breathing
	Rhinitis	Sneezing, stuffy nose, itchy nose
Systemic allergy	Anaphylaxis	Respiratory difficulties, low blood pressure, throat swelling, abdominal Pain, vomiting, and diarrhea, asphyxia, vascular collapse, irregular heartbeat, or myocardial infraction

regulation role between the two types of cells. The chemical mediators release during the degranulation process include histamines, granule proteins such as tryptase, lipid mediators such as leukotrienes, cytokines like IL-4, and all these chemical mediators are causes of allergic reactions (Bush 2001; Chu et al. 2005). Seafood allergy can range from mild urticarial or oral allergy syndrome to life-threatening anaphylactic reactions (Prester 2016). Depending on the location of the attack, seafood allergies can be divided into four categories: gastrointestinal allergy, dermatological allergy, respiratory allergy, and systemic allergy and the specific details are included in Table 1. Based on the mechanism of seafood allergy, the traditional and innovative detection methods for allergens are subdivided into three categories of DNA-based, protein-based and aptamer-based methods and their recent applications are as follows.

DNA-based detection methods

Highly specific amplification of target allergen DNA fragments using polymerase chain reaction (PCR) is the premise of most DNA-based allergen detection methods (Mafra, Ferreira, and Oliveira 2008). This technology has the characteristics of fast, simple, sensitive, and strong specificity in allergen detection. DNA-based methods can use species-specific DNA regions or allergen encoding genes as target analytes, thus allowing the direct identification of allergenic foods (Costa et al. 2017). Over the past few years, DNA-based methods have attained some recognition as excellent tools for allergen detection, being already implemented as routine analysis in some governmental laboratories (Holzhauser et al., 2018). Summary of DNA-based methods for detection of seafood allergens includes PCR, real-time PCR, multiplex PCR, and digital PCR, which are presented in Table 2.

PCR

PCR technology realizes DNA molecule amplification through artificial simulation of natural DNA synthesis conditions. PCR consists of three basic steps shown in Figure 2 (a). 1) Denaturation by heating: double-stranded DNA molecules open the double helix structure at high temperature

(93 °C) to become two single-stranded DNA; 2) Renaturation by cooling down: when the temperature drops to around 55 °C, the primers bind to single-stranded DNAs respectively following the principle of base pairing; 3) Primer extension: when the temperature rises to around 72 °C, the DNA-primer molecules become new double-stranded DNA molecules using free nucleotides under the effect of DNA polymerase (Garibyan and Avashia 2013). Repeating the same steps, the amount of DNA molecules increases exponentially. In general, the traditional PCR detection technology can perform a qualitative analysis of allergens; however, the exhaustion of reactants after repeated cycles and self-annealing of the accumulated product makes it difficult to perform quantitative analysis. In other words, no matter how many specific target DNA sequences there are before PCR, there may be the same amount of amplified DNA after PCR amplification, which results in no quantitative relationship between the initial DNA amounts and amplified DNA amounts (Valasek and Repa 2005). Some researchers tried to use PCR technology to detect allergens in seafood (mainly in fish). Ishizaki et al. (2012) designed the first PCR method to detect salmonid fish component in processed food, in which the detection limit was as low as 0.02 fg/μL. Subsequently, the PCR method developed by Rencova, Kostelnikova, and Tremlova (2013) showed high specificity and could be used to detect the major allergens of Atlantic and Pacific herring in fish products. It is noteworthy that PCR methods are specific to specific species, not specific allergens, thus it is more suitable for allergen primary screening.

Real-time PCR

Although PCR has been applied to achieve satisfactory detection of allergens, some shortcomings associated with the traditional PCR have led to the development of real-time PCR. Real-time PCR is a technology that measuring the total amount of DNA amplification product after each cycle of PCR using fluorescent chemicals as signal probe, of which target DNA sequences quantification of the sample can be achieved using internal or external references. During real-time PCR process, the CT value, that is, the number of cycles for a given sample to generate a signal, is determined at the early exponential stage of the reaction.

Table 2. Information on DNA-based detection methods regarding seafood allergens.

Species	Technique	Food matrices tested	Target gene	Limit of detection	References
Salmonid fish	PCR	11 kinds of processed food such as breakfast cereal	Cytochrome b	0.02 fg/ μ L	Ishizaki et al. (2012)
Atlantic and Pacific herrings	PCR	22 fish food products	pvalb 2 gene	10 pg/ μ L	Rencova, Kostelnikova, and Tremlova (2013)
Lobster	Real-time PCR	Seafood, deli foods, juices, dry noodles, and canned soups	12S rRNA	0.1-1 ppm	Eischeid (2016)
Penaeid shrimp and blue crab	Real-time PCR	5 kinds of food such as Canned soups	For the shrimp, 12S/16S rRNA; For the crab, cytochrome oxidase I/cytochrome b	0.1-1 ppm	Eischeid, Kim, and Kasko (2013)
Cod and pollock	Real-time PCR	3 kinds of food such as cooking oil	Mitochondrial cytochrome oxidase I	1-10 ppm	Eischeid (2019)
Shrimp crustacean	Real-time PCR	Binary model mixtures simulating a processed shrimp stuffing/filling	16S rRNA	0.1 pg	Fernandes et al. (2018b)
Crustacean	Real-time PCR-based screening assay	12 kinds of processed food such as prawn crackers	16S rRNA	0.04-2.5 pg	Zagon et al. (2017)
Fish	Fast real-time PCR	The elaborated products containing fish	18S rRNA	0.05 ng	Herrero, Vieites, and Espiñeira (2014)
Fish	Real-time PCR	All kinds of processed food such as fish sticks	16S rRNA	0.01 pg	Fernandes et al. (2018a)
Crustacean	Fast real-time PCR	The elaborated products containing crustaceans	16S rRNA	1.25 pg	Herrero, Vieites, and Espiñeira (2012)
Crustacean	Fast real-time PCR	6 kinds of food such as chocolate bars	16S rRNA	NR	Santaclara and Espiñeira (2017)
Shrimp	Ultrafast real-time PCR	9 kinds of processed food such as porridge, dumpling	Shrimp tropomyosin genes	3.2 pg	Kim et al. (2019)
Fish, cephalopods and shellfish	LPA-based multiplex PCR	Spiked sushi	Species-specific genes	20 mg/kg (scallop, fish and bivalve) ;100 mg/kg (cephalopod, gastropod and crustacean)	Unterberger et al. (2014)
oyster, mussel, abalone, and clam	Multiplex PCR	19 commercial fishery products	tropomyosin genes/ 18S rRNA	16 pg	Suh et al. (2020)
The snow crab and red snow crab	Multiplex PCR	23 different commercially processed crab products	COI/ITS genes	0.005 ng/ μ L.	Kang (2019)
Fish	Digital PCR	Fresh or frozen tissue of whole-animals	18S rRNA	0.18 pg	Daga et al. (2018)

NR, Not reported.

The template CT value maintains a quantitative relationship with the template initial copy number in the PCR exponential amplification stage. Therefore, plotting the CT value on the Y axis and the logarithm of the initial copy number of the template on the X axis forms a linear curve of real-time PCR, which provides a basis for quantification (Adams 2006; Heid et al. 1996).

The establishment of a real-time PCR method can be roughly divided into three steps: design and synthesis of primers and probes; design of PCR amplification system; method validation (Fernandes et al., 2018a; Herrero, Vieites, and Espiñeira 2012). In the first step, the selection of target genes is crucial. Common target genes for allergen detection include species-specific gene sequence, such as nuclear genes for parvalbumin and the mitochondrial genes such as 16S

rRNA, and mitochondrial cytochrome oxidase I. The most suitable target genes are selected according to the requirements of the test object and test method. An essential factor to consider is high copy number targets, such as mitochondrial genes, which are more sensitive than those lower copy number targets, such as nuclear genes in the actual application process (Eischeid 2019). In the second stage, PCR amplification is continuously optimized to achieve faster and more efficient detection. For example, microfluidic chips have been recently developed and could reduce the reaction time of thermal cycling in PCR process (Furutani, Hagihara, and Nagai 2017). The purpose of method validation is to verify its practicability and reliability, especially in food with severe processing conditions or complicated food matrices. Herrero, Vieites, and Espiñeira (2014) verified a developed

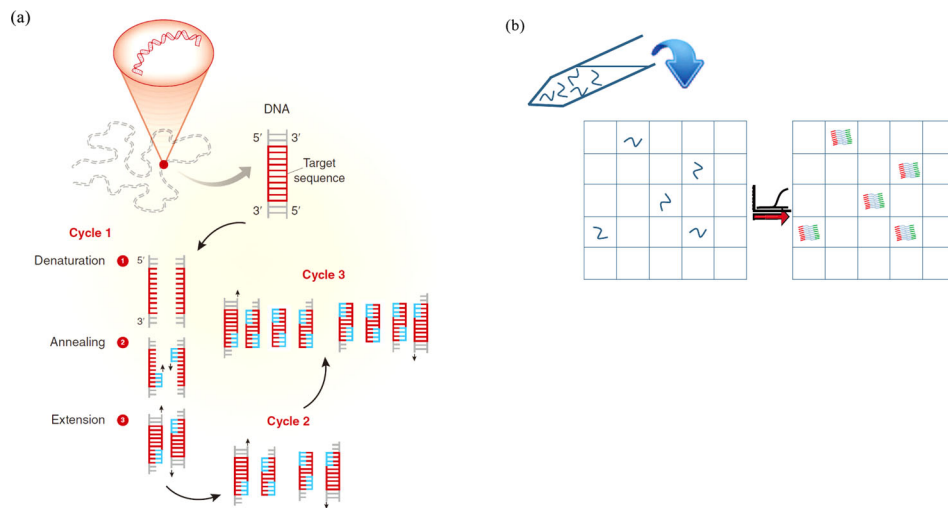


Figure 2. Schematic of DNA-based methods. (a) Detailed explanation of polymerase chain reaction (Garibyan and Avashia 2013). (b) Digital PCR is a detection method that derives the initial sample size by counting the total amount of reaction units after dilution (Baker 2012).

real-time PCR with the products elaborated with fish component and the results demonstrated the developed method could be used to detect fish allergens in various foods including processed products.

The specificity of the method is closely related to the accuracy of the detection results. If the specificity of the method is not high, false positives may occur. Eischeid (2016) developed a real-time PCR method and ensured that his method could specifically detect lobster without cross-reaction with crab and shrimp. A real-time PCR method, taking a region of the mitochondrial 16S rRNA gene as the target gene, was proved to be specific for crustacean species after silico analysis, assessment with primer-blast tool, and further assessment using the DNA from 62 food-related species, and achieved the detection and quantification down to 0.1 pg and 0.0001% (w/w) of shrimp DNA and shrimp in model mixtures, respectively (Fernandes et al., 2018b).

Although commercial kits based on real-time PCR to detect different allergens are available, they do not completely meet people's needs. They are required to be further improved in terms of characteristics, time, function, etc. (Herrero, Vieites, and Espiñeira 2014). Santaclara and Espiñeira (2017) designed a fast and real-time detection method, which reduced analysis time to 40 min and achieved trace detection even under extreme processing conditions. Similarly, Kim et al. (2019) developed a novel ultrafast PCR system, which eliminated the routine procedures for DNA extraction, providing on-site trace detection for shrimp allergen, and reduced the detection time to less than 30 min. Zagon et al. (2017) assembled PCR systems optimized for groups and species to create a 96-wells format small screening arrays, which managed to detect multiple crustacean species in one analytical step.

Multiplex PCR

Multiple PCR, also called multiple primer PCR, is a PCR reaction allowing for simultaneous amplification of two or more pairs of primers in the same PCR system. The reaction

conditions are the same as the general PCR. The advantage of this technique is that multiple objects can be detected simultaneously. For allergen detection, multiplex PCR is used to detect multiple allergens. Suh et al. (2020) firstly achieved the simultaneous analysis of allergenic mollusk species. They achieved combination of a multiplex PCR assay with capillary electrophoresis, making full use of the advantages of efficiently separating DNA fragments and expressing DNA concentration of capillary electrophoresis. Unterberger et al. (2014) also achieved simultaneous detection of allergens in fish, cephalopods, and shellfish components using a multiplex ligation-dependent probe amplification (LPA) system. Unlike traditional multiplex PCR systems, LPA, which involves using a single primer pair to detect different targets in a single assay simultaneously, was used to develop a cheap and flexible system that can be expanded by adding more hybridization probes to obtain higher multiplexing than traditional PCR methods.

Digital PCR

"Divide and conquer" is the best overview of digital PCR strategy shown in Figure 2 (b). The samples are diluted and dispersed into enough independent reaction chambers, ensuring each chamber contains one or no target sequence. After PCR amplification, each reaction chamber is carefully examined and defined as either positive (with PCR products) or negative (without PCR products). By counting the total amount of positive reaction chambers and negative reaction chambers using Poisson distribution, nucleic acid content can be determined (Baker 2012; Morisset et al. 2013). Compared with PCR technology mentioned above, digital PCR does not require the establishment of a standard curve and is considered to be an absolute quantitative method.

There are few literatures on the application of digital PCR to detect allergens, and most of them focus on the detection of allergens of peanuts and soybeans (Mayer et al.

2019; Pierboni et al. 2018; Temisak et al. 2019). So far, only one study reported the application of digital PCR technology for detecting fish allergens. Daga et al. (2018) designed suitable primers, optimized the concentration of primers and probes, determined the annealing temperature for droplet digital (ddPCR), and managed to analyze a small number of targets in a sample quantitatively. The results showed that ddPCR was well suited for qualitative and quantitative detection of fish allergens. Additional reaction products were observed during ddPCR reactions, which might result from intraspecies variability, single nucleotide polymorphism, or interspecies. Further studies to validate the use of ddPCR for analysis of specific fish species and common allergens are required.

In a word, Digital PCR is more accurate and sensitive under the condition of low amount of target DNA and more tolerance inhibitors, which is worthy of further research in allergen detection.

Protein-based detection method

Most protein-based detection methods are based on the specific binding of antibodies to allergen epitopes, which can be mainly divided into three categories, namely enzyme linked immunosorbent assay (ELISA), immunochromatography assay, and immunosensor. The first two methods appear early and have developed for a long time, and more mature products are already on the market. However, the immunosensor method with great development potential has a relatively short development time. It cannot be used for large-scale routine testing for the time being and is still some way from large-scale commercialization. Besides, another method of detecting allergenic proteins that cannot be ignored is mass spectrometry. This method requires precise instruments and professional operators, and has extremely high accuracy and sensitivity, which is often used in laboratory tests. Summary of protein-based detection of seafood allergens is provided in Table 3.

ELISA

The ELISA method was first described in a paper published by Engvall and Perlmann in 1971, and has become a well-known detection method widely used in medicine, biology, chemistry and other fields after decades of development (Lequin 2005). ELISA presents advantages such as high sensitivity and large detection scale, and simple equipment design (Clark, Lister, and Bar-Joseph 1986). There are also convenient and reliable ELISA kits for seafood allergen detection commercially (Fernandes et al. 2015).

ELISA reaction modes can be divided into competition, sandwich, direct, indirect method. The competition method is mainly used to detect small molecular substances such as toxins, while the sandwich method is commonly used to detect large molecular substances (such as globulin, viruses, enzymes, etc.). The difference between the indirect method and the direct method is whether to add HRP-conjugated second antibody. The addition of HRP-conjugated secondary

antibodies can increase the sensitivity of detection. For allergen analysis, sandwich type is normally used since the combination of a capture antibody with a recognition antibody increases the specificity and sensitivity of the method. However, indirect ELISA type is also commonly used due to high specificity/sensitivity, while decreasing the time per analysis and problem associated with antibody conjugation (Pereira et al. 2020).

Taking the work of Kamath et al. (2014) as an example, the specific procedures of indirect and sandwich ELISA include: 1) Coating. Coat the capture antibody diluted in buffer in a 96-wells plate and incubate at 4 °C overnight. 2) Blocking. Block wells with blocking solution after washing to avoid nonspecific binding. 3) Adding analytes. Add the test sample to the wells and incubate for 3 h. 4) Adding signal molecule. Add the detection antibody diluted in buffer to the wells after washing. Afterwards, add the streptavidin-horse radish peroxide conjugate diluted in buffer to the wells and incubate for 30 min. The wells are then washed five times and patted dry. 5) Color rendering. Add subtract to the wells to form a coloration in the blank wells and 1 N hydrochloric acid was used to stop reaction. 6) Reading. Read the corresponding result of the color change at 450 nm by a microplate reader.

In 2007, Werner, Faeste, and Egaas (2007) successfully constructed a quantitative sandwich ELISA to detect crustacean tropomyosin in five food matrices, one of the few quantitative methods used to detect tropomyosin in food. Faeste and Plassen (2008) achieved quantitative detection of parvalbumin in fish by using quantitative sandwich ELISA for the first time.

Furthermore, some commercial ELISA kits do not possess IgE linear epitope binding ability, hence their inability to detect allergenic peptides produced during food processing. Zhang et al. (2014) developed a sandwich ELISA detection method based on an antibody that can specifically recognize the linear epitope of tropomyosin, reducing the impact of processing to a certain extent.

During the detection of parvalbumin in fish and fish by-products, structural modification caused by processing and differences in parvalbumin content in fish species can sometimes affect the accuracy of detection. Hence, Chen and Hsieh (2014) took a 36 kDa thermal-stable fish muscle protein as the detection target instead of parvalbumin, established a sandwich ELISA detection method, and successfully accomplished the fish ingredients detection under various processing conditions.

Immunochromatography assay

Immunochromatography is a combination of immunology and chromatography for the detection of allergen. This technique was developed at the end of the 20th century and has found application in various fields in form of the lateral flow strip. Compared with other detection technologies such as ELISA, the lateral flow strip has the advantages of fast detection time (within 20 min), simple operation (no professionals required), and can achieve large-scale initial on-site

Table 3. Information on protein-based detection methods regarding seafood allergens.

Target allergen	Technique	Food matrices tested	Limit of detection	Working range	References
Tropomyosin in crustaceans	ELISA	Surimi, breaded codfish, fish cake, fish sauce, mayonnaise	1 $\mu\text{g/g}$	0.38 to 49 ng/mL	Werner, Faeste, and Egaas (2007)
Parvalbumin in fish	ELISA	5 kinds of processed food such as mushroom soup	0.02 mg/kg	0.49–31.3 $\mu\text{g/L}$	Faeste and Plassen (2008)
thermal-stable fish muscle protein (not allergen)	ELISA	Selected salted, smoked and canned Commercial fish products	0.1 ppm	NR	Chen and Hsieh (2014)
Tropomyosin in invertebrate	ELISA	10 kinds of processed food, such as shrimp cracker	Prawn tropomyosin: 0.09 ng/ml, squid tropomyosin: 0.64 ng/ml for	Prawn tropomyosin: 0.045–600 ng/ml, squid tropomyosin: 0.080–512 ng/ml	Zhang et al. (2014)
Tropomyosin in crustaceans	ELISA	NR	60 pg/mL	0.02–1.25 ng/mL	Kamath et al. (2014)
Shellfish Tropomyosin	Lateral flow immunoassay	30 food species	12.4 ng/mL	0.01–20 $\mu\text{g/mL}$	Shi et al. (2011)
Parvalbumin	Lateral flow immunoassay	Beef, Pork, Chicken	40 ng/mL	50–1000 ng/mL	Du et al. (2014)
crustacean protein	Lateral flow immunoassay	Tomato juice, Fish-meat sausage, Freeze-dried egg soup	25 $\mu\text{g/L}$	NR	Koizumi et al. (2014)
crustacean tropomyosin	Lateral flow immunoassay	4 kinds of food such as spice blends	0.05 $\mu\text{g/mL}$	NR	Wang, Li, et al. (2019)
Shrimp tropomyosin	Lateral flow immunoassay	Shrimp cracker, shrimp flakes, Cup noodles, Laver soup, Fish ball	0.5 ng/mL	NR	Zeng et al. (2019)
Parvalbumin in fish	Optical immuosensor	Sardine fish cake (tsunire) and dried skipjack tuna (katsuonut)	3.55 $\mu\text{g/L}$	NR	Lu, Ohshima, and Ushio (2004)
Tropomyosin in shellfish	Optical immuosensor	Shellfish	21 pg/mL	0.1–15 ng/mL	Wang, Li, et al. (2019)
Tropomyosin in shellfish	Optical immuosensor	Shellfish	1 $\mu\text{g/mL}$	3–50 $\mu\text{g/mL}$	Zhou et al. (2020)
Shrimp tropomyosin	Electrochemical immunosensor	Pork, beef, chicken, shrimp and squid	46.9 pg/mL	0–218.7 ng/mL	Angulo-Ibanez et al. (2019)
Tropomyosin	Electrochemical immunosensor	A shrimp-free cream cracker	0.01 pg/mL	0.01–100 pg/mL	Mohamad et al. (2020)
Fish major allergen parvalbumin	MS	Pork, shrimp, chicken muscle and beef	0.02 nM	0.1–1179.36 nM	Sun et al. 2019
Soy glycinin G2, milk α -S1-casein, egg ovalbumin	MS	Surimi products like fish ball, fish sausage, fish tofu, simulated crab meat	0.054 $\mu\text{g/g}$ for soy, 0.024 $\mu\text{g/g}$ for milk and 0.032 $\mu\text{g/g}$ for egg	0.5–200 ng/mL	Huang et al. 2020
Crustacean tropomyosin	MS	Chicken sausages, beef balls, and egg–milk biscuits.	1.6 mg/kg	1–1000 $\mu\text{g/L}$	Wang et al. 2021

NR, Not reported.

screening of samples (Du et al. 2014; Zeng et al. 2019). However, it generally achieves qualitative analysis or semi-quantitative analysis, but not quantitative analysis.

Two types of the lateral flow strip methods have been developed, namely, a sandwich-type and a competitive-type. Once the prepared sample solution is dropped on the sample pad, the antigen within the sample solution first binds with the detection antibody stored in the binding pad to form an antigen-detection antibody dimer. Then the dimer binds to the capture antibody coated on the T line to form a detection antibody-antigen-capture antibody trimer. Therefore, the detection antibody is indirectly fixed on the T line, and the T line macroscopically changes the color, which can be caught by the naked eye. When the antigen is in the sample, the T line develops color; otherwise, the T line remain the same. In addition, The C line must be colored in any case, otherwise the test result is invalid (Figure 3).

The colloidal gold immunochromatography, using colloidal gold-antibody conjugate as detection antibody, is the most commonly used, because of easy preparation, good biocompatibility of colloidal gold (Ambrosi et al. 2007). In 2014, a colloidal gold immunochromatography assay was developed to quantify crustacean protein in processed food, of which the limit of detection was 25 ng/mL, and the whole process took less than 20 min (Koizumi et al. 2014). Likewise, Zeng et al. (2019) developed a colloidal gold fast test strip applied to detect shrimp tropomyosin, and the limit of detection was lower (0.5 ng/mL) and time consumption was less (within 10 min). This may be related to the difference between the detection systems with different antibodies used and different optimization of detection systems.

In a bid to improve the performance of the detection method, a common research trend is to replace the signal molecules combined with antibody. Signal molecules

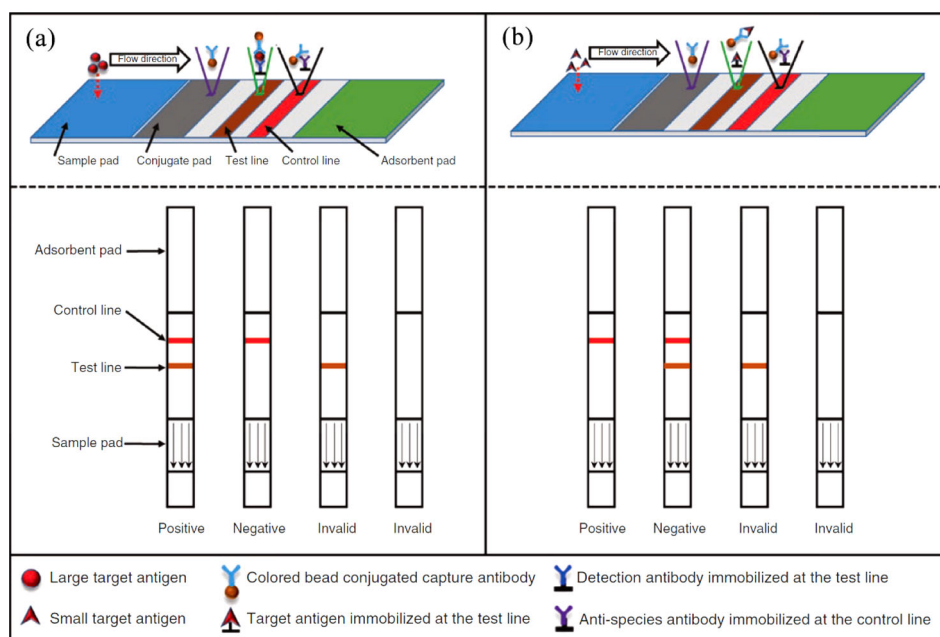


Figure 3. Classification of immunochromatographic test strips (a) sandwich-type (non-competitive) (b) competitive-type (Hsieh and Rao 2017).

reported in the literature include quantum dots, colloidal gold, latex microspheres, etc. (Anfossi et al. 2019; Wang, Li, et al. 2019). Shi et al. (2011) developed an immunochromatography assay based on superparamagnetic nanoparticle-labeled antibody for detection of shellfish tropomyosin. This method was more sensitive because the signal from superparamagnetic nanoparticle could completely be recognized by signal receiving system under the effect of food matrix. Quantum dots are ideal fluorescent probes with wide and continuous excitation spectrum, adjustable color, high photochemical stability and good biocompatibility. A quantum dots-based immunochromatography assay with excellent specificity, reproducibility, and reliability was developed to detect tropomyosin in crustacean (Wang, Li, et al. 2019).

Immunosensor

Biosensors can convert indiscernible, but highly sensitive and selective biological signals into other signals that can be recognized, and can well achieve allergen detection, which are considered to be a novel technology that can quickly and accurately analyze and track food allergens. (Pilolli, Monaci, and Visconti 2013). Additionally, biosensors have several advantages, such as increased sensitivity but they are more time-consuming than other techniques. The biosensor consists of three components, the bio-signal recognition unit (antibody, cell, aptamer, DNA, etc.), the transducer (electrochemical, mass, optical, thermal, etc.), and the signal processing unit (Alves et al. 2016). The biosensor that recognizes the antigen-antibody binding signal is called the immunosensor. At present, the most widely used biosensor type for the detection of food allergens is the immunosensor.

Currently, two main types of immunosensor used for seafood allergen detection have been developed, electrochemical immunosensor and optical immunosensor (according to transducer component) (Angulo-Ibanez et al. 2019; Lu,

Ohshima, and Ushio 2004; Mohamad et al. 2020; Wang, Li, et al. 2019; Zhou et al. 2020). All of them are listed in Table 3.

Due to the antigen-antibody binding reaction that ensues on the surface of the optical immunosensor certain optical properties change occur during immunosensor detection. Therefore, the measurement signal related to the antigen concentration in the sample can be established by changes in optical properties such as refractive index, light absorption, light reflection, or light scattering (Alves et al. 2016). Lu, Ohshima, and Ushio (2004) applied surface plasmon resonance (SPR) biosensor to detect fish Parvalbumin for the first time. This biosensor could detect the allergen for five min without labels, of which limit of detection was 10 folds lower than that of ELISA. Zhou et al. (2020) fabricated a SPR biosensor with gold patterned biochips to detect shellfish (mainly shrimp) tropomyosin within 3 min. they enhanced the sensitivity of detection using a gold patterned biochip covered by electrodeposited gold nanostructures. Wang, Li, et al. (2019) achieved ultrasensitive detection of shellfish (mainly shrimp) tropomyosin by using chiral biosensor based on gold nanoparticle trimer. The limit of detection of this biosensor was reduced by several orders of magnitude compared with the work of Zhou et al. (2020).

Electrochemical immunosensor are developed by the principle that the analytes with redox capability undergo a redox reaction on the electrode surface, which is affected by a predetermined pattern of fixed or varying potential. Changes in electron flux that caused by the antigen-antibody binding then produces a measurable electrochemical signal (Alves et al. 2016). Angulo-Ibanez et al. (2019) presented the first electrochemical immunosensor for detecting tropomyosin in shrimp. They overcame the effect of food matrix complexities by using magnetic particles to improve the capture of the tropomyosin. In a recent study, Gold-microrods/Pd-nanoparticles/polyaniline-nanocomposite-interface-

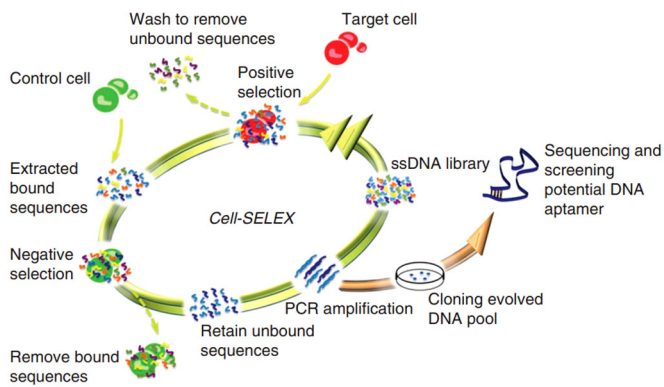


Figure 4. Aptamer screening mode with the cell SELEX strategy. Positive screening and negative screening are carried out respectively to obtain the aptamer that specifically binds to the target cell on the basis of the specific binding of the aptamer and the cell, and further cloning and screening are carried out to obtain the target aptamer (Sefah et al. 2010).

modified microelectrodes were prepared to enhance the sensitivity of tropomyosin achieved 0.01 pg/mL limit of detection, the lowest immunosensors detection of tropomyosin till date (Mohamad et al. 2020).

Mass spectrometry (MS)

Antibody-based detection methods are sometimes inadequate for qualitative and quantitative allergen detection because of matrix/processing effects and epitope masking. However, MS-based detection methods can well resolve it (López-Pedrouso et al. 2020). MS-based detection methods are usually used in determination and detection of food allergens, nevertheless, the workflows of MS-based methods are different with diverse aims. When determining a new allergen, MS is only one tool to determine protein species, and it is often combined with other techniques to achieve the goals. To be specific, Yang et al. (2017) determined a triosephosphate isomerase as a novel allergen in *Octopus fangsiao*. They firstly identified a 28 kDa allergenic protein by IgE-immunoblot analysis, and then digested the protein with trypsin to produce a mixture of peptides, which were then analyzed by MS to get a protein fingerprint for protein information comparison. Finally, they determined this 28 kDa allergenic as protein triosephosphate isomerase in *Octopus fangsiao* by comparing homologous proteins in the protein database. During allergen detection process, MS is used for marker peptides screening and target allergen detection. Marker peptides are some short amino acid sequences which can be regarded as allergen substitutes to qualify and quantify target allergens. Normally, at least 3 or 4 peptides of each allergen are needed to ensure the specificity of the MS method because of the exogenous and endogenous interferences in complex foodstuffs (Sun et al. 2019). Wang et al. (2021) detected crustacean tropomyosin in foods using high-performance liquid chromatography–tandem mass spectrometry method with a linear range of 1–1000 $\mu\text{g L}^{-1}$. They first predicted the sequence of marker peptides of crustacean tropomyosin by bioinformatics method, and obtained five predictive marker peptides. Then, the reliability of the predictive marker peptides was further verified by purified

Litopenaeus vannamei TM protein with MS. In the results of MS, peptides with high scores were considered to have good specificity and were determined as marker peptides, while the peptide among them with the highest intensity was determined as quantitative peptides. Finally, the detection conditions of MS were optimized, and the detection method based on MS was obtained on the premise that the marker peptides were reliable. In addition, simultaneous detection of multiple allergens by MS is possible. Huang et al. (2020) screened for marker peptides from different food origins, including EAFGVNMQIVR (soy glycinin G2), YLGYLEQLLR (milk α -S1-casein), GGLEPINFQTAADQAR (egg ovalbumin) and a stable isotope-labeled peptide EAFGVNMQI*(I*, 13C6, 15N) VR, to achieve four allergens detection in surimi products with a range of 0.5–200 ng/mL.

Aptamer-based detection method

Similar to the detection method based on specific allergen-antibody binding, the aptamer-based detection method is also based on specific binding of the aptamer and the allergen. However, aptamer-based detection methods can overcome some shortcomings of the protein-based detection methods, which result from the differences between antibodies and aptamers to some extent. Specifically, antibodies are expensive with long synthesis cycles, however, aptamer synthesis is relatively easy and the synthesis cycle is short (Dong et al. 2014). Aptamers are a small sequence of oligonucleotides or short peptides with specificity and affinity obtained by in vitro screening, which can specifically bind with the corresponding ligands and are easy to Synthes. In order to obtain suitable aptamers of target analytes, systematic evolution of ligands by exponential enrichment is usually used (shown in Figure 4). (Chinnappan et al. 2020).

Throughout literature, Aptamer-based detection of allergen detection is often performed using sensors, summary of aptamer-based detection of allergen is presented in Table 4.

Among the studies, Abdelli et al. (2019) designed a portable aptamer-based allergen sensing device, characterizing results with light intensity and the detection principle is shown in Figure 5. A high-sensitivity visible light driven photoelectrochemical aptasensor was successfully developed for shrimp tropomyosin detection for the first time, albeit there were some deficiencies in the limit of detection compared with ELISA (Tabrizi et al. 2017). Chinnappan et al. (2020) developed a graphene oxide labeled aptamer method for detection of major shrimp allergen tropomyosin with reference to Tabrizi et al. (2017). They increased the affinity of the aptamers by four times by removing unnecessary parts of the aptamers. Unfortunately, significant cross-reactions with other invertebrate tropomyosin were observed because of the high similarity of the protein amino acid sequences. For the first time, Zhang et al. (2017) selected an aptamer with high affinity and specific recognition of tropomyosin through a six-round screening process. This aptamer was combined with a fluorescent/graphene oxide sensor to successfully achieve the detection of tropomyosin.

Table 4. Information on aptamer-based detection methods regarding seafood allergens.

Target allergen	Food matrices tested	Limit of detection	Work range	References
Shrimp allergen tropomyosin	human serum (Not food)	0.23 ng/mL	1-400 ng/mL	Tabrizi et al. (2017)
Shrimp allergen tropomyosin	TM spiked chicken soup	2 nM	NR	Chinnappan et al. (2020)
Tropomyosin	3 kinds of processed food such as Heinz tomato ketchup	77 ng/mL	0.4-5 µg/mL	Zhang et al. (2018)
Shrimp allergen tropomyosin	NR	4.2 nM	0.5-50 µg/mL	Zhang et al. (2017)
shrimp allergen	Food sample solution	6.2 nM	25-1000 nM	Tah et al. (2018)

NR, Not reported.

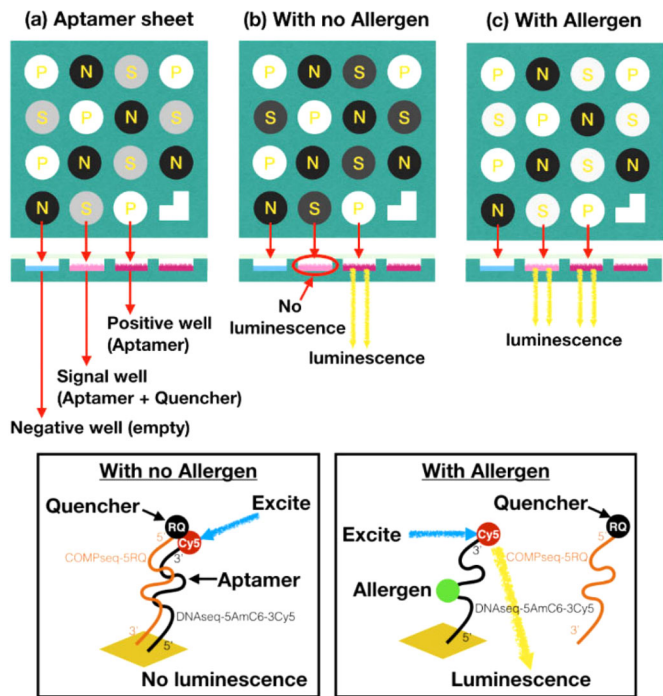


Figure 5. Schematic of aptamer-based detection film. (a) Composition of aptamer-based detection film: positive well containing only aptamer; signal well containing the aptamer and the quencher; and a negative well containing nothing. (b) Negative results, the signal well emits light similar to the negative well, because dye quenched by the quencher cannot produce emission light after excitation (c) Positive results, the signal well emits light similar to the positive well, because allergens prevent the binding of the quencher to the aptamer, making the dye luminesces after excitation (Abdelli et al., 2019).

Subsequently, Zhang et al. (2018) designed a detection method for tropomyosin, combining functionalized magnetic nanoparticles with aptamer-based fluorescent assay, which reduced the interference of the reaction system and simplified the detection process. This method can be used to detect other proteins by changing the aptamer on the probe. So far, there are few related studies on the aptamer-based detection methods of seafood allergens, and further research is needed.

Advantages and disadvantages of detection methods

DNA-based detection methods have the following advantages:

- Strong stability. DNA molecules are widely distributed in biological tissues and are relatively more stable than

protein molecules, which reduces the difficulty of allergen detection in processed foods or complex food matrices.

- High selectivity. For example, it is easy to distinguish the same allergens from shrimp and crab with the high homology of crustacean allergens by DNA detection.
- Low cost. The primers and probes used for DNA-based detection methods can be chemically synthesized at low cost unlike the highly-cost antibodies (Holzhauser 2018).

For DNA-based detection methods, some disadvantages cannot be ignored. Protein expression was not correlated with the presence of the gene, which lead to incorrect results using DNA-based methods. As example, there are many allergens that are expressed only in special conditions (e.g., PR-10 proteins), like responses to abiotic and biotic factors. In addition, DNA-based detection methods are not suitable for identifying allergens with unknown gene sequences.

The advantages of the protein-based detection methods are as follows.

- High reliability. It directly uses allergens as the detection object, reducing the possibility of false positives in indirect detection methods.
- High sensitivity. As a relatively mature method, it has been deeply studied and can achieve a low detection limit.

For protein-based methods, the structure of allergens directly affects the detection results because protein-based methods directly detect allergens as target objects. Structural modifications of allergens under modern food processing conditions will affect the test results, and highly homologous allergens from different species pose a challenge to the protein-based detection method.

In recent years, aptamer-based detection methods have been developed rapidly with the following advantages:

- High sensitivity. This is because the aptamer has a large number of receptor binding sites and can better bind to allergens.
- Low cost. Aptamers can be extensively and easily screened and synthesized in vitro, which presents an excellent opportunity for commercialization.

At present, most studies on aptamer-based detection methods are combined with biosensors, which increases the cost of detection. In addition, the reliability of aptamer-based detection method needs to be further verified.

Although these methods are flawed, they can complement each other. The DNA-based, protein-based and aptamer-based detection methods can be used simultaneously and mutually verified to obtain more reliable detection results.

Conclusions and future prospects

Seafood allergy is growing into a troublesome problem as seafood consumption increasing. Efficient, convenient and low-cost allergen detection methods are urgently needed to protect consumers' health. In this review, the existing seafood allergen detection methods can fall into three categories in the light of detection principles: the DNA-based, protein-based and aptamer-based methods, respectively. DNA-based methods are used to test the DNA of allergic species. Simply put, if the DNA of a particular species is detected in a food, it means that the food contains an ingredient belonging to that species, but it cannot be identified as a specific allergen, such as tropomyosin. Protein-based detection methods, which take a certain allergen as the direct detection object, are mostly realized based on the binding ability of antigen and antibody. However, the detection accuracy is easily affected by the sample substrate, processing and other factors. As a protein-based detection method, mass spectrometry can overcome interference factors to some extent, but it is time-consuming and requires expensive equipment. Similar to protein-based tests, aptamer-based tests are based on specific binding between aptamers and allergens, but aptamers are easier to synthesize and lower in cost than antibodies. Relatively more information on the protein-based and DNA-based detection methods are elaborated, because they are current mainstream detection methods. Relatively few studies of aptamer-based detection methods on allergen detection, it is expected to become the mainstream detection method in future due to their unique advantages.

There are still some knowledge gaps in the detection of seafood allergens from the current research findings. There are many applications of real-time PCR in DNA-based methods, but few about digital PCR. In addition, the non-thermal amplification technology used in other allergens detection has not been reported in seafood allergen detection. Its application in seafood allergen might present scope of improvement. In protein-based methods, there are few studies on immunosensors of seafood allergen detection. Studies on detection methods based on aptamers are also few.

The combination of multiple detection methods is the development trend of allergen detection in the future, which can combine the advantages of different methods, make up for the disadvantages, and improve the accuracy and reliability of detection to the greatest extent. In a word, the research on novel technology in the detection of seafood allergens should be strengthened in future.

Author contributions

Jilin Li wrote and revised the original manuscript, prepared the tables and all the figures. Huifen Wang revised the manuscript. Jun-Hu

Cheng conceived the idea, supervised the work and revised and the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors are grateful to the National Natural Science Foundation of China (31972205) for its support. This research was also supported by Guangdong Basic and Applied Basic Research Foundation (2021A151010644), the International S&T Cooperation Projects of Guangdong Province (2020A0505100007), the Fishery Development Project of Guangdong (2019B9), the Contemporary International Collaborative Research Centre of Guangdong Province on Food Innovative Processing and Intelligent Control (2019A050519001) and the Common Technical Innovation Team of Guangdong Province on Preservation and Logistics of Agricultural Products (2020KJ145).

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