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# Methods for the detection of anisakid material in different food matrices: A systematic review

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ABSTRACT

Anisakid nematodes are common foodborne parasitic nematodes with an important implication for seafood safety and public health. On top of usual symptoms resulting from infestation with live larvae, these nematodes have allergenic proteins that may cause allergic reactions even in the absence of live larvae. Current methods of detection in industrial settings are mainly limited to gross visual detection and candling, as required by EU legislation. These have a limited sensitivity and do not detect macroscopically invisible allergens that persist after larval removal. In scientific settings, highly sensitive methods such as UV-press and artificial digestion are commonly used, but also don't detect allergens. Alternative detection methods may be useful in seafood processing industry, as well as in scientific settings. A systematic review of detection methods for trace anisakid material has been performed. PubMed, Web of Science, Scopus, and Google Scholar were used as databases. Only English papers with a focus on anisakid detection in food matrices were included, while methods based on visual detection were excluded. A total of 25 articles were identified, and methods were categorized by detection principle. Out of the detection methods, 10 were DNA-based, 14 immunochemistry-based, and 5 mass spectrometry-based (MS-based) methods. There was a lack of standardized reporting of the detection performance parameters which hinders thorough cross comparison. We recommend a chemiluminescent sandwich ELISA method for routine analysis of allergens in food. For larval DNA detection, a LAMP method seems the most promising for fast routine DNA detection. When trace detection of anisakid material is needed, MS-based methods could be most effective.

## 1. Introduction

Anisakid nematodes, comprising genera such as *Anisakis*, *Phocanema* (previously referred to as *Pseudoterranova*) and *Contracaecum*, are zoonotic parasites with a global distribution in marine ecosystems (Levsen et al., 2016; Bao et al., 2023). These parasites are commonly found in a wide range of fish and cephalopod species, many of which are integral to human consumption (Chai et al., 2024; Herrero et al., 2011). Traditionally, these nematodes are associated with gastrointestinal infestations through the ingestion of live larvae (anisakidosis) due to consumption of raw or undercooked seafood. According to the European Food Safety Authority, an estimated 20,000 cases of anisakidosis have been reported worldwide up to 2010 with over 90% of those cases being in Japan (EFSA, 2010). A retrospective survey in France identified 37 actual cases from 2010 to 2014 as reported by parasitology laboratories of

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university hospitals (Yera et al., 2018). These numbers are probably an underestimation, as another study made a mathematical prediction estimating that yearly there are up to 8000 cases of anisakidosis requiring medical attention in Spain alone (Bao et al., 2017). Globalization and popularization of traditional dishes containing raw seafood, such as sushi and sashimi, are making this issue become increasingly relevant. On top of this, a growing body of evidence indicates that anisakids, with *Anisakis simplex* as a prime example, can also elicit allergic responses due to their thermostable proteins, posing additional food safety concerns (Armentia et al., 2006; Audicana and Kennedy, 2008; Nieuwenhuizen, 2016). Up until now, 14 allergens have been officially recognized by the World Health Organization and International Union of Immunological Sciences (WHO/IUIS) (Fæste et al., 2014).

Detection of anisakid nematodes is considered an important component in safeguarding seafood safety and quality, and European legislation is in place to make sure seafood is treated. For example, EU regulation (EC) No. 853/2004 mandates treatment of fishery products intended to be consumed raw or lightly processed, with methods such as freezing to ensure no live larvae are ingested by the consumer (Bao et al., 2019; EFSA, 2024). However, these processes are not always adequate in inactivating allergenic proteins. Indeed, some of these proteins are thermostable and largely retain their allergenic properties after treatment. Reports have described instances where sensitized individuals have developed allergic reactions after consuming processed seafood (Mehrdana and Buchmann, 2017; Rahmati et al., 2020). Due to the possibility of hypersensitivity to anisakid allergens, monitoring the presence of biological contaminants in fish is becoming an important issue (Carrera et al., 2016).

Up until recently, it was believed that anisakid material was only present in wild-caught fish, as aquaculture products were considered to be *Anisakis*-free. However, recent evidence challenges this assumption, as a 2.5% *Anisakis* prevalence was determined in market-size Norwegian farmed cod (Bao et al., 2025). Additionally, a study by Polimeno et al. (2021) demonstrated that allergens could be transmitted through contaminated feed like fishmeal. Other studies have also shown proof of concept of allergen transmission through feed (Fæste et al., 2015a; Saelens et al., 2023).

Typically, detection of visual parasites in industrial settings relies on visual inspection methods such as candling, as described in European Regulation (EC) No 2074/2005, which is labour-intensive and has limited sensitivity (EFSA, 2010; Mercken et al., 2021). However, as described above, detection of anisakid material cannot be solely based on visual detection methods, as allergens can be transmitted through feed. Without the presence of a larva, this would be undetectable. Additionally, it has been established that parasitic nematodes leave distinct molecular footprints within the host tissues they inhabit. Throughout their lifecycle, they actively release excretory-secretory proteins and extracellular vesicles into the surrounding matrix. These persistent traces allow for the diagnosis of infestations without the necessity of locating an intact worm (Palomba et al., 2023). Although currently there are no regulations which mandate the detection of anisakid traces, this could potentially constitute a health risk. In the case of additional regulations, there is a need for more sensitive, rapid, and scalable techniques. In scientific and research settings, detection of anisakid trace material is an important topic, and a range of analytical methods, including immunological assays, molecular diagnostics (e.g., PCR, qPCR, LAMP), proteomics-based approaches, and next-generation sequencing (NGS) technologies have been developed.

These methods vary widely in terms of their detection limits, specificity, throughput, and applicability to different sample matrices. These range from raw and processed seafood, to other animal matrices such as chicken products (Saelens et al., 2023). As regulatory bodies and industry stakeholders increasingly recognize the importance of detecting both viable parasites and their molecular or protein residues, a systematic evaluation of available detection methods becomes essential.

This systematic review aims to provide a comprehensive overview of current methodologies used for the detection of anisakid nematode traces, such as genomic material and proteins. It evaluates the strengths and limitations of each technique, contextualizes their application in food safety and surveillance, and identifies knowledge gaps and opportunities for method harmonization and improvement. By consolidating existing evidence, this review seeks to inform both regulatory practice and future research directions in the detection and risk assessment of anisakid parasites.

## 2. Methods

### 2.1. Search strategy

A systematic search of published literature on 'Methods for the detection of anisakid material in different food matrices' was conducted by implementing the PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis) guidelines (Page et al., 2021). For a first identification phase of relevant articles, a search strategy was developed with the inclusion of Boolean operators (AND, OR, *) and key words referring to the detection of material, either DNA, proteins or peptides, from *Anisakis* spp., *Phocanema* spp., *Pseudoterranova* spp. and *Contraceacum* spp.. This was performed blinded, and systematically applied in four electronic search engines (PubMed, Web of Science, Scopus, and Google Scholar). To ensure the accuracy and reproducibility, the electronic search strategy was peer-reviewed by a second, independent researcher/reviewer. Discrepancies in retrieved records were discussed and resolved through consensus. Searches were limited to English language and studies published from January 2006 to June 2025. Hence, only peer-reviewed and indexed literature -with the exception of one paper by Stobbeleir (2022), which was not peer reviewed- was retrieved using the following search syntax: ((((Anisaki*) OR (Anisakidae) OR (Pseudoterranova) OR (Phocanema)) AND (antigen* OR allergen*)) AND (mass spectrometry OR ELISA OR immunoassay OR PCR)) AND (detection).

### 2.2. Study selection

Following the initial identification phase, the selection of relevant articles for the literature review proceeded through three consecutive screening stages. Each stage was conducted independently by two researchers. The first phase involved removing

duplicate records after merging results from the four search engines using the reference management tool Zotero, along with Rayyan, a web-based tool designed to streamline the early stages of systematic reviews through semi-automated screening (Ouzzani et al., 2016) (https://www.zotero.org/; https://www.rayyan.ai/). In the second phase, titles and abstracts were screened to assess their relevance to the study question. This step was carried out using Rayyan. Two authors performed this screening independently and in a blinded manner, applying predefined exclusion criteria: (i) record not available in English, (ii) record with no available full text, (iii) record focusing on the wrong parasite or species, (iv) record focusing on detection and/or diagnosis in humans, (v) record with a focus on detecting seropositivity in animals (e.g. presence of antibodies against Anisakidae, not the presence of anisakid material), (vi) records with a focus on characterization of anisakid proteins outside of a matrix, (vii) records that use visual methods for the detection of Anisakidae (candling, UV-press, artificial digestion), and finally, (viii) record is a review.

Next, a final screening phase was conducted, this time involving full-text evaluation of the remaining records. In addition, potentially relevant studies not captured by the initial search strategy were identified by reviewing the reference lists of the selected articles. These additional records were also assessed for relevance based on their full-text content. Additionally, two relevant studies were identified which were not found by the search syntax (Godínez-González et al., 2021, 2017).

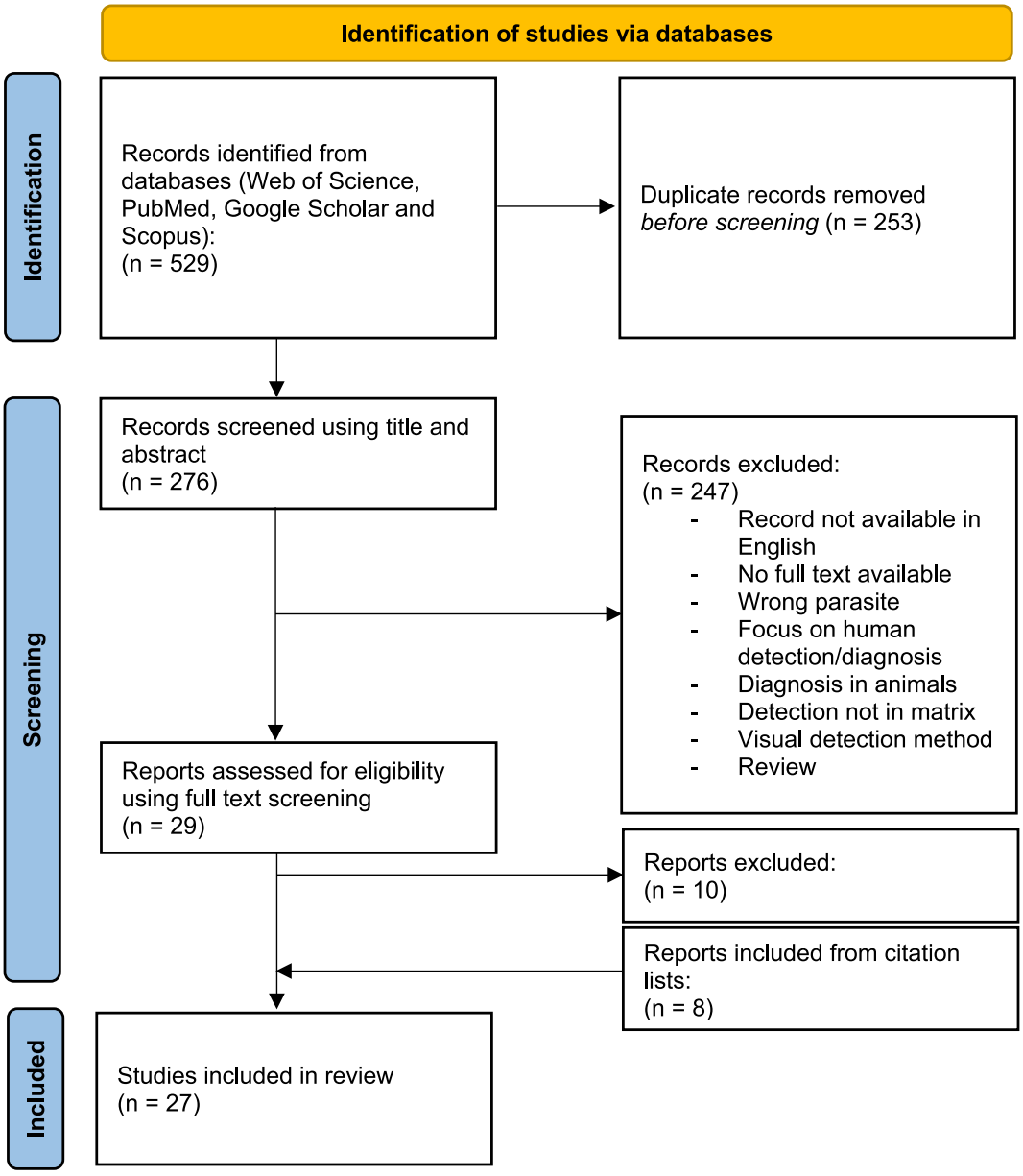


**Fig. 1.** PRISMA 2020 flow diagram for new systematic reviews which included searches of databases.

### 2.3. Data extraction

The final set of included studies was then categorized according to their underlying detection principles (i.e., DNA-based, immunochemistry-based, and mass spectrometry-based) in order to extract the following data: techniques or methods used, which parasite species is targeted, sample type or matrix, sample size, amount of sample (concentration or weight), qualitative or quantitative method, quantitative range, detection limit, intra- and inter-assay coefficient of variation or repeatability, prevalence of parasite material in the matrix, sensitivity, specificity, if matrices were spiked, the protein or DNA extraction method, pre-processing steps, which assay reagents and detection equipment were used, and finally technical advantages and disadvantages. For DNA-based detection methods, data on the targeted DNA region and used primers were extracted. Specifically for immunochemistry-based methods, following data were extracted: sample storage method, identification method (specific monoclonal antibody, SDS-PAGE, etc.), ELISA type (competitive, direct, indirect, sandwich, etc.) or other test formats, allergen/protein targeted, use of polyclonal or monoclonal antibodies, cross-reaction. In the case of mass spectrometry-based articles, following data was additionally extracted: use of discovery versus targeted proteomics, which peptides are targeted, proteotypicity (i.e., uniqueness) of targeted peptides, peptide separation method, amount of injected material, data analysis programs and databases used, and finally, standards used in case of quantitative methods.

## 3. Results

In total, 529 records were identified over all databases. For Web of Science, PubMed, Google Scholar and Scopus, respectively 30, 61, 100 and 338 records were found. After a first screening phase in Zotero and subsequently Rayyan, 253 duplicate records were removed, resulting in 276 unique records which were eligible for title and abstract screening. After this second screening phase, a total of 247 records were excluded due to the exclusion criteria: 96 records had a focus on detection or diagnosis in humans, 68 records were removed due to detection or identification out of matrix. Another 34 records were excluded due to a focus on wrong parasite or species protein or DNA material, 20 records focused on diagnostics in animals, another 22 were review papers. Finally, five records were not available in English, one did not have the full text available, and one was based on visual detection. For the last phase where the full text was screened, 10 papers were excluded, while six papers identified in citation lists and two by a reviewer were included, bringing the total of included papers in this systematic review to 27 (See Fig. 1).

The included records were then divided according to their detection principles, with 10 in the DNA-based detection category, 11 in the immunochemistry-based, three in mass spectrometry-based category, and three papers described a combination of methods.

### 3.1. DNA-based methods

DNA-based methods detect allergenic sources by targeting species-specific genetic material. Although these methods do not detect allergens directly, they can indicate indirectly that allergens could be present. These approaches can be useful when highly specific detection at the species level is required, as well as in processed products where disintegrated larvae could not be detected. Out of all studies identified, 12 described the use of DNA-based methods.

### 3.2. Summary of DNA-based methods

The DNA-based methods used in the identified records can be divided into 4 categories, namely conventional polymerase chain reaction (PCR), real-time polymerase chain reaction (RT-PCR), high throughput sequencing (HTS) and loop-mediated isothermal amplification (LAMP) (Table 1). Molecular methods were applied to the following matrices: fresh, frozen and processed fish, cephalopods, and fishmeal. Two studies were identified where a conventional PCR method was used for the detection of anisakid DNA in seafood. The aim of these studies was to detect the presence of anisakid nematodes in seafood, not to detect allergenic material. The reporting on the limit of detection (LOD) and spiking was not performed in a uniformed way. While Santos et al. (2006) reported an LOD of 1 part *Anisakis* spp. DNA/100,000 fish DNA, Espiñeira et al. (2010) reported an LOD of 1 part larvae tissue to 6250 parts of fish tissue. Another difference between the two methods was the targeted DNA region. While Santos et al. (2006) targeted the 18S ribosomal RNA gene, Espiñeira et al. (2010) targeted the internal transcribed spacer I (ITS-1) region. Both studies reportedly had good specificity, although no quantifiable numbers are reported. Santos et al. (2006) describe 5 primer pairs, 3 of which amplify the Anisakidae species *Anisakis simplex* and *Phocanema decipiens*, and the Raphidascarididae species *Hysterothylacium aduncum*, while no cross-reaction with fish is observed. Another eight different papers used RT-PCR as DNA-based method. The limit of detection (LOD) varied significantly across studies, and again reported in different manners, sometimes comparing amount of DNA, or comparing total tissue used. Mossali et al. (2010) demonstrated an LOD of 1:10,000 dilution of *Anisakis* spp. and *Phocanema* spp. DNA in fish DNA. Similarly, Cavallero et al. (2017) reported detection thresholds of 0.00032 ng/μL for *Anisakis* species (*A. pegreffii*, *A. simplex* sensu stricto, hybrid *A. simplex/A. pegreffii*, *A. typica*, *A. physeteris*) and 0.0016 ng/μL for *Phocanema* species (*P. decipiens*, *P. krabbei*, *P. azarasi*, *P. cattani*). In contrast, Herrero et al. (2011) reported a detection limit of 1 ppm nematode tissue (genera *Anisakis*, *Phocanema*, *Contracaecum* and *Hysterothylacium*) using 300 mg of fish muscle tissue. Lopez and Angel Pardo (2010) confirmed detection down to 40 ppm per 25 g of seafood. Fæste et al. (2015a) described a lower limit of application (LLA) of 0.0004 ng/100 ng in fish feed DNA. Godínez-González et al. (2017) reported the detection limit of their technique as 1.50 ng of *A. simplex* (s.l.) DNA per gram of sample, while in the 2020 study quantification was reported using the number of *Anisakis* larval equivalents (NLE) calculated using the descriptive function based on amount of larvae detected with visual inspection (Godínez-González et al., 2021). Bald et al. (2024)

**Table 1**
Overview of DNA-based detection techniques of anisakid material.

| Method | Number of studies | Sample Type | Spiking | LOD | Specificity | DNA region targeted | Ref. and year |
|---|---|---|---|---|---|---|---|
| Conventional PCR | 2 | | | | | | |
| | | Eviscerated *Sardina pilchardus* | 1 g *Anisakis*/96870 g fish to 1 g/294 g | 1/100,000 *Anisakis* DNA/ fish DNA | All the sequences were correctly assigned to the anisakid species that were used in each mixture | 18S rRNA gene | Santos et al. (2006) |
| | | Fishes: *Thunnus alalunga*, *Euthynnus lineatus*, *Sarda orientalis*, *Rastrelliger kanagurta*, *Scomber colias*, *Auxis thazard*, *Engraulis encrasicolus*, *Limanda aspera*, *Microstomus pacificus*, *Brama australis*, *Salmo salar*, *Oncorhynchus mykiss*, *Salilota australis*, *Decapterus macarellus*, *Gadus morhua*, *Merluccius merluccius* Cephalopods: *Loligo vulgaris*, *Octopus vulgaris*, *Todarodes sagittatus*, *Sepia officinali* | 0.1–100% | 1 part larvae/ 6250 part fish | No amplification from non-anisakid parasites or fish host DNA | ITS-1 | Espiñeira et al. (2010) |
| RT-PCR | 8 | | | | | | |
| | | Hake silage | No spiking | Not described | Not described | COII | Bald et al. (2024) |
| | | commercial zebrafish feed | No spiking | $4*10^{-4}$ ng/ 100 ng extracted DNA | Only detected in spiked fishmeal | COII | Fæste et al. (2015a) |
| | | Commercial fish species: *Coryphaenoides rupestris*, *Engraulis encrasicolus*, *Engraulis anchoita*, *Gadus macrocephalus*, *Gadus morhua*, *Katsuwonus pelamis*, *Merlangius merlangius*, *Merluccius capensis*, *Molva molva*, *Pollachius pollachius*, *Sarda sarda*, *Scomber japonicus*, *Theragra chalcogramma*, *Thunnus albacares*, *Thunnus obesus*, *Thunnus thynnus*, and *Trisopterus luscus*; farmed turbot (*Psetta maxima*); terrestrial species | 25 and 50 g turbot spiked with 1 mg *A. simplex* | 40 ppm in 25 g sample | Some cod samples gave signal, author suggests presence of *A. simplex* | COII | Lopez and Angel Pardo (2010) |
| | | Spiked fish fillets, raw unspiked marine tissue (fish, crustacean, squid) | Spiked with purified *Anisakis* and *Phocanema* DNA | *Anisakis*: 0.00032 ng/µl *Phocanema*: 0.0016 ng/µl | High specificity: no amplification for negative samples | ITS-region | Cavallero et al. (2017) |
| | | Fishes and cephalopods | Spiked with 100% to 0.016% anisakid tissue | 0.016% in 300 mg fish tissue | High specificity: no amplification in fish species, cephalopods or other non-anisakid parasites | COI | Herrero et al. (2011) |
| | | Experimental samples: Hake fish tail (*Merluccius merluccius*) Commercial samples: Atlantic blue whiting | Hake spiked with 0 to 50 *A. simplex* (s.l.) L3 larvae | 1.50 ng of *A. simplex (s. l.)* DNA per gram of sample | Specific for *A. simplex*, no detection of *Hysterothylacium* when present | COII | Godínez-González et al. (2021) |

(*continued on next page*)

**Table 1** (*continued*)

| Method | Number of studies | Sample Type | Spiking | LOD | Specificity | DNA region targeted | Ref. and year |
|---|---|---|---|---|---|---|---|
| | | (*Micromesistius poutassou*) | | | | | |
| | | Crab sticks (surimi), gulas (surimi), croquettes (hake), burger (hake, salmon) | Crab sticks with different concentrations ranging from $1.36*10^{4}$ to $1.36*10^{-3}$ ng of *A. simplex* (s.l.) DNA per gram of processed food | 1.50 ng of *A. simplex (s. l.)* DNA per gram of sample | *Pseudoterranova* and *Hysterothylacium* DNA was not amplified | COII | Godínez-González et al. (2017) |
| | | Fresh and frozen fish fillets; fish-derived food products (baby food, surimi, fish sticks, canned tuna products) | 1 mg of anisakid larvae spiked in 1, 10, and 100 g fresh cod | 1:10,000 anisakid DNA: Fish DNA | No cross-reaction observed | ITS1–1 gene (ANIKIT primers) 18S rRNA gene (EUKA primers) | Mossali et al. (2010) |
| HTS | 1 | | | | | | |
| | | Degreased fish feed | No spiking | Not described, detected trace amounts in fishmeal | Not described | COI | Abollo et al. (2024) |
| LAMP | 1 | | | | | | |
| | | Farmed trout, sea bream, salmon<br>Infested anchovy and sardine products | The number of *Anisakis* spp. larvae used for artificial contamination was inferred from the prevalence of infestation of the fish species as described in the literature | 0.00022 ng /µL | No amplification products were detected in uninfested samples, giving a very high specificity rate<br>Only *Anisakis* spp. DNA was amplified | ITS-2 | Cammilleri et al. (2020) |

COI = Mitochondrial cytochrome *c* oxidase I gene, COII = Mitochondrial cytochrome *c* oxidase II gene, HTS = high throughput sequencing, ITS = internal transcribed spacer, LOD = Limit of detection, LAMP = loop-mediated isothermal amplification.

provided only qualitative assessments of *Anisakis* presence in hake silage with no precise detection limits, and identification of larvae only to the genus level. The studies targeted a variety of genetic markers. The mitochondrial cytochrome oxidase subunit II (COII) gene was most commonly used (Lopez and Angel Pardo, 2010; Fæste et al., 2015a; Godínez-González et al., 2017, 2021; Bald et al., 2024), while COI was employed by Herrero et al. (2011). The LOD reported by Lopez and Angel Pardo (2010) and Fæste et al. (2015a) could be compared if we assume the amount of DNA extracted per gram of larval tissue is equal to that of DNA per gram of matrix. Fæste et al. (2015a) reported an LOD which was around 10 times lower, with 4 parts larvae that could be detected in $10^{6}$ parts matrix. However, the matrices in which this was tested differed between the two, with Fæste et al. (2015a) testing fish feed instead of fish tissue. Detection of *Anisakis* and *Phocanema* species through the ITS1 region was utilized in two studies (Mossali et al., 2010; Cavallero et al., 2017). The LOD of the two studies targeting ITS-1 could not be compared, as the LOD reported by Cavallero et al. (2017) was determined in the absence of fish matrix. When comparing all RT-PCR methods, the ones targeting COII seem to be the most sensitive. Remarkably, the LOD of a method using conventional PCR described above (Santos et al., 2006) was reported to be 10 times lower than an RT-PCR method using a similar approach targeting the 18S rRNA gene (Mossali et al., 2010). Six out of eight RT-PCR studies employed highly specific primer-probe systems, typically TaqMan-based, some using commercial kits with proprietary sequences (e.g., PATHfinder) (Cavallero et al., 2017). Two RT-PCR studies described the use of SYBR Green, where no probe system was used (Godínez-González et al., 2021, 2017). Sample types ranged from isolated parasite DNA and spiked tissues to highly processed fish products. Most assays demonstrated high specificity, with no cross-reactivity observed in negative controls or non-target tissues. Cavallero et al. (2017) noted false positives in herring and mackerel, likely due to undetected *Anisakis* spp. DNA in fish tissue rather than probe cross-reactivity. Similarly, Lopez and Angel Pardo (2010) detected some positive signals for cod, which they also accredit to undetected presence of *A. simplex* in the fish sample. All TaqMan-based systems showed excellent discrimination between closely related species.

The LAMP DNA-based detection method was described in one identified paper (Cammilleri et al., 2020). Naturally negative fish samples ($n = 120$) from aquaculture were tested as negative controls, while processed infested anchovy ($n = 80$) and sardines ($n = 40$) were used as positive samples. No quantitative range was described, and hence this method was considered as used for qualitative detection. A sensitivity of 100% was reported, as DNA from *A. simplex* s.s., *A. pegreffii*, *A. physeteris*, *A. ziphidarum*, and *A. typica* could be detected in all positive controls. There was no amplification in any of the negative controls, nor in samples contaminated with *Contracaecum* sp., *Phocanema* sp., and *Hysterothylacium* sp., so the authors described the specificity rate as being very high. The LOD was established as the lowest concentration of DNA of *Anisakis* species that provided a significantly different signal to the negative

control. This LOD of 0.00022 ng DNA/µl was thus determined in the absence of a fish matrix. This is slightly lower compared to the LOD reported by Cavallero et al. (2017) for their RT-PCR method (0.00032 ng/µl for *Anisakis* spp.). This LAMP method used the ITS-2 gene as target and was able to amplify *Anisakis* spp. DNA in processed fish samples. A significant advantage compared to RT-PCR is the ease of use combined with its speed, making it a valid alternative for routine examination in field settings.

Finally, one paper describes the use of high throughput sequencing (HTS) for analysing fish feed composition (Abollo et al., 2024). Mini-barcoding primers Uni MinibarF1 and Uni MinibarR1 for the COI region were used. Genetic material from the phylum Nematoda was detected in 80% of the feeds analyzed. The anisakid species *Phocanema krabbei* was identified in 2 out of 10 samples, while the Raphidascarididae species *Hysterothylacium aduncum* was found in 6 out of 10 samples. The implementation of HTS technologies allows profiling the composition of complex samples at low cost, such is the case of the analysis of animal feeds carried out in this work, on top of detecting anisakid material, other ingredients of fish feed can be identified. Because no spiking was performed, the LOD could not be determined and compared to the other DNA-based methods. However, it can be assumed that trace amounts of anisakid DNA were detected, and further validation of this method with positive controls could lead to a highly valuable detection method.

Different DNA extraction systems were used for the DNA-based detection methods. For both conventional PCR methods, the cetyltrimethylammonium bromide (CTAB) phenol-chloroform protocol was used for extraction. In the method described by Santos et al. (2006), there was either a prior lyophilisation step (2.0–4.0 g of material was sub sampled) or boiling in a water-bath at 100 °C (supernatant divided in 10 mL sub samples), while the method described by Espiñeira et al. (2010) used processed samples, where thermal treatments were applied for validation. Additionally, Santos et al. (2006) also applied a Qiagen extraction on 25 mg of material. However, the authors recommended CTAB extraction to test total original material instead of a small amount. Concerning the RT-PCR methods, three out of eight methods described some sort of pre-treatment. This included acid autolysis at a pH of 3.5 (Bald et al., 2024), defatting and salt removal by washing and blotting with a filter paper (Lopez and Angel Pardo, 2010), and transformation processes such as canning, smoking, freezing, frying, cooking, salting and drying (Herrero et al., 2011). Multiple DNA extraction systems were mentioned for this category. These included the Wizard DNA Clean-Up System (Lopez and Angel Pardo, 2010; Fæste et al., 2015a), the Wizard Magnetic DNA Purification System (Promega, Madison, WI) (Lopez and Angel Pardo, 2010; Mossali et al., 2010), the Wizard Genomic DNA Purification Kit (Godínez-González et al., 2021, 2017), the CTAB phenol-chloroform protocol (Herrero et al., 2011), and the REDExtraction-N-Amp tissue PCR Kit (Sigma-Aldrich, St. Louis, MO) (Mossali et al., 2010). The DNA extraction for the LAMP method described by Cammilleri et al. (2020) used a ready-to-use buffer contained in the *Anisakis* Screen Glow kit (Enbiotech S.r.l., Palermo, Italy). After extraction, 250 mg of sample was incubated in 4 mL of buffer for 40 min at room temperature. Finally, for the HTS method (Abollo et al., 2024) two grams of each fish feed were degreased. Hereafter, a QIAamp DNA Mini Kit (Qiagen) was used for DNA-extraction.

### 3.3. Immunochemistry-based methods

Immunochemical methods are commonly used for protein or allergen detection, and are based on the antigen-antibody interactions of specific allergens in various matrices. In this review, enzyme-linked immunosorbent assays (ELISA), immunostaining, immunoblotting, and immunohistochemistry detection methods are described, which vary in complexity, sensitivity and application scope. Out of all studies identified, 14 described the use of immunochemistry-based methods.

### 3.4. Summary of Immunochemistry-based methods

The identified methods can be divided into 3 different categories. These methods are the enzyme-linked immunosorbent assays (ELISA), immunostaining methods, and immunohistochemistry methods (Table 2). Eight papers were identified where an ELISA method was employed to detect anisakid antigens and allergens. These are differentiated by the selectivity of the used antibodies. Indeed, when a polyclonal antiserum is used targeting crude *Anisakis* spp. extract, a positive signal does not conclusively mean allergens are present. In contrast, when antisera targeting 1 specific allergen are used, a positive signal implies the presence of allergens. These detection methods were employed on a range of matrices, mainly on fresh fish, but also on highly processed fish such as hake silage, fishmeal and fish balls (Bald et al., 2024). In five of these studies, matrices were spiked with intact *A. simplex* larvae or antigen (Xu et al., 2010; Werner et al., 2011; Fæste et al., 2015a; Fæste et al., 2015b; Kochanowski et al., 2020). In the other studies, samples were either naturally infested or the infestation status was unknown (Arilla et al., 2008; Stobbeleir, 2022; Bald et al., 2024). Polyclonal rabbit anti-*A. simplex* antibodies were the most commonly used detection antibodies, being used in five studies (Xu et al., 2010; Werner et al., 2011; Fæste et al., 2015a; Fæste et al., 2015b; Kochanowski et al., 2020). Bald et al. (2024) used sera of patients sensitized to *A. simplex* to detect the presence of anisakid material in hake silage. Two papers described the use of monoclonal antibodies, with Arilla et al. (2008) using an 4F2 monoclonal capture antibody in combination with an anti-Ani s 1 polyclonal tracer antibody. Another method used unlabeled monoclonal UA3 antibodies (specific for Ani s 7) for antigen capture, and FITC-labeled UA3 antibodies for detection (Stobbeleir, 2022). Concerning the 5 immunostaining methods described, the matrices on which they were applied were similar to those described for the ELISA methods. Matrices were spiked with *A. simplex* material for the immunostaining methods in 3 studies (Fæste et al., 2015a; Fæste et al., 2015b; Rodríguez-Mahillo et al., 2010). For the other immunostaining methods, no spiking was performed and hence no LOD could be determined (Polimeno et al., 2021; Bald et al., 2024). All these methods deployed patient sera with anti-*Anisakis*-specific antibodies, with either IgE or IgG antibodies. Some methods additionally used self-produced specific polyclonal anti-*A. simplex* antibodies (Rodríguez-Mahillo et al., 2010; Fæste et al., 2015a; Fæste et al., 2015b).

ELISA-based methods were consistently reported to offer high sensitivity for allergen detection. However, direct comparison of detection limits across studies was complicated by significant variability in experimental design. One key issue was the diversity of

**Table 2**
Overview of immunochemistry-based detection techniques of anisakid material.

| Method | No. of studies | Sample Type | Spiking | LOD | Specificity | Monoclonal/ polyclonal | Ref. and year |
|---|---|---|---|---|---|---|---|
| ELISA | 8 | | | | | | |
| | | Raw and boiled seabream, monkfish, sardine, anchovy | No | 1.8 ng/mL or 2.5 larvae/kg fish | *Ascaris lumbricoides*: detected 0.04 Ani s 1-like protein per gramme of extract | 4F2 monoclonal antibody (capture); anti-Ani s 1 polyclonal antibody (tracer) | Arilla et al. (2008) |
| | | Hake silage | No | N.D. | N.D. | Polyclonal patient sera | Bald et al. (2024) |
| | | Fish feed<br>Zebrafish | 20% F1 zebrafish spiked | **Zebrafish:** 2 mg/kg<br>**Fish feed:** 5 mg/kg | N.D. | Rabbit polyclonal antibodies | Fæste et al. (2015b) |
| | | Mackerels (*Scomber scombrus*), Sardines (*Sardina pilchardus*), Herring (*Clupea harengus*), Anchovy (*Engraulis encrasicolus*), Pollack (*Pollachius pollachius*), Haddock (*Melanogrammus aeglefinus*), Salmon (*Salmo salar*), Cod (*Gadus morhua*), Products containing shrimp (*Pandalus borealis*), Crabs (*Cancer pagurus*) and Scallops (*Pecten maximus*) | Spiked cod muscle (50 *A. simplex* larvae) | 0.3 mg protein/kg fish | N.D. | Rabbit polyclonal antibodies | Fæste et al. (2015b) |
| | | Heated Spiked and Non-Spiked Trout Samples<br><br>Spiked Autoclaved Canned Fish Products<br><br>Seafood Products from the Market | Spiked trout: native *A. simplex* antigen: (0.05–400 ng/mL) autcoclaved fish products (1–8 larvae/ 200 g fish muscle) | **CL-S-ELISA**: 0.5 ng/mL<br>**CL-C-ELISA**: 5 ng/mL | No cross-reaction with the different fish matrices and products | Rabbit polyclonal antibodies | Kochanowski et al. (2020) |
| | | Fishmeal (5 different manufacturers) | No | 40 ng/mL | Specific for Ani s 7, no cross-reactions described | Monoclonal UA3 antibody (Specific for Ani s 7) | Stobbeleir (2022) |
| | | fish balls and mackerel in tomato sauce<br><br>cod meat | 1, 10, or 100 µg/g | **In codfish and mackerel with tomato sauce:** 0.6 mg *A. simplex* protein/kg food<br>**In fish balls:** 0.5 mg *A. simplex* protein/kg food | Cross-reaction assessed for fish, crustaceans, molluscs and insects: none >0.002% | Rabbit polyclonal antibodies | Werner et al. (2011) |
| | | *Pseudopleuronectes yokohamae* (plaice) *Scomberomorus niphonius* (mackerel) *Ommastrephes bartrami* (squid) | 20–100 larvae/kg | 5 *A. simplex* larvae/kg food | high cross-reactivity (75.4%) to *Phocanema* larvae | Rabbit polyclonal antibodies | Xu et al. (2010) |

(*continued on next page*)

**Table 2** (*continued*)

| Method | No. of studies | Sample Type | Spiking | LOD | Specificity | Monoclonal/ polyclonal | Ref. and year |
|---|---|---|---|---|---|---|---|
| Immunostaining | 5 | | | | | | |
| | | Hake silage | No | N.D. | N.D. | Patient sera with anti-*Anisakis*-specific IgE antibodies (polyclonal) | Bald et al. (2024) |
| | | Fish feed Zebrafish (a F4 generation of the "Tupfel long-fin" wild-type strain line) | 20% F1 zebrafish spiked | < 2 mg/kg | No cross-reaction to shrimp or mite | Polyclonal anti-*A. simplex* antibodies (IgG); Serum of a patient with *A. simplex* allergy (IgE) | Fæste et al. (2015a) |
| | | Mackerels (*Scomber scombrus*), Sardines (*Sardina pilchardus*), Herring (*Clupea harengus*), Anchovy (*Engraulis encrasicolus*), Pollack (*Pollachius pollachius*), Haddock (*Melanogrammus aeglefinus*), Salmon (*Salmo salar*), Cod (*Gadus morhua*), Products containing shrimp (*Pandalus borealis*), Crabs (*Cancer pagurus*) and Scallops (*Pecten maximus*) | Spiked cod muscle (50 *A. simplex* larvae) | 0.3 mg protein/kg food | N.D. | Polyclonal anti-*A. simplex* antibodies (IgG); Serum of a patient with *A. simplex* allergy (IgE) | Fæste et al. (2015b) |
| | | Fresh hakes (*Merluccius merluccius*), Anchovies (*Engraulis encrasicholus*), Sardines (*Sardina pilchardus*), Red mullets (*Mullus barbatus*), Blue whitings (*Micromesistius potassou*), Clams (*Tapes decussatus*), Shrimps (*Parapanaeus longirostris*), Cockles (*Cerastoderma edule*) from local markets Hake stock | 10 g fillets injected with 20 µL *A. simplex* crude extract (0–200 µg/mL) or recombinant Ani s 4 (0–10 µg/mL) | **Spiked blue whiting:** 200 ng *A. simplex* crude extract/g of fish; 2.5 ng of Ani s 4/g fish **Spiked anchovies:** 625 ng *A. simplex* crude extract/g of fish; 5 ng of Ani s 4/g fish | No cross-reaction with shellfish such as shrimps, cockles, or clams | polyclonal anti–*A. simplex* crude extract or anti recombinant Ani s 4 rabbit antisera pool of *A. simplex*–sensitized human sera | Rodríguez-Mahillo et al. (2010) |
| | | Aquacultured sea bream Infested horse mackerel Commercial fishmeal | No | N.D. | All selected serum samples for the study showed undetectable sIgE to fish and in particular to cod, shrimp and dust mite | Patient sera (polyclonal human IgE) used as primary antibody; HRP-conjugated anti-human IgG as secondary antibody. | Polimeno et al. (2021) |
| Immunohistochemistry | 4 | | | | | | |
| | | Steaks of frozen-thawed dorsal muscle of hake (*Merluccius merluccius*), anchovy (*Engraulis encrasicolus*) | Unspecified number of larvae in hake steaks and anchovy fillets | N.D. | N.D. | Polyclonal rabbit anti–Ani s 4 or anti–crude extract antisera | Solas et al. (2008) |
| | | Anchovy (*Engraulis encrasicolus*) | 50 g of fillets artificially infested with | N.D. | N.D. | Polyclonal rabbit anti–Ani s 4 or | Solas et al. (2009) |

(*continued on next page*)

**Table 2** (*continued*)

| Method | No. of studies | Sample Type | Spiking | LOD | Specificity | Monoclonal/ polyclonal | Ref. and year |
|---|---|---|---|---|---|---|---|
| | | | 12 live *A. simplex* larvae | | | anti–crude extract antisera | |
| | | Hake (*Merluccius merluccius*) | 12 live *A. simplex* larvae between 2 hake steaks | N.D. | N.D. | Polyclonal rabbit anti–Ani s 4 antiserum | Vidaček et al. (2009) |
| | | Hake (*Merluccius merluccius*) | 12 live *A. simplex* larvae between 2 hake steaks | N.D. | N.D. | Polyclonal rabbit anti–Ani s 4 antiserum | Vidaček et al. (2011) |

CL-C-ELISA = Chemiluminescent competitive ELISA, CL-S-ELISA = Chemiluminescent sandwich ELISA, LOD = Limit of detection, N.D. = Not described.

sample matrices, ranging from processed food products to fluids such as hake silage, which can influence extraction efficiency and assay performance. For Arilla et al. (2008), there was no clear mention on how the extracts of fish tissue were obtained, but they were added at 1 mg/mL to the ELISA wells, and spiked with Ani s 1 extract. Xu et al. (2010) described spiking 20 to 100 larvae/kg fish muscle, followed by incubation and protein extraction in 1:5 w:v PBS, then adjusted the volume to 500 mL and diluted this 5 times in 1% BSA in PBS. From this solution, 100 µL was used in the ELISA microplates. Werner et al. (2011), as well as (Fæste et al., 2015b), used homogenized samples of 2 g, with proteins extracted in 10 mL of a Tris-glycin solution. Prior to ELISA, the protein extracts were diluted at least 1:20 in PBS. A PBS extraction method with 2 g of matrix was also used in the studies by Fæste et al. (2015a, 2015b), and Stobbeleir (2022). Kochanowski et al. (2020) homogenized 200 g of food sample in 600 mL of PBS. The preprocessing steps described by Bald et al. (2024) were more elaborate. In short, approximately 1 kg of hake viscera was ground, and the pH was adjusted to 3.5, to perform silage for 11 days. The ELISA tests were performed straight on the liquid fractions which were obtained after centrifugation.

Additionally, studies reported detection limits using differing units, such as mass of allergen per mass of food (e.g., mg/kg), mass per volume (e.g., ng/mL), or based on biological equivalents (e.g., number of larvae or amount of larval protein). LODs of 0.5–5 mg *A. simplex* protein per kg of fish were reported (Werner et al., 2011; Fæste et al., 2015a). Alternatively, 2.5–5 larvae/kg of fish could be detected (Arilla et al., 2008; Xu et al., 2010). Another reported metric ranged from 0.5 ng/mL to 40 ng/mL (Arilla et al., 2008; Kochanowski et al., 2020; Stobbeleir, 2022). This lack of standardization hinders straightforward cross-study comparison and obscures broader conclusions about ELISA's relative sensitivity. Harmonizing reporting standards and matrix-specific validations would enhance the comparability and reliability of ELISA-based allergen detection. The reporting of LODs for the immunostaining methods was done more uniformly. Here, LODs ranged 0.2 µg crude *Anisakis* spp. extract per gram of food and 2 µg/g. Alternatively, when targeting Ani s 4 specifically, the LOD was lower (2.5–5 ng/g).

Finally, four studies employed immunohistochemistry-based (IHC) methods to detect *A. simplex* proteins (Solas et al., 2008, 2009; Vidaček et al., 2009, 2011). All studies performed spiking on either hake or anchovy fillets by placing live larvae between two steaks. Polyclonal rabbit anti-Ani s 4 or anti-crude extract antisera were employed for detection. A notable limitation across all studies employing immunohistochemistry (IHC) was the absence of reported detection limits. Allergen presence was assessed qualitatively through microscopic visualization of antibody binding, without quantitative data. This contrasts with the other immunochemistry-based methods described above, which typically report specificity metrics or an LOD. The lack of quantitative data in IHC limits its utility for determining trace allergen levels and comparing sensitivity across methods. While IHC provides valuable spatial and cellular context, especially for localizing excretory/secretory (E/S) proteins in an *A. simplex* infested sample, its subjective nature and qualitative output highlight the need for complementary quantitative techniques in allergen detection workflows. Additionally, these methods were only performed on unprocessed fish samples. Its usefulness on more heavily processed samples is unknown and could warrant further investigation.

### 3.5. Mass spectrometry-based methods

In the last years, mass spectrometry (MS) has emerged as a powerful tool for the detection and quantification of food allergens, offering high sensitivity, and multiplexing capabilities. Unlike traditional immunoassays, which rely on antibody–antigen interactions, MS-based methods identify allergenic proteins or their peptide markers directly through their mass-to-charge ($m/z$) signatures (Monaci and Visconti, 2009). By a prior separation step, for example liquid chromatography (LC), it is possible to detect multiple allergens or proteins in complex matrices (Heick et al., 2011). Out of all studies identified in this systematic review, five described the use of MS-based methods to detect anisakid proteins (Table 3).

### 3.6. Summary of mass spectrometry-based methods

In this review, the MS-base methods are divided into two categories, either untargeted proteomics, or targeted. Here, two studies make use of untargeted proteomics, using a nano-electrospray LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), acquiring mass spectra in positive ion mode in the mass range of m/z 200–2000, which was followed by MS/MS for the most

**Table 3**
Overview of mass spectrometry-based detection techniques of anisakid material.

| Method | No. of studies | Sample Type | Spiking | LOD | Targeted vs Discovery Proteomics | Specificity | Mass Spectrometry device | Ref. and year |
|---|---|---|---|---|---|---|---|---|
| Mass Spectrometry | 5 | | | | | | | |
| | | Fish feed<br>Zebrafish<br>Control samples: naturally-contaminated cod liver, naturally contaminated salmon muscle, spiked cod muscle | Fish feed F1 consists of 20% Anisakis larvae | Not mentioned | Untargeted Proteomics | Two specific marker peptides of *A. simplex* haemoglobin: HSWTTIGEEFGHEADK and LFAEYLDQK | nano-electrospray LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) | Fæste et al. (2015a) |
| | | *Salmo salar* filets | Spiked with concentrations (*w*/w) of 0, 5, 10, 20, 30, 40, 50, 75, 100, 125, and 250 mg larvae/50 g fish | Method 1:<br>LLA: 10 µg/mL.<br>Method 2:<br>LOD: 0.5 µg/mL for LFAEYLDQK and 1 µg/mL for DAFAALAQTFK LLA: 2 µg/mL for both peptides | Targeted proteomics | The two best measurable peptides from both haemoglobin (LFAEYLDQK and HSWTTIGEEFGHEADK) and SXP/RAL-2 (DAFAALAQTFK and IVQTFESLPPAVK) were selected as URPs for *A. simplex*. Homology searching in the NCBI protein database revealed that both proteins were very specific for the genus *Anisakis*. | LC-TripleQ-MS/MS: Agilent 1260 Infinity Binary HPLC System (Agilent Technologies, Palo Alto, CA, USA) with vacuum degasser and thermostatted column department (20 °C) interfaced by electrospray ionization (ESI) with an API 5000 TripleQ MS/MS with Analyst 1.5.2. Software (AB Sciex) | Fæste et al. (2016) |
| | | Mackerels (*Scomber scombrus*), sardines (*Sardina pilchardus*), herring (*Clupea harengus*), anchovy (*Engraulis encrasicolus*), pollack (*Pollachius pollachius*), haddock (*Melanogrammus aeglefinus*), salmon (*Salmo salar*), cod (*Gadus morhua*), products containing shrimps (*Pandalus borealis*), crabs (*Cancer pagurus*) and scallops (*Pecten maximus*) | 20 ng *A. simplex* PBS-extracted standard protein/µL salmon extract | Not mentioned | Untargeted proteomics | Two specific marker peptides of *A. simplex* haemoglobin: HSWTTIGEEFGHEADK and LFAEYLDQK | nano-electrospray LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) | Fæste et al. (2015b) |
| | | Chicken meat and blood, larvae meal, plant-based feed | Gram of larvae administered per chicken per day: Week 1: 0,82 g, Week 2: 1,11 g, week 3: 1,46 g | Not mentioned | Targeted proteomics | Specificity was ensured by targeting proteotypic peptides | IonKey source connected to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Massachusetts, USA) | Saelens et al. (2023) |
| | | Fish muscle extract (*European hake*) | Heavy peptide biomarkers spiked at 0–500 fmol/µL into hake extract and buffer | Not explicitly stated; but sensitivity shown to be down to low fmol/µL range | Both: Shotgun (Untargeted) and PRM (Targeted), only targeted on the spiked hake samples | High specificity: specific to Ani s 9 peptides, validation via heavy peptide spiking | LTQ Velos-Pro mass spectrometer (Thermo Fisher Scientific) | Carrera et al. (2016) |

LLA = Lower limit of application, LOD = Limit of detection, MRM = Multiple reaction monitoring, N.D. = Not described, PRM = Parallel reaction monitoring.

intense peptides using collision-induced dissociation (CID) (Fæste et al., 2015a, 2015b). Two other studies used targeted proteomics methods, more specifically multiple reaction monitoring (MRM), where previously identified target peptides can be identified through their unique precursor-peptide m/z signature (Fæste et al., 2016; Saelens et al., 2023). This makes it possible to quantify peptides, and enhances both sensitivity and reproducibility of the detection method. The paper written by Fæste et al. (2016) describes two targeted proteomics methods, one using label-free semi-quantitative nLC-nESI-Orbitrap-MS/MS, and the other heavy-peptide-applying absolute-quantitative (AQUA) LC-TripleQ-MS/MS. Both of these methods target unique reporter peptides derived from the *A. simplex* allergens Ani s 8 and Ani s 13. The targeted proteomics method described by Saelens et al. (2023) does not include quantification, and is therefore a qualitative detection method. The method targeted all WHO-recognized *A. simplex* allergens with their transitions predicted by *in-silico* digestion using the Skyline application (ver. 20.6, 64-bit, Seattle, USA, www.skyline.ms). The proteomics setup was an IonKey source connected to a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters Corporation, Massachusetts, USA). The last mass spectrometry-based paper described a targeted method, along with an untargeted discovery method which was used for the development of the targeted method (Carrera et al., 2016). The discovery method employs LC-MS/MS using a Proxeon EASY-nLC II liquid chromatography system (Thermo Fisher Scientific, San Jose, CA, US) coupled to a LTQ-Orbitrap Elite (Thermo Fisher Scientific). The targeted method, a parallel reaction monitoring (PRM) analysis, was performed using a Dionex UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) coupled to a LTQ Velos-Pro mass spectrometer (Thermo Fisher Scientific). Four peptides from the SXP/RAL-2 protein Ani s 9 were identified, and used as targets for PRM.

The protein extraction steps could be differentiated by extraction buffer. The most frequently used buffer was PBS in which 2 g of tissue was incubated (Fæste et al., 2016, 2015a, 2015b; Saelens et al., 2023). Additionally, the commercial buffers - TPER (Thermofisher, Massachusetts, USA) and Minute Total Protein Extraction Kit for Muscles (Invent Biotechnologies, Plymouth, USA) - were used to compare to the performance of the PBS buffer (Saelens et al., 2023). Carrera et al. (2016) used a Tris-HCl based lysis buffer for protein extraction on 5 g of fish tissue. For upstream processing, 10–50 μg of the protein samples were digested with trypsin overnight (Fæste et al., 2016, 2015a, 2015b; Saelens et al., 2023). Again, Carrera et al. (2016) utilized a different approach compared to the other studies, with 20 μg of protein supernatants were ultrafast digested with trypsin during simultaneous high-intensity focused ultrasound (HIFU).

Concerning the LODs of the MS-based methods, only one study explicitly stated this metric (Fæste et al., 2016). Out of the two methods described here, the lower limit of application (LLA) was 10 μg/mL for the semi-quantitative label-free method, and 0.5 μg/mL or 1 μg/mL for the AQUA method when targeting peptide LFAEYLDQK and DAFAALAQTFK respectively. Carrera et al. (2016) did not mention a specific LOD, but according to the amount of biomarkers spiked (0–500 fmol/μL), the sensitivity was shown to be down to around 100 fmol/μL for peptide biomarker sequences AEAAHQASLTR and GGAVQAEFNK.

## 4. Discussion

Overall, there seems to be a lack of standardization on reporting performance of the detection methods. This can be expected due to the wide range of different methods, but even within groups the reporting is not standardized. Therefore, we propose that future studies adopt standardized reporting guidelines. For DNA-Based Methods such as RT-PCR methods, MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) can be used. This requires full disclosure of sample processing, primer/probe sequences, PCR efficiency, reference genes, and most importantly, reporting the Limit of Detection (LOD) in standardized units (e.g., copies per μL or ng DNA/reaction) and with a defined confidence level. Additionally, there is limited taxonomic reporting on which anisakid species the DNA detection method is targeting. For example, taxonomic clarity is required when citing *A. simplex*. Authors should explicitly distinguish between *A. simplex* sensu stricto and the broader *A. simplex* complex (sensu lato). Furthermore, authors should adopt the recently resurrected genus *Phocanema*, as a genus independent from *Pseudoterranova* (Bao et al., 2023). For immunochemistry methods (ELISA), STARD (STAndards for the Reporting of Diagnostic accuracy studies) can be used. This is a general guideline for studies evaluating diagnostic accuracy. It requires cross-tabulation of results (True/False Positives/Negatives), clear reporting of the reference standard used, and clear definition and rationale for the units, cutoffs, and categories of the test results. For Mass Spectrometry (MS), ProteomicsMIAPE (Minimum Information About a Proteomics Experiment) are relevant guidelines. Here, key recommendations include detailing the experimental design (e.g., number of replicates, use of internal standards), clear descriptions of the quantification method used (e.g., label-free, AQUA, MRM), and explicit reporting of confidence intervals for measured values.

For the DNA-based detection methods, the main problem in the reporting of the LOD was that either the amount of DNA was reported, or the amount of total larval or fish tissue. Some advantages of the DNA detection methods include the ability to distinguish between anisakid genera and species, and compared to immunochemistry-based methods the chances of false positives are lower. However, some drawbacks limit its usefulness when taking into account the allergenic potential of anisakid proteins. When larvae are removed from the matrix, allergens may persist. This is especially the case for E/S proteins, which are excreted in the host tissue. Moreover, some of these proteins are highly pepsin-resistant and can withstand thermal processing (Kobayashi et al., 2007; Mehrdana and Buchmann, 2017). For example, Ani s 4 and Ani s 9 are resistant allergens, and are targeted in the IHC methods (Ani s 4), and MS-based methods (Ani s 9). Positive samples detected by these methods could be overlooked by DNA detection, as thermal processing could lead to DNA degradation. Nonetheless, the HTS method did detect trace amounts of *Phocanema* and *Hysterothylacium* DNA (Abollo et al., 2024).

Comparatively, immunochemistry-based detection methods have some advantages, but also drawbacks. In contrast to DNA-methods, anisakid proteins are directly detected and provide stronger evidence of allergen contamination. It is nonetheless known that some allergens such as Ani s 2 and Ani s 3 are highly conserved panallergens which show cross-reactivity with other nematodes,

arthropods, and dust-mite species (Johansson et al., 2001). It is therefore important that the test specificity is thoroughly tested. Although some of the methods mentioned above did test specificity - for example Polimeno et al. (2021) tested cross reaction to fish, shrimp and dust mite – most did not report any specificity metric. This cross-reaction can be avoided by targeting *Anisakis* specific allergens such as Ani s 1, and Ani s 7, as described in two ELISA methods (Arilla et al., 2008; Stobbeleir, 2022). In addition, Ani s 4 is also a specific allergen to *Anisakis* species and used as a target, as described in the immunostaining and IHC methods (Solas et al., 2008, 2009; Rodríguez-Mahillo et al., 2010; Vidaček et al., 2011, 2009).

Finally, mass spectrometry proves to be a promising technique for the detection of allergens and other contaminant proteins in food products (Picariello et al., 2011). With limits of detection down to the low fmol/μL range it outperforms the immunochemistry- and DNA-based tests (Carrera et al., 2016; Fæste et al., 2016). Moreover, the possibility of relative or absolute quantification of the contaminant makes it an attractive method. It is also possible to carry out multiplex analysis by targeting multiple peptides and proteins. Some disadvantageous aspects are the high equipment cost and maintenance, the need for trained personal, delicate sample preparation, and resource intensive method development. A new method needs to be developed for different MS setups, which makes it less straightforward to use compared to DNA- and immunochemistry-based detection methods. Its commercial potential would therefore likely be limited.

## 5. Conclusion

Although more research and standardization is needed, for more routine analyses of anisakid allergens in seafood the chemiluminescent sandwich ELISA method described by Kochanowski et al. (2020) seems to be the most sensitive and straightforward method. The use of DNA-based techniques would only be recommended in products where *Anisakis* larvae are prevalent, and in particular the LAMP method described by Cammilleri et al. (2020) due to the low LOD and fast and straightforward protocol. When allergen detection in the context of scientific research is needed and the lowest detection limits are needed, we believe that using an MS-based approach is warranted, either targeting *Anisakis* haemoglobin peptides, or peptides from SXP/RAL-2 proteins like Ani s 8 or 9.

## CRediT authorship contribution statement

**Faust Schotte:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ganna Saelens:** Writing – review & editing, Validation, Methodology, Investigation. **Brecht Devleesschauwer:** Supervision. **Sarah Gabriël:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition.

## Declaration of competing interest

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

Faust Schotte reports financial support was provided by Federal Public Service Health Food Chain Safety and Environment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## References

Abollo, E., Ramilo, A., Pascual, S., 2024. Fish feed composition by high-throughput sequencing analysis: parasite risk assessment. Int. J. Food Microbiol. 415, 110633. https://doi.org/10.1016/j.ijfoodmicro.2024.110633.

Arilla, M.C., Ibarrola, I., Martinez, A., Monteseirin, J., Conde, J., Asturias, J.A., 2008. An antibody-based ELISA for quantification of Ani s 1, a major allergen from Anisakis simplex. Parasitology 135, 735–740. https://doi.org/10.1017/S0031182008004332.

Armentia, A., Martín-Gil, F., Pascual, C., Martín-Esteban, M., Callejo, A., Martínez, C., 2006. Anisakis simplex allergy after eating chicken meat. J. Invest. Allergol. Clin. Immunol. 16.

Audicana, M.T., Kennedy, M.W., 2008. Anisakis simplex: from obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbiol. Rev. 21, 360–379. https://doi.org/10.1128/CMR.00012-07.

Bald, C., Lavilla, M., Abaroa, C., Aboitiz, X., Díez, G., Iñarra, B., 2024. Inactivation of Anisakis simplex allergens in fish viscera by acid autolysis. Appl. Sci. 14, 10650. https://doi.org/10.3390/app142210650.

Bao, M., Pierce, G.J., Pascual, S., González-Muñoz, M., Mattiucci, S., Mladineo, I., Cipriani, P., Bušelić, I., Strachan, N.J., 2017. Assessing the risk of an emerging zoonosis of worldwide concern: anisakiasis. Sci. Rep. 7, 43699. https://doi.org/10.1038/srep43699.

Bao, M., Pierce, G.J., Strachan, N.J.C., Pascual, S., González-Muñoz, M., Levsen, A., 2019. Human health, legislative and socioeconomic issues caused by the fish-borne zoonotic parasite Anisakis: challenges in risk assessment. Trends Food Sci. Technol. 86, 298–310. https://doi.org/10.1016/j.tifs.2019.02.013.

Bao, M., Giulietti, L., Levsen, A., Karlsbakk, E., 2023. Resurrection of genus Phocanema Myers, 1959, as a genus independent from Pseudoterranova Mozgovoĭ, 1953, for nematode species (Anisakidae) parasitic in pinnipeds and cetaceans, respectively. Parasitol. Int. 97, 102794. https://doi.org/10.1016/j.parint.2023.102794.

Bao, M., Cipriani, P., Giulietti, L., Levsen, A., 2025. Occurrence of *Anisakis simplex* in Norwegian farmed Atlantic cod (*Gadus morhua*). Aquaculture 609, 742814. https://doi.org/10.1016/j.aquaculture.2025.742814.

Cammilleri, G., Ferrantelli, V., Pulvirenti, A., Drago, C., Stampone, G., Quintero Macias, G.D.R., Drago, S., Arcoleo, G., Costa, A., Geraci, F., Di Bella, C., 2020. Validation of a commercial loop-mediated isothermal amplification (LAMP) assay for the rapid detection of Anisakis spp. DNA in processed fish products. FOODS 9. https://doi.org/10.3390/foods9010092.

Carrera, M., Gallardo, J.M., Pascual, S., Gonzalez, A.F., Medina, I., 2016. Protein biomarker discovery and fast monitoring for the identification and detection of Anisakids by parallel reaction monitoring (PRM) mass spectrometry. J. Proteomics 142, 130–137. https://doi.org/10.1016/j.jprot.2016.05.012.

Cavallero, S., Bruno, A., Arletti, E., Caffara, M., Fioravanti, M.L., Costa, A., Cammilleri, G., Graci, S., Ferrantelli, V., D'Amelio, S., 2017. Validation of a commercial kit aimed to the detection of pathogenic anisakid nematodes in fish products. Int. J. Food Microbiol. 257, 75–79. https://doi.org/10.1016/j.ijfoodmicro.2017.06.011.

Chai, J.-Y., Sohn, W.-M., Jung, B.-K., 2024. Anisakidosis in humans and animals and detection of anisakid larvae in fish and cephalopods in Korea: a literature review (1971-2022). Annals of Clinical Microbiology 27, 93–130. https://doi.org/10.5145/ACM.2024.27.2.6.

EFSA, 2010. Scientific opinion on risk assessment of parasites in fishery products. EFSA J. 8, 1543.

EFSA, 2024. Re-evaluation of certain aspects of the EFSA scientific opinion of April 2010 on risk assessment of parasites in fishery products, based on new scientific data. Part 1: ToRs1–3. EFSA Journal 22, e8719.

Espiñeira, M., Herrero, B., Vieites, J.M., Santaclara, F.J., 2010. Detection and identification of anisakids in seafood by fragment length polymorphism analysis and PCR–RFLP of ITS-1 region. Food Control 21, 1051–1060. https://doi.org/10.1016/j.foodcont.2009.12.026.

Fæste, C.K., Jonscher, K.R., Dooper, M.M.W.B., Egge-Jacobsen, W., Moen, A., Daschner, A., Egaas, E., Christians, U., 2014. Characterisation of potential novel allergens in the fish parasite Anisakis simplex. EuPA Open Proteom. 4, 140–155. https://doi.org/10.1016/j.euprot.2014.06.006.

Fæste, C.K., Levsen, A., Lin, A.H., Larsen, N., Plassen, C., Moen, A., Van Do, T., Egaas, E., 2015a. Fish feed as source of potentially allergenic peptides from the fish parasite Anisakis simplex (s.l.). Anim. Feed Sci. Technol. 202, 52–61. https://doi.org/10.1016/j.anifeedsci.2015.01.006.

Fæste, C.K., Plassen, C., Lovberg, K.E., Moen, A., Egaas, E., 2015b. Detection of proteins from the fish parasite Anisakis simplex in Norwegian farmed Salmon and Processed fish products. Food Anal. Methods 8, 1390–1402. https://doi.org/10.1007/s12161-014-0003-8.

Fæste, C.K., Moen, A., Schniedewind, B., Anonsen, J.H., Klawitter, J., Christians, U., 2016. Development of liquid chromatography-tandem mass spectrometry methods for the quantitation of Anisakis simplex proteins in fish. J. Chromatogr. A 1432, 58–72. https://doi.org/10.1016/j.chroma.2016.01.002.

Godínez-González, C., Roca-Geronès, X., Cancino-Faure, B., Montoliu, I., Fisa, R., 2017. Quantitative SYBR green qPCR technique for the detection of the nematode parasite Anisakis in commercial fish-derived food. Int. J. Food Microbiol. 261, 89–94. https://doi.org/10.1016/j.ijfoodmicro.2017.05.012.

Godínez-González, C., Roca-Geronès, X., Montoliu, I., Fisa, R., 2021. Estimation of the number of Anisakis larvae in commercial fish using a descriptive model based on real-time PCR. J. Sci. Food Agric. 101, 1085–1090. https://doi.org/10.1002/jsfa.10718.

Heick, J., Fischer, M., Pöpping, B., 2011. First screening method for the simultaneous detection of seven allergens by liquid chromatography mass spectrometry. J. Chromatogr. A 1218, 938–943. https://doi.org/10.1016/j.chroma.2010.12.067.

Herrero, B., Vieites, J.M., Espiñeira, M., 2011. Detection of anisakids in fish and seafood products by real-time PCR. Food Control 22, 933–939. https://doi.org/10.1016/j.foodcont.2010.11.028.

Johansson, E., Aponno, M., Lundberg, M., van Hage-Hamsten, M., 2001. Allergenic cross-reactivity between the nematode Anisakis simplex and the dust mites Acarus siro, Lepidoglyphus destructor, Tyrophagus putrescentiae, and Dermatophagoides pteronyssinus. Allergy 56, 660–666. https://doi.org/10.1034/j.1398-9995.2001.00798.x.

Kobayashi, Y., Shimakura, K., Ishizaki, S., Nagashima, Y., Shiomi, K., 2007. Purification and cDNA cloning of a new heat-stable allergen from Anisakis simplex. Mol. Biochem. Parasitol. 155, 138–145. https://doi.org/10.1016/j.molbiopara.2007.06.012.

Kochanowski, M., Różycki, M., Dąbrowska, J., Karamon, J., Sroka, J., Antolak, E., Bełcik, A., Cencek, T., 2020. Development and application of novel Chemiluminescence immunoassays for highly sensitive detection of Anisakis simplex proteins in thermally processed seafood. Pathogens 9, 777. https://doi.org/10.3390/pathogens9100777.

Levsen, A., Paoletti, M., Cipriani, P., Nascetti, G., Mattiucci, S., 2016. Species composition and infection dynamics of ascaridoid nematodes in Barents Sea capelin (Mallotus villosus) reflecting trophic position of fish host. Parasitol. Res. 115, 4281–4291. https://doi.org/10.1007/s00436-016-5209-9.

Lopez, I., Angel Pardo, M., 2010. Evaluation of a real-time polymerase chain reaction (PCR) assay for detection of Anisakis simplex parasite as a food-borne allergen source in seafood products. J. Agric. Food Chem. 58, 1469–1477. https://doi.org/10.1021/jf903492f.

Mehrdana, F., Buchmann, K., 2017. Excretory/secretory products of anisakid nematodes: biological and pathological roles. Acta Vet. Scand. 59, 42. https://doi.org/10.1186/s13028-017-0310-3.

Mercken, E., Van Damme, I., Šoba, B., Vangeenberghe, S., Serradell, A., Lumain, J.P.L., De Sterck, T., Lalle, M., Gabriël, S., 2021. High occurrence of Anisakidae at retail level in cod (Gadus morhua) belly flaps and the impact of extensive candling. Food and Waterborne Parasitology 22, e00108. https://doi.org/10.1016/j.fawpar.2020.e00108.

Monaci, L., Visconti, A., 2009. Mass spectrometry-based proteomics methods for analysis of food allergens. TrAC Trends Anal. Chem. 28, 581–591. https://doi.org/10.1016/j.trac.2009.02.013. Applying combinations of chemical analysis and biological effects to environmental and food samples - I.

Mossali, C., Palermo, S., Capra, E., Piccolo, G., Botti, S., Bandi, C., D'Amelio, S., Giuffra, E., 2010. Sensitive detection and quantification of Anisakid parasite residues in food products. Foodborne Pathog. Dis. 7, 391–397. https://doi.org/10.1089/fpd.2009.0428.

Nieuwenhuizen, N.E., 2016. Anisakis – immunology of a foodborne parasitosis. Parasite Immunol. 38, 548–557. https://doi.org/10.1111/pim.12349.

Ouzzani, M., Hammady, H., Fedorowicz, Z., Elmagarmid, A., 2016. Rayyan—a web and mobile app for systematic reviews. Syst. Rev. 5, 210. https://doi.org/10.1186/s13643-016-0384-4.

Page, M.J., Moher, D., Bossuyt, P.M., Boutron, I., Hoffmann, T.C., Mulrow, C.D., Shamseer, L., Tetzlaff, J.M., Akl, E.A., Brennan, S.E., Chou, R., Glanville, J., Grimshaw, J.M., Hróbjartsson, A., Lalu, M.M., Li, T., Loder, E.W., Mayo-Wilson, E., McDonald, S., McGuinness, L.A., Stewart, L.A., Thomas, J., Tricco, A.C., Welch, V.A., Whiting, P., McKenzie, J.E., 2021. PRISMA 2020 explanation and elaboration: updated guidance and exemplars for reporting systematic reviews. BMJ, n160. https://doi.org/10.1136/bmj.n160.

Palomba, M., Rughetti, A., Mignogna, G., Castrignano, T., Rahimi, H., Masuelli, L., Napoletano, C., Pinna, V., Giorgi, A., Santoro, M., et al., 2023. Proteomic characterization of extracellular vesicles released by third stage larvae of the zoonotic parasite Anisakis pegreffii (Nematoda: Anisakidae). Front. Cell. Infect. Microbiol. 13, 1079991.

Picariello, G., Mamone, G., Addeo, F., Ferranti, P., 2011. The frontiers of mass spectrometry-based techniques in food allergenomics. J. Chromatogr. A 1218, 7386–7398. https://doi.org/10.1016/j.chroma.2011.06.033.

Polimeno, Lorenzo, Lisanti, M.T., Rossini, M., Giacovazzo, E., Polimeno, Lucrezia, Debellis, L., Ballini, A., Topi, S., Santacroce, L., 2021. Anisakis allergy: is Aquacultured fish a safe and alternative food to wild-capture fisheries for Anisakis simplex-sensitized patients? Biology 10, 106. https://doi.org/10.3390/biology10020106.

Rahmati, A.R., Kiani, B., Afshari, A., Moghaddas, E., Williams, M., Shamsi, S., 2020. World-wide prevalence of Anisakis larvae in fish and its relationship to human allergic anisakiasis: a systematic review. Parasitol. Res. 119, 3585–3594. https://doi.org/10.1007/s00436-020-06892-0.

Rodríguez-Mahillo, A.I., González-Muñoz, M., de las Heras, C., Tejada, M., Moneo, I., 2010. Quantification of Anisakis simplex allergens in fresh, long-term frozen, and cooked fish muscle. Foodborne Pathog. Dis. 7, 967–973. https://doi.org/10.1089/fpd.2009.0517.

Saelens, G., Planckaert, S., Devreese, B., Gabriël, S., 2023. Transmissibility of anisakid allergenic peptides from animal feed to chicken meat: proof of concept. J. Food Compos. Anal. 115, 104939. https://doi.org/10.1016/j.jfca.2022.104939.

Santos, A.T., Sasal, P., Verneau, O., Lenfant, P., 2006. A method to detect the parasitic nematodes from the family Anisakidae, in Sardina pilchardus, using specific primers of 18 S DNA gene. Eur. Food Res. Technol. 222, 71–77. https://doi.org/10.1007/s00217-005-0052-8.

Solas, M.T., García, M.L., Rodriguez-Mahillo, A.I., Gonzalez-Munoz, M., de las Heras, C., Tejada, M., 2008. Anisakis antigens detected in fish muscle infested with Anisakis simplex L3. J. Food Prot. 71, 1273–1276. https://doi.org/10.4315/0362-028X-71.6.1273.

Solas, M.T., Garca, M.L., De Las Heras, C., Rodriguez-Mahillo, A.I., Gonzalez-Munoz, M., Moneo, I., Mendizbal, A., Tejada, M., 2009. Anisakis simplex antigens in fresh and frozen-thawed muscle of anchovies in vinegar. Food Sci. Technol. Int. 15, 139–148. https://doi.org/10.1177/1082013208105171.

Stobbeleir, S., 2022. Detection of Anisakidae Allergens in Fishmeal (Master Thesis). Ghent University.
Vidaček, S., de las Heras, C., Solas, M.T., Rodriguez Mahilloc, A.I., Tejada, M., 2009. Effect of high hydrostatic pressure on mortality and allergenicity of Anisakis simplex L3 and on muscle properties of infested hake. J. Sci. Food Agric. 89, 2228–2235. https://doi.org/10.1002/jsfa.3712.
Vidaček, S., De Las Heras, C., Solas, M.T., García, M.L., Mendizábal, A., Tejada, M., 2011. Viability and antigenicity of anisakis simplex after conventional and microwave heating at fixed temperatures. J. Food Prot. 74, 2119–2126. https://doi.org/10.4315/0362-028X.JFP-11-108.
Werner, M.T., Fæste, C.K., Levsen, A., Egaas, E., 2011. A quantitative sandwich ELISA for the detection of Anisakis simplex protein in seafood. Eur. Food Res. Technol. 232, 157–166. https://doi.org/10.1007/s00217-010-1373-9.
Xu, X., Sui, J., Cao, L., Lin, H., 2010. Direct competitive enzyme-linked immunosorbent assay (ELISA) for rapid screening of anisakid larvae in seafood. J. Sci. Food Agric. 90, 877–881. https://doi.org/10.1002/jsfa.3898.
Yera, H., Fréalle, É., Dutoit, E., Dupouy-Camet, J., 2018. A national retrospective survey of anisakidosis in France (2010-2014): decreasing incidence, female predominance, and emerging allergic potential. Parasite 25, 23. https://doi.org/10.1051/parasite/2018016.