A quantitative sandwich ELISA for the detection of *Anisakis simplex* protein in seafood

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Abstract There is a growing awareness by consumers and food safety authorities regarding the possible presence of parasites or parasite-related potentially hazardous substances in seafood. *Anisakis simplex* is among the most frequently occurring parasites in wild-caught marine fish. Except for various visual inspection techniques and PCR-based methods for the detection of more or less intact worms or parasite DNA, respectively, there are at present no validated methods for the quantification of *A. simplex* proteins in processed fish products. This work describes the development and validation of a sandwich enzyme-linked immunosorbent assay (ELISA) for the quantification and analysis of proteins from *A. simplex* in seafood products. The ELISA is based on a polyclonal rabbit anti-*A. simplex* antibody for capture and a biotinylated conjugate of the same antibody for detection. The ELISA is specific for *A. simplex* and does not cross-react with other species. Recoveries ranged from 72–101% in typical food matrixes, while intra- and inter-assay precisions were <11 and <25%, respectively. With a limit of detection of 1.1 µg *A. simplex* protein/g of sample, the sensitivity of the *A. simplex* sandwich ELISA appears to be sufficient to detect even low levels in seafood products.

Keywords *Anisakis simplex* · Allergen · ELISA · Food allergy

Introduction

Wild-caught marine fish seems to be the only industrially produced food, which is at risk to carry parasites when put on the market [1]. In this respect, the larvae of the parasitic nematode *Anisakis simplex*, also known as the herring or whale worm, are among the most frequently occurring parasites in virtually all of the commercially exploited fish stocks in temperate seas around the globe.

The apparently most prevalent *Anisakis* species, *A. simplex*, has been shown to actually comprise a complex of closely related siblings in many areas of the North Atlantic and adjacent areas [2]. However, in the North Sea and Norwegian Sea, *A. simplex sensu stricto* appears to be the only species present [2, 3]. The *Anisakis* life cycle involves various marine mammals as definitive host, planktonic crustaceans—mainly krill—as intermediate host, and fishes and possibly squids as transport host, transferring the larvae from krill to whales. In fish, the majority of the *Anisakis* larvae occur encapsulated as flat tight spirals in and on the visceral organs and the peritoneum. However, a minor proportion of the larvae usually migrate from the visceral cavity into the flesh, sometimes penetrating deeply into the epaxial musculature of the fish host [4].

Besides the considerable quality-reducing effect of *Anisakis* larvae with respect to seafood products, they are of direct human health concern, especially regarding the increasing interest in Asian-inspired seafood dishes based on undercooked, brined or marinated, or even raw fish meat (e.g. sushi, sashimi). Thus, anisakiasis, i.e. human infection with live *Anisakis* larvae, is reported most frequently from Japan, Korea and some southern European countries, especially Spain, where raw or lightly salted or marinated fish is part of the everyday diet. If accidentally ingested by humans, live worms may bore into the intestinal mucosa

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and elicit an immune reaction which again may cause considerable distress. The clinical manifestations of anisakiasis include epigastric pain, nausea, vomiting, diarrhea, and urticaria. The condition may resemble several other gastrointestinal disorders, such as gastric ulcer, tumors, acute appendicitis or rectal carcinoma [5]. Additionally, the potential of the worm—both dead and alive—to induce hypersensitivity reactions in humans has received increased attention [6–9].

The European Food Safety Authority (EFSA) has recently released a scientific opinion on risk assessment of parasites in fishery products including the elicitation of allergic reactions in consumers [1]. It was assessed that human fishery product-borne parasitic diseases are mainly caused by cestodes, trematodes, and nematodes. Furthermore, it was concluded that only the nematode *Anisakis simplex* has been found to have the potential to induce IgE-mediated reactions. Patient sensitisation presumably occurs by the ingestion of seafoods infected with live larvae. It has also been demonstrated that occupational exposure of fish processing employees with *Anisakis* allergens may lead to increased levels of airway hyperactivity and dermatitis [10].

Currently, eleven allergens of *A. simplex* have been identified, of which most have been expressed as recombinant proteins. Allergic individuals may be exposed either to somatic antigens from dead larvae in food or to excretory-secretory (ES) antigens as a consequence of the expulsion or surgical removal of intact larvae. In some cases, both may apply when the larvae has penetrated the tissue, is killed by the host, and subsequently degenerates inside the host [11]. Several of the identified *A. simplex* allergens seem to retain their allergenic potential even after freezing and/or heating [12–14] and appear to be pepsin resistant [15]. In a study on the progression of allergens in the food chain, it was suggested that *Anisakis*-allergic consumers were affected by the intake of meat from chicken that had been fed with a high proportion of *Anisakis*-containing fishmeal [16]. The somatic antigens are the most abundant, and some of these proteins show cross-reactivity with other ascarid proteins [17]. The muscle protein tropomyosin is mainly responsible for allergenic cross-reactivities in other invertebrates; but, whether *Anisakis* tropomyosin (Ani s 3) is a clinically relevant allergen is under discussion. In an allergenicity study using sera from *Anisakis*-sensitive patients and crude *Anisakis* extract, IgE-binding to Ani s 3 was undistinguishable [18]. However, Guarneri et al. [19] have suggested that *Anisakis* tropomyosin could play an important role in eliciting food allergy after the ingestion of cooked seafood, since it closely resembles the heat-stable shrimp tropomyosin, which is an important seafood allergen.

The presence of *A. simplex* in seafood products may present a health risk for consumers. At the same time, it is also

regarded as an economic problem because esthetical aspects may lead to a reduced attractiveness of fish consumption. Therefore, food safety authorities and seafood manufacturers are likewise interested in improved methods for the detection of *Anisakis* in foods. So far, only visual inspection methods of more or less intact worms [20, 21] or PCR methods for the determination of parasite DNA [22, 23] in fish products are available. However, there are at present no validated methods for the quantification of *Anisakis* protein in processed fish products and seafood. Recently, a sandwich ELISA for the detection of *Anisakis* in different fish using recombinant *A. simplex* allergen Ani s 1 has been published [24]. The ELISA has a limit of detection corresponding to about 25 *Anisakis* larvae per 100 g fish. The applicability of the assay for the quantification of *Anisakis* proteins in processed foods was not reported. In contrast, several PCR-based methods have been developed that are intended for the quantification of parasite residues in food products [22, 23]. PCR- and ELISA-based methods can be regarded as complementary techniques with respect to allergen detection. However, PCR results can only depict the presence of *Anisakis*-DNA, which may not be correlated to the amount of allergenic protein, especially in processed food [25].

The aim of the current study was therefore to develop and validate a sensitive, specific, and quantitative sandwich ELISA method for the detection of *Anisakis* proteins in seafood products.

Materials and methods

Anisakis species identification

A. simplex 3rd stage larvae were collected from freshly caught blue whiting (*Micromesistius poutassou*) during a research cruise in the Northeast Atlantic. The *A. simplex* larvae were manually harvested from the muscle tissue of the fish, and the capsules were opened and removed. For *Anisakis* species identification, polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis was used, as described before [26]. DNA from *Anisakis* larvae ($n = 30$) from blue whiting was prepared and digested using the restriction enzyme *HinfI* resulting in typical *Anisakis simplex* s.s.-specific RFLP patterns in agarose gel analysis. The results of this pre-study will be published separately (by H. Lin and A. Levsen).

Protein extraction from *A. simplex*

A protein extract was prepared from larvae that were freed from host tissue remains, rinsed in physiological saline, and subsequently homogenized in 0.1 M tris(hydroxymethyl)aminoethane (Tris)/0.5 M glycine buffer (pH 8.7)/1 mM

dithioerythriol (DTE) (Sigma-Aldrich) extraction buffer with a rod homogenizer (Braun Vario, Kronberg, Germany). After overnight extraction at 45 °C in a shaking water bath (OLS 200, Grant, Cambridge, UK) and centrifugation for 25 min at 4 °C and 18,000g (J2-MC, Beckman Instruments, Palo Alto, CA), the supernatant was transferred to a glass beaker. Protein was precipitated with ammonium sulfate (Merck, Whitehouse Station, NJ), reaching a saturation of 75%, under continuous stirring at room temperature (RT) followed by centrifugation (25 min, 18,000g, 4 °C). The pellet was resuspended in 50 mM Tris/1 M DTE (pH 8.0) and dialyzed against distilled water in a dialysis tube with 6–8 kDa μ m pore size (Spectrapor, Spectrum Medical industries, Los Angeles, CA) and freeze-dried (Heto, Allerød, Denmark) over night. The protein was dissolved in phosphate-buffered saline (PBS) (pH 7.4). Total protein content was determined by Lowry protein assay (BioRad Laboratories, Hercules, CA). Aliquots were stored at –20 °C until use. Additionally, a second extract was prepared using fewer purification steps to prevent potential protein loss. Therefore, *A. simplex* larvae were added PBS and homogenized by Ultra-Turrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany). The homogenate of the raw extract was centrifuged at 39,200g for 25 min at 4 °C. Total protein content was again determined by Lowry protein assay, and aliquots were stored at –20 °C.

Production, purification, and labeling of anti-*A. simplex* antibody

A polyclonal antiserum was raised against the $(\text{NH}_4)_2\text{SO}_4$ —precipitated extract from *A. simplex* larvae in rabbit using the same procedure as previously described [27]. The crude rabbit serum was desalted on PD-10 columns (Amersham Biosciences, Uppsala, Sweden), and purified by using a HiTrap r-Protein A FF-column (Amersham Biosciences), which had previously been washed with 0.1 M sodium citrate buffer pH 4.0 (elution buffer) and reequilibrated with 20 mM sodium phosphate buffer pH 7.0 (binding buffer). After washing with binding buffer, the bound IgG-antibodies were eluted in 0.5 mL fractions from the column with elution buffer, neutralized with 1 M Tris-base pH 8.5, and stored at 4 °C. The fractions were tested for binding activity using indirect ELISA, and total protein was determined by the Lowry method (DC Protein Assay, BioRad, Hercules, CA). The purity of the IgG in the collected fractions was tested on SDS–PAGE under reducing conditions. The purest antibody fractions were pooled prior to buffer exchange to 0.1 M sodium phosphate, 0.15 M NaCl, 0.1% sodium azide (pH 7.4), using a PD-10 column, and finally stored at –80 °C until use. In total, 30 mg of IgG had been obtained from 10 mL of crude rabbit serum.

For covalent conjugation with biotinamido-hexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (Sigma-Aldrich, Steinheim, Germany), the buffer was changed to 0.05 M carbonate-bicarbonate buffer (pH 9), and the antibody concentration was adjusted to 1 mg/mL. The biotin salt was then dissolved in deionized H₂O to a concentration of 1 mg/mL and added to the antibody solution at a ratio of 1–6.67. The mixture was vortexed and rotated at RT for 4 h, and subsequently neutralized with 1 M NH₄Cl under rotation at RT for 10 min. The total protein concentration of the biotinylated anti-*A. simplex* antibody was measured after a buffer change to 0.1 M sodium phosphate, 0.15 M NaCl, 0.1% sodium azide, pH 7.4, using PD-10 columns, and the antibody was stored at –20 °C until use.

Protein extraction and sample preparation

All protein extracts and food samples, unless otherwise stated, were prepared as previously described [28]. In brief, homogenized samples (2 g) were extracted with 10 mL of 0.1 M Tris, 0.5 M glycine (pH 8.7) under shaking, overnight at 45 °C and centrifuged at 39,200g for 25 min at 4 °C. Protein extracts were stored at –20 °C, at 4 °C or were used freshly. Prior to analysis using the sandwich ELISA, extracts were diluted at least 1:20 in phosphate-buffered saline (PBS) (Oxoid, Basingstoke, UK) containing 1% bovine serum albumin (BSA).

Evaluation of extraction buffers for seafood samples

The following extraction buffers were tested in the study (Table 1): urea 6 M, pH 8.7; Tris-glycine, 0.1 M Tris, 0.5 M glycine, pH 8.7; HSB, 20 mM NaH₂PO₄·H₂O, 1 M NaCl, pH 7.5; high-salt buffer (HSB), 20 mM NaH₂PO₄·H₂O, 1 M NaCl, pH 4.5; Citrate, 0.5 M citric acid monohydrate, 0.5 M sodium citrate dehydrate, pH 4; sodium carbonate, 50 mM, pH 11; sodium borate, 7 mM disodium tetraborate decahydrate, pH 9.2; PBS, 0.172 M, pH 7.4 (Oxoid). Extractions were performed as described above. Ionic strengths were high (>1 M), medium (0.5–1), and low (<0.5 M).

Western blotting

The NuPage Gel System (Invitrogen, Carlsbad, CA) was used for electrophoretic separation of protein samples by SDS–PAGE, in accordance with the manufacturer's instructions. All protein samples were applied in equal amounts (10 μ g). Samples were prepared with lithium dodecyl sulfate (LDS) sample buffer and dithiothreitol (DDT) reducing agent (all from Invitrogen). Separation was performed under reducing conditions for 40 min at 200 V in 2-(*N*-morpholino) ethane sulfonic acid (MES) SDS running

Table 1 Effect of extraction buffers with different pH and ionic strengths on the total protein and *A. simplex* protein content measured in cod contaminated with *A. simplex* larvae

Buffer	pH	Ionic strength	Total protein (mg/mL)	<i>A. simplex</i> protein (pg/g)
Urea	8.7	High	15.5	1.1
Tris-glycine	8.7	Medium	26.9	13.3
HSB	7.5	Medium	2.8	nd
HSB	4.5	Medium	28.5	nd ^a
Citrate	4.0	Medium	1.2	nd ^a
Sodium carbonate	11.0	Low	14.9	3.1
Sodium borate	9.2	Low	6.6	5.3
PBS	7.4	Low	30.4	nd

nd not detected

^a Precipitation during color development

buffer, using 4–12% Bis-Tris gels and SeeBluePlus2 pre-stained reference standard with a range of 3–188 kDa.

Proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) for 60 min at 30 V with transfer buffer in an XCell II Blot Module (Invitrogen). Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.6) was used as washing buffer and TBS-T containing 1% BSA was used as blocking and assay buffer for the Western blots. After blocking for 30–60 min, the blot was incubated at 4 °C overnight, with the unlabelled purified anti-*A. simplex* antibody (3.3 mg/mL) diluted 1:30 000 in assay buffer. The blot was washed (3 × 15 min) and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Zymed, San Francisco, CA) diluted 1:5,000 in assay buffer. After washing (3 × 10 min), the membrane was developed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Zymed) until bands of satisfactory intensity appeared (2–10 min). All washing and incubation steps were performed under gentle shaking at RT.

Sandwich ELISA procedure

Ninety-six-well flat-bottom polystyrene microtiterplates (Corning Inc., Corning, NY) were coated overnight at 4 °C with 100 µL/well of purified rabbit anti-*A. simplex* polyclonal antibody diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) (Sigma-Aldrich) to 10 µg/mL. Then, the plates were washed with PBS containing 0.05% Tween-20 (PBS-T, pH 7.6) three times, using a programmable automatic plate washer (Skatron Instruments, Lier, Norway). All consecutive washing steps performed between each operation in the tropomyosin sandwich ELISA were the same. PBS containing 1% BSA and 0.05% Tween-20 was used as blocking and assay buffer for the ELISA. All dilutions, except for the initial coating step, were performed in assay buffer, and the plates were sealed with plate-sealing film during the incubations. Unsaturated binding sites on the polystyrene surface of the microtiterplate were blocked by incubation with 250 µL/well of the blocking/assay buffer for 1 h at RT. After washing, a

twofold serial dilution of the *A. simplex* raw extract, prepared as described above, was added in the concentration range 0.98–1,000 ng/mL and served as assay standard. Incubation of standards, buffer blanks, and sample extracts at a minimum dilution of 1:20 was performed in triplicate on each plate, for 1 h at RT using an electronic plate shaker (IKA®-Werke, GmbH & Co. KG, Staufen, Germany) at low speed. After washing, bound *A. simplex* protein was detected by adding 100 µL/well of biotinylated rabbit anti-*A. simplex* antibody diluted 1:800 and incubated for 1 h at RT under gentle shaking. Following the next wash, the plates were incubated for 1 h at RT with 100 µL/well of HRP-streptavidin conjugate (Zymed), 1:5,000 dilution. After a final wash, each well was incubated with 75 µL of K-Blue TMB substrate (Neogen, Lexington, KY, USA). After 20 min, the color development was stopped by the addition of 50 µL/well of 2 M H₂SO₄. Absorbance was read at 450 nm using a 1420 VICTOR² multilabel plate counter (Wallac, Turku, Finland).

Assay validation: specificity, accuracy, precision, limit of detection, and limit of quantification

The specificity of the assay was evaluated through cross-reactivity studies using extracts of different fish and seafood species, in addition to some potentially food-contaminating arthropods. Extracts from Atlantic salmon (*Salmo salar*), Atlantic cod (*Gardus morhua*), bluefin tuna (*Thunnus thynnus*), mackerel (*Scomber scombrus*), Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), blue whiting (*Micromesistius poutassou*), rainbow trout (*Oncorhynchus mykiss*), edible frog (*Rana esculenta*), European squid (*Loligo vulgaris*), common cockle (*Cerastoderma edule*), Northern shrimp (*Pandulus borealis*), blue mussel (*Mytilus edulis*), edible crab (*Cancer pagurus*), giant tiger prawn (*Panaeus monodon*), European brown snail (*Helix aspersa aspersa*), German cockroach (*Blattella germanica*), European house dust mite (*Dermaphagoides pteronyssinus*), and dried fruit mite (*Carapoglyphus lactis*) were all diluted 1:20 in ELISA assay buffer, which corresponds to a portion of 100% in a typical

food matrix. The apparent *A. simplex* contents obtained from the ELISA assay were compared to the total protein content of the corresponding extract, and recovery rates were calculated.

The accuracy of the method was assessed by performing recovery studies. Two previously measured *A. simplex*-free food items (fish balls and mackerel in tomato sauce) were homogenized as described above and spiked with *A. simplex* protein standard at levels of 1, 10, and 100 μg *A. simplex*/g sample in a total volume of 5 mL extraction buffer. After vortexing and 15-min incubation at RT, an additional portion of 5 mL extraction buffer was added, and the extraction procedure was performed as previously described. For the evaluation of recovery rates, extractions were performed in triplicate, the resulting extracts were analyzed by the *Anisakis* sandwich ELISA, and the mean values for the recoveries and the standard error of the mean were calculated.

The precision of the method was evaluated as within (intra-assay) and between (inter-assay) assays, by using extracts from cod meat that had been spiked with two, ten, twenty-five, and fifty intact *A. simplex* larvae, which had been obtained from infected fish caught on research missions. The spiked samples were homogenized together with uninfected cod fish. After extraction, the samples were stored at -20°C , and each analysis was performed with freshly thawed extract. The intra-assay precision was determined as the mean coefficient of variation (CV) of 7 replicates in one assay. The inter-assay precision was calculated as the mean coefficient of variation (CV) on the basis of triplicate analysis on 7 different days.

The limit of detection (LOD) of the *A. simplex* sandwich ELISA was calculated from 13 experiments as the mean of the measured content of triplicate buffer blank samples plus three times the standard deviation (SD) of the mean value. The limit of quantification (LOQ) was calculated as the buffer blank mean value plus 10 times the standard deviation (SD) of the mean value in 13 experiments. Additionally, the limit of detection was determined for three food matrixes free from *A. simplex* protein, i.e. cod-fish, fish balls, and mackerel with tomato sauce diluted 1:20 in assay buffer, on the basis of 7 experiments with triplicate samples each.

Results

Characterization of the anti-*A. simplex* antibody

The binding characteristics of the purified anti-*A. simplex* antibody were evaluated by Western blot analysis, using the raw and purified *A. simplex* larvae protein extract, and extracts from several fish, seafood, and insects (Fig. 1).

The antibody binds specifically to three major *A. simplex* protein bands at about 41, 67, and 97 kDa, and in addition multiple weaker bands in the entire blot from 14–190 kDa in both extracts from *A. simplex*. However, there were slight differences in the observable binding patterns of the raw protein extract (lane 2) and the purified extract that had been thermally treated at 45°C (lane 1). In the raw preparation, the protein band at 97 kDa was less distinct, and fewer protein bands were visible between 14 to 40 kDa, whereas after heating several bands with molecular weight above 67 kDa became visible.

The Western blot analysis (Fig. 1, lanes 6–17) of possible cross-reacting proteins of salmon, cod, tuna, mackerel, herring, and trout revealed only unspecific background signals.

Sandwich ELISA standard curve

The raw *A. simplex* protein preparation was used as protein standard in the sandwich ELISA in concentrations ranging from 0.98 to 1,000 ng/mL. The working range of the assay was defined as the part of the curve that had a correlation coefficient of $r^2 > 0.99$. This was achieved both with 2nd degree polynomial regression ($r^2 = 0.9998$) and linear regression ($r^2 = 0.9935$) for an eight-point calibration curve ranging from 0.98–125 ng/mL, corresponding to 0.1–12.5 μg *A. simplex* protein/g food sample (Fig. 2). Therefore, the more user-friendly linear transformation was used for the determination of *A. simplex* protein concentrations in food. If necessary, serial dilutions of extracts were performed, and those that gave optical density (OD) values closest to the midpoint of the linear part of the

Fig. 1 Western blot analysis of various protein extracts of *A. simplex* larvae and potential cross-reacting species of seafood and insects using purified polyclonal anti-*A. simplex* antibody. *M* Molecular weight marker. Protein sizes [kDa] are indicated on the left side of the blot. *Lane 1* *A. simplex* purified extract; *lane 2* raw *A. simplex* extract; *lane 3* *A. simplex*-spiked cod (HSB (pH 4.5) extract); *lane 4* *A. simplex*-spiked cod (PBS extract); *lane 5* *A. simplex*-spiked cod (Tris-glycine extract); *lane 6* salmon; *lane 7* cod; *lane 8* bluefin tuna; *lane 9* mackerel; *lane 10* herring; *lane 11* haddock; *lane 12* blue whiting; *lane 13* trout; *lane 14* shrimp; *lane 15* cockroach; *lane 16* dried fruit mite; and *lane 17* house dust mite

standard curve were used to calculate the *A. simplex* protein concentration.

Influence of buffers on sample extraction

The extraction efficiency was evaluated by extracting homogenized codfish artificially contaminated with 25 *A. simplex* larvae, with various buffers of different pH and ionic strength (Table 1). Both, the total protein content and *A. simplex* protein content, were measured in the resulting extracts. The collected data suggested that the extraction buffer used had a vast impact on the extracted amount of total protein and *A. simplex* protein (Table 1). For total protein extraction, the best efficiencies were achieved by PBS, Tris-glycine, and HSB (pH 4.5), although no correlation could be observed between the yield of total protein and ionic strength or pH. *A. simplex* proteins appeared to be more extractable under basic than neutral or acidic conditions. It was observed that the most acidic extracts led to precipitation. Furthermore, *A. simplex* proteins were not detected in the PBS-based extract at all, even if the recovery of total protein was highest in this sample. The three extracts with the highest total protein concentrations were accessed by SDS-PAGE (data not shown) and Western blot (Fig. 1, lanes 3–5). The blot analysis of the Tris-glycine extract (Fig. 1, lane 5) showed two major bands at 41 kDa and about 70 kDa, which resembled the major protein bands in the raw and purified *A. simplex* extracts in lane 1 and 2. The profiles for the PBS and HSB-4.5 protein extracts were slightly different, with broader, more undefinable protein bands at lower molecular weights. The PBS and HSB-4.5 protein extracts seemed to have the 41-kDa

band as well, but less prominent. The HSB extract (lane 3) showed comparable bands at about 65 and 97 kDa, whereas the PBS extract (lane 4) showed multiple weak bands in the range from 30 to 70 kDa. Tris-glycine buffer seemed to be the most effective with respect to *Anisakis* protein extraction and recovery in the ELISA and was therefore chosen for all subsequent studies.

Specificity

A number of seafoods were analyzed for cross-reactivity in the *A. simplex* sandwich ELISA. A preliminary screening of extracts from scallop, snail, mussel, squid, salmon, mackerel, herring, bluefin tuna, cod, and sprat in a non-optimized competitive ELISA showed no inhibition with assay concentrations of 10 µg/mL of total protein. The *A. simplex* protein standard gave total inhibition at the same concentration (data not shown).

Subsequently, extracts from different potentially cross-reacting species of fish, crustaceans, molluscs, and insects were analyzed with the *A. simplex* sandwich ELISA under real sample conditions (Table 2). The recoveries of *A. simplex* protein equivalents were calculated and none of the extracts gave rise to ELISA results exceeding 0.002%.

Fig. 2 Representative eight-point calibration curve obtained using the raw *A. simplex* protein standard in the *A. simplex* sandwich ELISA. The graph shows the average values of triplicate measurements, the standard deviation in each point, and the 2nd degree polynomial regression curve and coefficient r^2

Table 2 Specificity of the *A. simplex* sandwich ELISA

Food ingredient ^a	Protein content ^b (mg/mL)	<i>A. simplex</i> protein equivalents ^c (%)
Atlantic salmon	6.1	<0.002
Atlantic cod	4.1	<0.002
Bluefin tuna	6.5	<0.002
Mackerel	5.5	<0.002
Herring	2.9	<0.002
Haddock	4.5	<0.002
Blue whiting	6.9	<0.002
Trout	5.4	<0.002
Frog	4.3	<0.002
Squid	5.6	<0.002
Common cockle	2.4	<0.002
Shrimp	2.0	<0.002
Blue mussel	3.7	<0.002
Edible crab	4.3	<0.002
Scampi	5.1	<0.002
European brown snail	5.6	<0.002
German cockroach	8.0	<0.002
House dust mite, linn	36.7	<0.002
House dust mite, trous	26.8	<0.002

^a All food ingredients were extracted like real samples in the assay

^b Values were obtained by the Lowry method

^c Recovery of *A. simplex* protein equivalents

Table 3 Recovery of *A. simplex* protein from blank food samples spiked with 1, 10, or 100 µg/g *A. simplex* protein standard

Blank food	Recovery ^a (%) at indicated amount of <i>A. simplex</i> protein standard added in µg/g		
	1	10	100
Fish balls	88 ± 12	80 ± 5	72 ± 8
Mackerel in tomato sauce	100 ± 10	101 ± 9	84 ± 10

^a Values represent the average of three spiking experiments and are reported as mean ± standard error of the mean (SEM)

Accuracy

Recovery studies were performed to assess the accuracy of the *A. simplex* sandwich ELISA. Two blank matrixes were spiked with low, intermediate, and high concentration amounts of *A. simplex* protein standard before sample extraction and used to determine recovery rates (Table 3). In fish balls, the recoveries ranged from 72 to 88%, and in mackerel in tomato sauce from 84 to 101% with the standard errors of less than 12%.

Precision

The intra- and inter-assay precision for the *A. simplex* sandwich ELISA was assessed by analyzing codfish spiked with different numbers of *A. simplex* larvae prior to homogenization and extraction (Table 4). The intra-assay precision, expressed as the coefficient of variation (%CV) ranged from 5 to 11%, and the inter-assay precision ranged from 16 to 25% with the highest uncertainties for the lowest concentrations. The precision studies were performed on relatively moderate concentrations with the highest level corresponding to 30 µg *A. simplex* protein/g food. Considering, the amount of larvae added to the sample, this can probably be regarded as a quite heavy *A. simplex* infection and an appropriate level of validation.

Table 4 Intra- and inter-assay variances determined for the *A. simplex* sandwich ELISA using cod contaminated with *A. simplex* larvae

Cod spiked with <i>A. simplex</i> larvae	Measured <i>A. simplex</i> protein (mean µg/g)	Intra-assay variance (%CV, <i>n</i> = 7)	Inter-assay variance (%CV, <i>n</i> = 7)
2 larvae	2	11	25
10 larvae	6	6	22
25 larvae	10	7	23
50 larvae	30	5	16

Limit of detection and limit of quantification

The LOD of the ELISA corresponded to 0.003 µg/mL measured in assay buffer, equivalent to 0.3 µg *A. simplex* protein/g food, considering the dilution. The LOQ was 0.01 µg/mL measured in the assay buffer and 1.1 µg *A. simplex* protein/g food. Additionally, the LOD was assessed in blank samples of codfish, fish balls, and mackerel with tomato sauce resulting in varying LODs in the different matrices. The LOD in codfish and mackerel with tomato sauce was 0.6 µg *A. simplex* protein/g food and 0.5 µg *A. simplex* protein/g food in fish balls.

Discussion

The potential contamination of seafood with the fish parasite *A. simplex* is concerning health authorities, food manufacturers, and consumers. Therefore, survey studies assessing the prevalence of *A. simplex* in fish and seafood products should be conducted. However, a reliable and sensitive method for the quantitative analysis of *A. simplex* in foods is not available so far.

ELISA is the most commonly used immunological method for the quantitative detection of food allergens. The principal advantages of these fully quantitative assays are their robustness and sensitivity. Several formats are in use, but all are based on the use of a species-specific antibody. Antibodies may be monoclonal, detecting specifically a single peptide sequence in the target protein, or polyclonal, reacting to many epitopes in the target. Antibodies, in particular polyclonal ones, may also react to similar peptide structures in other related proteins, resulting in cross-reactivity, which generally is limiting the applicability of the assay. However, proteins can be altered by food processing techniques, destroying epitopes, or exposing formerly hidden ones [29, 30]. Therefore, a broader specificity of the antibody seems to be advantageous for the analysis of processed food. In the development of an ELISA method for the detection of *A. simplex* proteins, we therefore chose to use a polyclonal antibody expecting that proteins and fragments of the dead parasite larvae as well as excretory proteins would be detectable.

In the present study, a sensitive and specific sandwich ELISA for the determination of *A. simplex* in foods was developed and validated with two different food matrixes. By this method, extractable *A. simplex* proteins were successfully detected and quantitated in commercially available food products and in fish spiked with *A. simplex* larvae. A pilot survey on different Norwegian fish products was performed. Several of the tested products contained small amounts of *A. simplex* protein. These results will be

published elsewhere (M.T. Werner et al., 124th AOAC International Meeting and Exposition, Sep. 2010).

Since the *A. simplex* sandwich ELISA constructed using a purified polyclonal anti-*A. simplex* antibody, extensive studies on cross-reactivity was performed during the assay validation. The specificity of the anti-*A. simplex* antibody used in the ELISA was studied by cross-reaction experiments with different fish, molluscs, and insects. The test conditions used in the ELISA to assess the assay specificity were based on the protein concentrations of the raw food ingredients. These conditions would clearly overestimate the real situation in commercial food products, which usually consist of a considerable number of mixed ingredients. None of the species tested showed any cross-reactivity in the *A. simplex* sandwich ELISA. Furthermore, several species of fish, shrimp, cockroach, and mite [31] were analyzed with Western blot. A number of weak bands in the range of 40–70 kDa were considered to be a result of unspecific binding to the rather high protein amounts applied on the gel and the high anti-*A. simplex* antibody concentration used. None of these species produced any positive signals in the ELISA.

Additionally, the detection characteristics of the anti-*A. simplex* antibody were evaluated by Western blot, comparing the binding patterns to two different *A. simplex* protein extracts. The antibody bound apparently to multiple proteins in the range from 14 to 190 kDa. Three pronounced bands were visible in both the purified and the raw *A. simplex* extract at about 41, 67 and 97 kDa. The protein bands at 41 and 97 kDa could be identical with the *A. simplex* allergens Ani s 3 (tropomyosin) [18] and Ani s 2 (paramyosin) [32], respectively, which are the only major somatic allergens described so far [19, 33]. They show extensive homology to tropomyosins and paramyosins from other species causing probably the considerable cross-reactivity of anti-*A. simplex*-IgE with e.g. German cockroach, chironomids, and dust mites that has been clinically observed [28, 34, 35]. The protein band at 67 kDa might be connected to a thermolabile protein of the same size that had shown antibody binding in mammalian cell cultures [36]. However, to our knowledge, this protein has not been further characterized or regarded as an important immunogen concerning *A. simplex* allergy. Several other proteins have been identified as allergens from *A. simplex*. Ani s 1 is a secretory protein of 21 kDa and the only major allergen described with no described function or homology to date [37, 38]. Two allergens, Ani s 4 (9 kDa) and Ani s 6 (7 kDa) [13], are both protease inhibitors [14, 39]. Ani s 5 (15 kDa) [13], Ani s 8 (15 kDa) [40], and Ani s 9 (15 kDa) [41] are all proteins in the SXP/RAL-2 family, whose function is still unclear. Ani s 7 has recently been described as the most important excretory-secretory allergen for diagnosis of infected patients [42].

The key parameters of the *A. simplex* sandwich ELISA were evaluated and determined in an intra-laboratory validation. The standard curve was linear at concentration levels of 0.98 to 125 ng *A. simplex* protein/mL, or 0.1 to 12.5 µg *A. simplex* protein/g food if the sample dilution in the assay is considered. The assay performed well with 1:20 dilution of all tested matrixes. Due to the restricted availability of purified *A. simplex* protein standard, the method validation had to be modified in some aspects. After determining the optimal ELISA conditions and dilutions of all components in the sandwich ELISA and the initial cross-reactivity testing, the assay performance of the purified *A. simplex* protein standard and the raw *A. simplex* protein standard were compared. Since the difference of the observed OD 450 nm absorbance values was approximately 5% at corresponding concentrations and both preparations demonstrated similar antigenicity of the major proteins on Western blot, we decided to switch to the raw *A. simplex* protein standard in the further validation of the ELISA assay. Regarding the lack of reference materials and standards, and considering errors from sampling and sample preparation, we considered the difference between the two *A. simplex* protein extracts as insignificant and neglectable. For a further development of the *A. simplex* sandwich ELISA, the purification of a large amount of *A. simplex* protein could be reconsidered. Additionally, protein characterization by mass spectrometry should be aspired.

The choice of an appropriate extraction buffer has great impact on the protein composition in the extracts and the recovery from the sample. ELISA performance, however, was not affected by the extraction buffers due to the at least 20-fold sample dilution. Of the eight buffers examined in the present study including a range of different pH and ionic strengths, PBS was shown to be the most effective in terms of total protein yield from the *A. simplex*-spiked cod samples. However, the amount of total protein extracted was not correlated to the amount of *A. simplex* protein measured by the ELISA. Extraction by HSB (pH 4.5) and Tris-glycine resulted in similar total protein yield as for PBS. With regard to *A. simplex* protein, the extractability and ELISA-detectability appeared to be greatly dependent on the pH-values of the buffers. Measurable *A. simplex* proteins were only obtained under basic conditions, which is congruent to an observation made for other allergens in a previous study [43]. The Tris-glycine extraction buffer was found to be optimal considering the yield of detectable *A. simplex* proteins from thermally treated samples. This might be connected to the amino acid content in the Tris-glycine buffer, possibly increasing the extraction efficiency of *A. simplex* proteins. In contrast, PBS was apparently only suited for the extraction of raw samples according to the Western blot results in this study.

The accuracy of the *A. simplex* sandwich ELISA was determined in recovery experiments, using the raw *A. simplex* protein standard for two different blank food matrixes. The recovery rates were over 72% for all test concentrations, and variances were low ($SEM < 15$). The food products analyzed in this study were composed of a variety of ingredients representing authentic seafood products commonly used in Norway. Optimally, more matrixes should have been evaluated in the *A. simplex* sandwich ELISA to demonstrate the methods general applicability. However, *A. simplex* protein standard was a limited resource during the method validation and experiments had to be confined to the most relevant ones. In the meantime, we were able to increase the supply of *A. simplex* protein considerably, so that further studies could be performed. Evaluating the available accuracy data of the *A. simplex* sandwich ELISA, the method performance was found sufficient for the intended applications. Due to the lack of certified reference materials and standardized allergen protein preparations, the trueness of the method could not be determined.

The repeatability of the *A. simplex* sandwich ELISA as determined by intra-assay precision was $<11\%$ and the reproducibility as measured by inter-assay precision $<25\%$ using cod that was artificially infected with *A. simplex* larvae. The method worked reliable both for low and high numbers of larvae, corresponding to different levels of extracted *A. simplex* proteins. The numbers of added larvae and the levels of measured protein were not directly correlated reflecting the differences in size and protein content from larva to larva [44]. Because *A. simplex* larvae can grow in their fish hosts they will vary considerably depending on how many cycles in fish they have passed. However, the method performance considering precision was regarded as satisfactory for the assessed levels, although further investigations including additional food matrixes and concentrations would be desirable.

The sensitivity of the *A. simplex* sandwich ELISA was evaluated on the basis of buffer blank samples, two blank food matrices, and cod meat without *A. simplex* protein spike. The assay was sufficiently sensitive to allow quantitative determination of *A. simplex* protein in seafoods at levels of $1 \mu\text{g A. simplex protein/g sample}$. Furthermore, matrix had rather little influence on the measured LODs, ranging from $0.3 \mu\text{g A. simplex protein/g food sample}$ in blank buffer to $0.6 \mu\text{g A. simplex protein/g sample}$ in both mackerel in tomato sauce and cod. In terms of *A. simplex* larvae count, the *A. simplex* sandwich ELISA was able to detect *A. simplex* protein originating from only 1 larva in 100 g of fish meat.

For routine analysis, however, the LOD of the *A. simplex* sandwich ELISA was set to $1 \mu\text{g A. simplex protein/g sample}$ to reduce the probability of false-positive results

and increase the reliability of the assay, which is important for e.g. survey studies commissioned by food authorities. This operative LOD is in agreement with the assay specifications considered generally necessary for the protection of food allergic consumers. Although threshold concentrations and assay requirements are still under discussion [25], there is some kind of consensus that LODs of methods detecting allergens in food matrixes should be somewhere between 1 and $100 \mu\text{g/g}$.

In conclusion, a sensitive *A. simplex* ELISA in sandwich format for the quantitative determination of *A. simplex* larvae in foods was successfully developed and validated in two different complex food matrixes. This is the first ELISA method for the detection of *A. simplex* protein that is suited for food analysis. The *A. simplex* sandwich ELISA could be used for the quality control of raw materials and end products and for food surveys by food safety authorities.

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