Detection of Proteins from the Fish Parasite *Anisakis simplex* in Norwegian Farmed Salmon and Processed Fish Products

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Abstract Wild-caught marine fish are potentially carrying parasites. Larvae of the nematode *Anisakis simplex* (herring or whale worm) occur in almost all commercially exploited fish stocks in temperate seas. The presence of *A. simplex* in fish and fish products is not only an economic concern but represents a significant consumer health risk. Anisakiasis, human infection with live larvae, can occur by consuming raw or undercooked fish while allergy symptoms can also be elicited by the presence of *A. simplex* proteins in processed seafood. The European Food Safety Authority (EFSA) has concluded in a scientific opinion that routine testing of seafood products for *A. simplex* is needed. In the present study, we have determined *A. simplex* proteins in farmed salmon intended for use in sushi and fish products from the Norwegian market by quantitative sandwich ELISA, immunostaining and mass spectrometry. Analytical methods detecting anisakid proteins at the single-digit milligram level appear to be sufficiently sensitive for the protection of allergic consumers. Only trace amounts (<10 mg/kg) were detected in a few samples showing that contamination with *A. simplex* is apparently not an immediate health problem given the results from this survey.

Keywords *Anisakis simplex* · Contamination · ELISA · Farmed salmon · Fish products · Mass spectrometry · Survey

Introduction

The marine nematode *Anisakis simplex* (herring or whale worm) has a complex life cycle involving planktonic crustaceans, fish and marine mammals (Sakanari and McKerrow 1989). In fish, the intermediate host, third-stage larvae are mainly situated in the visceral cavity encapsulated as flat tight spirals; however, a minor proportion (<5 %) may migrate deeply into the fillets (Levsen and Lunestad 2010; Karl et al. 2011). The parasite occurs in pelagic fish in all major oceans and seas (Sakanari and McKerrow 1989). Infestation frequencies increase apparently due to a rise in marine mammal populations (Klimpel and Palm 2011).

The occurrence of *A. simplex* in fishery products is not only unsavoury and causes economic problems but represents a significant health risk for consumers (Audicana and Kennedy 2008). Anisakiasis is a fish-transmitted zoonosis resulting from the accidental infection with live larvae through the consumption of raw or undercooked fish (Audicana et al. 2002). More than 90 % of the reported anisakiasis cases resulted from infection with a single larva (Daschner et al. 2000). The majority of anisakiasis cases occur in Japan, Spain, Italy, South America, the USA (Hawaii), the Netherlands and Germany, where traditional raw, marinated or brined fish dishes (sushi and sashimi, pickled anchovies, ceviche, lomi-lomi and salted herring) are consumed (Audicana and Kennedy 2008; Pravettoni et al. 2012; AAITO-IFIACI 2011; Torres-Frenzel and Torres 2014). In Norway, a country with proportionally high per-capita fish consumption, the number of anisakiasis incidents is low, possibly because mainly cooked fish products are eaten (Lin et al. 2012; Levsen et al. 2013; Vidaček et al. 2011). However, this might change due to a more globalised cuisine, especially with regard to Asian-inspired seafood, the trend to avoid overcooked food for vitamin preservation and a generally higher consumption of fish for health reasons (Audicana and Kennedy 2008).

A. simplex is the only known fish parasite so far that can provoke clinical allergic responses by the ingestion of processed seafood containing anisakid proteins (de Corres et al. 1996; Daschner and Pascual 2005). The *A. simplex* proteome is complex and contains a considerable number of proteins

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with allergenic potential (Fæste et al. 2014). Several allergens have been shown to be relatively resistant to digestion or heat treatment and might renature under cooling (Moneo et al. 2005; Caballero and Moneo 2004).

The European Food Safety Authority (EFSA) has concluded in a scientific opinion that routine testing of seafood products for the presence of *A. simplex* is needed (EFSA-BIOHAZ 2010). As safeguard measures for the prevention of anisakiasis, regulations for raw, cold smoked, marinated or salted fishery products have been implemented in the European Union requiring freezing or heat treatments (European Commission 2004; Adams et al. 2005), and similar regulations exist in the USA and Canada (FDA 2012; Weir 2005). These measures have been adopted by the fish industry as part of their Hazard Analysis and Critical Control Points (HACCP) systems (FDA 2011). Manufacturers of fish products have also to ensure the absence of visible parasites by visual inspection (FDA 2011, 2012) or artificial digestion (Codex Alimentarius 2005). However, in contrast to wild-caught fish, EFSA has evaluated the risk for *A. simplex* contamination in Atlantic salmon farmed in floating cages and fed on artificial diet to be negligible (EFSA-BIOHAZ 2010; Wootten et al. 2010), and thus, this product has been exempted from mandatory freezing since November 2006 (European Commission 2011). Comparably, *A. simplex* larvae have been detected in American wild-caught salmon but not in pen-reared fish that had been fed from hatchery to harvest only with commercially prepared feed (Deardorff and Kent 1989).

Several methods for the detection and characterisation of anisakid larvae, DNA and proteins in fish and fish products have been developed. The identification of *A. simplex* and related anisakid nematodes in seafood has been achieved by amplification of parasite DNA fragments using specific primers (Espiñeira et al. 2010) followed by a restriction fragment length polymorphism (RFLP) analysis and multiplex polymerase chain reaction (PCR) (Umehara et al. 2008). Parasites embedded in fish fillets can be detected after slicing and de-skinning by shining through of bright light (candling) or hyperspectral imaging (Sivertsen et al. 2012) or, more efficiently, by UV illumination after pressing and deep freezing (Levsen and Lunestad 2010; EFSA-BIOHAZ 2010). Digestion by pepsin under acid conditions with subsequent sieving of the incubation slush leads to the recovery of virtually all *A. simplex* but is rather time-consuming and destroys the fish (EFSA-BIOHAZ 2010; Petrie et al. 2007). However, these techniques are only suitable for the detection of intact larvae or visible pieces whereas DNA-based methods and immunochemical assays are also useful for the detection of *A. simplex* traces in fishery products. PCR and real-time PCR have been successfully applied for the specific quantitation of *A. simplex* (Mossali et al. 2010; Lopez and Pardo 2010) or the simultaneous quantitation of different anisakid species (Herrero et al. 2011) with a limit of detection (LOD) of about

1 ppm anisakid DNA in fish and seafood. Since the presence of *A. simplex* DNA in a sample is not necessarily correlated with the amount of protein present and only the larval proteins are responsible for allergic reactions in sensitised consumers, immunochemical assays for the direct measurement of anisakid proteins have been recently developed. *A. simplex* proteins have been quantified using monoclonal (Arilla et al. 2008) or polyclonal antibodies (Rodríguez-Mahillo et al. 2010; Werner et al. 2011) in dot blot (Rodríguez-Mahillo et al. 2010) and sandwich enzyme-linked immunosorbent assays (ELISAs) (Arilla et al. 2008; Werner et al. 2011) with a limit of quantitation (LOQ) of about 1 mg/kg anisakid protein in fish for both techniques. Additionally, the ELISA method (Werner et al. 2011) applying polyclonal antibodies raised against a protein extract from total larvae has been validated for different seafood products.

In the present study, we have used this method (Werner et al. 2011) for a survey on fish products popular in Norway and for the analysis of farmed Atlantic salmon (*Salmo salar*) intended for use in sushi. Furthermore, results were evaluated by high-resolution liquid chromatography-tandem mass spectrometry (LCMSMS) (Fæste et al. 2014) detecting specific *A. simplex* marker proteins.

Materials and Methods

Survey of Seafood Products on the Norwegian Market

In total, 105 seafood products were collected in retail stores in Oslo in 2010 to 2012 in yearly surveys. The samples included differently processed popular Norwegian fish products (Table 1) containing mackerels (*Scomber scombrus*), sardines (*Sardina pilchardus*), herring (*Clupea harengus*), anchovy (*Engraulis encrasicolus*), pollack (*Pollachius pollachius*), haddock (*Melanogrammus aeglefinus*), salmon (*Salmo salar*) and cod (*Gadus morhua*) and a few products containing shrimps (*Pandalus borealis*), crabs (*Cancer pagurus*) and scallops (*Pecten maximus*). All samples were analysed for content of *A. simplex* protein using a sandwich ELISA. Additionally, representative samples with high and low content of *A. simplex* protein were analysed by immunostaining using polyclonal anti-*A. simplex* antibodies or serum of a patient with *A. simplex* allergy and by mass spectrometry.

Survey of Atlantic Salmon Sampled from Sushi Restaurants in Norway

Raw fish intended for use in sushi (Table 2A) were sampled by the Norwegian Food Safety Authority in two rounds in different sushi-serving restaurants in Norway in 2011 and 2012. In total, 48 Atlantic salmon and three Atlantic halibut (*Hippoglossus hippoglossus*) samples, as well as one tuna (*Thunnus thynnus*),

Table 1 Survey (2010–2012) of seafood products from the Norwegian market

Product	Sample no.	No. >LOD (>0.3 mg/kg)	ELISA (mg/kg)	No. >LLA[b] (>3 mg/kg)
2010				
Mackerel in tomato sauce	18	5	0.3–0.4	0
Mackerel fillet	2	2	1.3–2.1	0
Sardines in tomato sauce	4	0	<0.3	0
Sardines in oil	4	0	<0.3	0
Herring fillet	2	0	<0.3	0
Cod roe and liver pie	4	0	<0.3	0
2011				
Mackerel in tomato sauce	20	0	<0.3	0
Sardines in oil	11	2	1.1–1.9	0
Surimi (crab sticks)	1	1	0.8	0
2012				
Mackerel in tomato sauce	5	0	<0.3	0
Mackerel fillet	4	4	1.9–9.3	**2**
Sardines in tomato sauce	1	1	1.0	0
Sardines in oil	1	0	<0.3	0
Herring fillet	5	2	0.7–2.6	0
Herring pickled	15	5	0.9–6.6	**3**
Salmon in oil	2	2	2.4–2.9	0
Haddock fillet	1	1	2.6	0
Haddock balls and pudding	16	3	4.4–8.6	**3**
Pollack fillet	1	0	<0.3	0
Pollack fish fingers	1	1	1.1	0
Cod fillet	1	1	1.6	0
Cod liver[a]	1	1	**14.5**	**1**
Cod roe and liver pie	1	0	<0.3	0
Surimi (crab sticks)	2	1	1.1	0
Anchovy fillet	1	1	1.8	0
Scallop	1	0	<0.3	0
Shrimps	2	1	1.0	0
Crab	2	0	<0.3	0
Salmon smoked	1	0	<0.3	0

[a] Cod liver was used as control sample in ELISA assays (see Table 4). The *A. simplex* content was also confirmed by immunostaining (Fig. 1) and LCMSMS (see Fig. 3a)

[b] Number of samples with A. simplex protein content > 3 mg/kg is marked bold

one Kingfish (*Seriola lalandi*) and one shrimp tail (*Litopenaeus vannamei*) sample of unknown geographical origin were collected and analysed with sandwich ELISA.

Survey of Slaughter-Ready Atlantic Salmon from a Norwegian Fish Farm

Belly flaps (91) and loins (25) from Atlantic salmon (Table 2B) produced at a fish farm in Western Norway in 2013 were analysed with the sandwich ELISA for contents of

Table 2 Survey of (A) salmon and other fish intended for use in sushi (2011–2012) and (B) belly flaps and loins from slaughter-ready farmed salmon (2013)

Sample	Sample no.	No. >LOD (>0.3 mg/kg)	ELISA (mg/kg)	No. >LLA (>3 mg/kg)
A				
Salmon[a]	48	8	0.3–7.1	2
Halibut	3	0	<0.3	0
Tuna	1	0	<0.3	0
Kingfish	1	0	<0.3	0
Shrimp tail	1	0	<0.3	0
B				
Salmon belly flaps[b]	91	38	0.35–8.9	21
Salmon loins	25	0	<0.3	0

[a] The salmon sample with the highest concentration was used as control sample in ELISA assays (see Table 4). The *A. simplex* content was also confirmed by LCMSMS (see Fig. 3b)

[b] The respective presence or absence of *A. simplex* in a belly flap and loin sample was also confirmed by immunostaining (Fig. 1). Additionally, five belly flap samples were analysed by LCMSMS (see Fig. 3b)

A. simplex protein. Additionally, some of the samples were analysed by immunostaining and mass spectrometry.

Sample Preparation for ELISA Assay

Fish and food samples (2 g) were homogenised with a mechanical blender (Retsch GmbH & Co, Haan, Germany) and extracted with 10 mL 0.1 M Tris/0.5 M glycin (pH 8.7) overnight at 45 °C in a shaking water bath (OLS 200, Grant, Cambridge, UK), centrifuged at 39,200×g (J2-MC; Beckman Instruments, Palo Alto, CA, USA) for 25 min at 4 °C and stored at −20 °C. Alternatively, sample homogenates were extracted by shaking with phosphate-buffered saline (PBS, pH 7.4) (Oxoid, Basingstoke, UK) at room temperature for 1 h. The extraction with PBS resulted in a larger amount of extracted protein whereas the Tris-glycine method recovered proteins also from more difficult matrices. Both approaches have been validated for the ELISA assay (Werner et al. 2011). Generally, PBS extraction was used in the present study with comparative use of Tris-glycine for some complex food products. Extracts were diluted at least 1:20 in PBS before analysis. Further dilution was performed if required to reach the working range of the ELISA.

Standard Protein for ELISA and LCMSMS Analysis

A. simplex third-stage larvae collected from freshly caught blue whiting (*Micromesistius poutassou*) and identified by RFLP were homogenised and extracted either with Tris-glycine buffer and semi-purified by ammonium sulphate precipitation, dialysis and freeze-drying or with PBS at room

temperature (Werner et al. 2011). Total protein contents were determined using Lowry assay (DC Protein Assay, Bio-Rad, Hercules, CA, USA). Both protein preparations were characterised by gel electrophoresis, immunostaining and mass spectrometry. The semi-purified protein had been used for the production of polyclonal antibodies in rabbits (Werner et al. 2011).

Gel Electrophoresis and Immunostaining

The standard proteins (10 μg per lane) and several survey samples (30 μg per lane) were analysed by gradient gel electrophoresis and subsequent immunostaining with either polyclonal anti-*A. simplex* antibodies or serum of a patient with *A. simplex* allergy (Fæste et al. 2014). The patient, a 60-year-old Spanish man with gastro-allergic anisakiasis, had a high IgE serum level (class 4) to *A. simplex* proteins, was positive in skin-prick testing and showed no cross-reactivity to arthropod proteins (shrimp, mite). The experiments were performed as described before (Fæste et al. 2014; Werner et al. 2011), only that the blocking buffer contained 5 % horse serum instead of 1 % BSA. For the immunostaining, the polyclonal antibody was diluted 1:250,000 and patient serum was diluted 1:20.

Polyclonal Sandwich ELISA for *A. simplex* Protein Detection in Fish and Seafood

Food products and fish samples were analysed using a previously developed polyclonal sandwich ELISA (Werner et al. 2011) that specifically detects *A. simplex* proteins. The assay had been validated for sensitivity, specificity, accuracy and precision and had a LOD of 0.3 mg protein/kg food and a LOQ of 1 mg protein/kg; however, the lower limit of application (LLA) was set to 3 mg protein/kg to have a safety margin for potential matrix interferences. The standard curve of the ELISA was constructed with 12 concentrations of PBS-extracted total *A. simplex* protein ranging from 0 to 1,000 μg/L. The working range of the ELISA was from 1 to 250 μg/L using polynomial regression for the standard curve.

Assessment of the Long-Term Stability of the Sandwich ELISA

An additional short validation was performed before applying the ELISA in the survey studies. The long-term inter-assay precision of the standard curve was assessed by determining a mean coefficient of variation (CV) from the individual inter-assay CV for each of the 12 concentrations in the standard curve for five subsequent years. The intra-assay CVs were represented by the maximum values among the standard concentrations of the respective years. Furthermore, precision

data were calculated for three control samples (cod muscle spiked with 50 *A. simplex* larvae (Werner et al. 2011), naturally contaminated cod liver (from the product survey, Table 1) and naturally contaminated salmon muscle (from the sushi survey, Table 2A) for 5 years. The three control samples were also included as performance controls in all experiments. The assay recovery was evaluated at three concentrations of spiked *A. simplex* proteins in two typical fish product matrices (whitefish pudding, pepper mackerel). Extractions were performed in triplicate, the resulting extracts were analysed by the sandwich ELISA, and the mean values for the recoveries and the standard error of the mean (SEM) were calculated.

Sample Preparation for LCMSMS Analysis

Protein sample extracts (50 μL, 1 mg/mL) were directly absorbed on ultrafiltration filters (Nanosep® centrifugal devices, 10-kDa cut-off, Pall Life Sciences, Ann Arbor, MI, USA) without previous separation by gel electrophoresis. After centrifugation (Eppendorf Centrifuge, Hamburg, Germany) for 5 min at 13,000×g and 4 °C, proteins were digested with trypsin (3 μg/150 μL 50 mM $(NH_4)_2CO_3$, pH 7.8; Trypsin Gold, mass spectrometry grade, Promega, Madison, WI, USA) overnight at 37 °C. Peptides were eluted by centrifugation for 10 min at 13,000×g, dried in a SpeedVac centrifuge (Heto, Allerød, Denmark) and re-dissolved in 20 μL of 0.1 % formic acid. After 30-s sonication and 10-min centrifugation at 13,000×g, 10 μL of each sample was transferred into a 0.3-mL mass spectrometry vial with inner cone (Macherey-Nagel, Düren, Germany).

LCMSMS for the Detection of *A. simplex* Proteins in Fish and Seafood

Reversed-phase (C18) nano-LCMSMS analysis of proteolytic peptides was performed using a system consisting of two Agilent 1200 HPLC binary pumps (nano and capillary) with autosampler, column heater and integrated switching valve (Agilent, Santa Clara, CA, USA). This LC system was coupled via a nano-electrospray ion source to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For the analyses, 3 μL of peptide solution was injected into the 5×0.3-mm extraction column filled with Zorbax 300 SB-C18 of 5-μm (diameter) particle size (Agilent). Samples were washed with mobile phase (97 %/ 0.1 % formic acid/3 % acetonitrile). The flow rate was 10 μL/ min provided by the capillary pump. After 5 min, the integrated switching valve was activated, and peptides were eluted in the back-flush mode from the extraction column onto a 150× 0.075 mm C18, 3-μm resin column (GlycproSIL C18–80 Å, Glycpromass, Stove, Germany). The mobile phase consisted of acetonitrile and MS grade water, both containing 0.1 %

formic acid. Chromatographic separation was achieved using a binary gradient from 5 to 55 % of acetonitrile in water in 68 min with a flow rate of 0.2 µL min^{-1} provided by the nano-flow pump.

Mass spectra were acquired in the positive ion mode, applying a data-dependent automatic switch between survey scan and tandem mass spectra (MS/MS) acquisition. Peptide samples were analysed with a collision-induced dissociation (CID) fragmentation method, acquiring one Orbitrap survey scan in the mass range of m/z 200–2,000 followed by MS/MS of the most intense ion on the parent mass list with a 10-ppm accuracy relative to the parent mass and 3-m/z isolation width. The target value in the LTQ Orbitrap was 1,000,000 for survey scans at a resolution of 100,000 at m/z 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Target ions on the parent mass list were fragmented three times in the Iontrap by CID at a resolution of 30,000 at m/z 400. The MS/MS target value was set to 5,000 ions and ion trap fill time to 500 ms. The ion selection threshold was 1,000 counts with selected ions dynamically excluded for 15 s. Data analysis was performed by Xcalibur V2.0. Previously identified marker peptides (Fæste et al. 2014) of the *A. simplex* protein haemoglobin were extracted with 10-ppm accuracy, and spectra were manually verified.

Results

Performance of the ELISA for the Detection of *A. simplex* in Fish and Food Products

All samples in this study were analysed with a previously developed sandwich ELISA using rabbit polyclonal anti-*A. simplex* antibodies (Werner et al. 2011). The long-term stability of the standard curve expressed in terms of intra-assay and inter-assay precision coefficients over five subsequent years (Table 3) was better than 19 % CV for intra-assay variation and 45 % for inter-assay variation; however, the latter value was calculated for the year with the fewest assays performed, and CVs for other years were considerably better. The evaluation of raw data showed that the mean absorbance (Abs$_{450\ nm}$) decreased over several years for the highest standard concentration and increased for one close to the LOD at 0.3 mg/kg, whereas it was almost stable for a concentration in the middle range of the standard curve. Thus, the curve had slightly flattened over time justifying the decision to set a LLA of 3 mg/kg with a safety margin for the ELISA.

The intra-assay and inter-assay precision for the three control samples confirmed the long-term stability of the sandwich ELISA (Table 4). The intra-assay and inter-assay CVs of the spiked cod muscle (Werner et al. 2011) (mean concentration determined as 26.5 mg/kg) were less than 11 and 41 %, respectively, over a period of 5 years. The precision was

poorest in 2010, the year in which the least number of assays was performed. In other years, the CVs were less than 5.4 and 18 %. The naturally contaminated cod liver control sample (mean concentration 14.5 mg/kg) achieved intra- and inter-assay CVs of less than 3.3 and 39 %, respectively, and the naturally contaminated salmon muscle control sample (mean concentration 7.1 mg/kg) achieved intra- and inter-assay CVs of less than 6.8 and 37 %, respectively.

The assay recoveries of *A. simplex* proteins from two typical fish product matrices using the PBS extraction method were ranging from 59 to 74 % in whitefish pudding and from 106 to 110 % in pepper mackerel (Table 5).

A. simplex Contamination of Seafood Products from the Norwegian Market

The analysis of 105 fish-containing products by ELISA showed that the presence of *A. simplex* proteins was low (Table 1). Concentrations above the LLA were only detected in four product types including mackerel fillet, pickled herring, haddock balls and pudding, and cod liver. The latter was the only product exceeding a content of 10 mg *A. simplex*/kg and was afterwards used as a positive control in all ELISA assays. Additionally, the presence of *A. simplex* was confirmed by immunostaining (Fig. 1) and measurement of an anisakid marker peptide (Fig. 2) by LCMSMS (Fig. 3a). Low-level contamination with *A. simplex* proteins between LOD (0.3 mg/kg) and LLA was found in additional 11 product groups.

Presence of *A. simplex* Proteins in Atlantic Salmon and Other Fish Intended for Use in Sushi

Analysis by ELISA revealed trace amounts of *A. simplex* protein (>LOD) in eight of 48 salmon samples, of which two had contents above the LLA but less than 10 mg/kg (Table 2A). Nothing was detected in other fish (Table 2A). The salmon muscle sample with the highest measured *A. simplex* concentration was used as a low-level positive control in all ELISA assays. Additionally, the presence of *A. simplex* in this sample was confirmed by LCMSMS (Fig. 3b).

Occurrence of *A. simplex* in Belly Flaps and Loins from Slaughter-Ready Atlantic Salmon

In total, 91 belly flaps sampled from a fish farm in Western Norway were analysed by ELISA, of which 38 contained detectable *A. simplex* proteins (>LOD), and 21 of these had contents higher than the LLA (Table 2B). However, none of the samples contained more than 10 mg/kg. None of the 25 salmon loins analysed by ELISA contained measurable *A. simplex* proteins. The respective presence or absence of

Table 3 Stability over time of the ELISA standard curve

Year	Number[a]	Mean RSD (%)[b] (inter-assay)	SD (%)	Mean Abs$_{450\,nm}$			RSD (%) (n)[c] (intra-assay)
				1,000 µg/L	62.5 µg/L	3.9 µg/L	
2009	8	16.6	3.93	2.647	0.605	0.148	<5.3 (8)
2010	6	44.5	18.1	2.225	0.814	0.165	<18 (2)
2011	10	14.8	1.83	2.323	0.898	0.159	<19 (2)
2012	9	25.3	7.98	2.267	0.545	0.185	<4.8 (4)
2013	18	27.7	9.01	2.270	0.606	0.281	<5.6 (2)

[a] Number of standard curves in the respective year

[b] Mean inter-assay coefficient of variation (CV) calculated from the individual inter-assay CV for each of the 12 concentrations (0–1,000 µg/L) in the standard curves

[c] Considering sample dilution (1:100) the standard curve concentrations are equivalent to 0.39 mg/kg, 6.25 mg/kg, and 100 mg/kg protein in matrix

[d] Maximum value for coefficient of variation among standard concentrations of a number of (n) standard curves measured on 1 day

A. simplex protein in a belly flap and loin sample was confirmed by immunostaining (Fig. 1). Additionally, five belly flap samples with *A. simplex* contents <LOD, <LLA and >LLA as measured by ELISA were analysed by LCMSMS, confirming the results (Fig. 3b).

Immunostaining as a Confirmative Method for the Presence of *A. simplex* in Fish

Immunostaining experiments were performed using the polyclonal rabbit anti-*A. simplex* IgG antibodies and IgE-containing serum of a patient with confirmed *A. simplex* allergy with the aim to compare the efficiency of the two extraction methods used and to characterise the ELISA control samples and salmon belly flap and loin samples with and without *A. simplex* protein contamination (Fig. 1). The comparison of protein patterns from differently extracted samples in gel electrophoresis and antibody-binding patterns in immunostaining revealed that the more stringent Tris-glycine extraction resulted in less gel bands (lane At) and immunostaining signals (1r; 1p) than the rather mild PBS extraction that produced a typical gel band pattern in the range of 98 to 38 kDa (lane A) and consistently more immunosignals (2r; 2p). The polyclonal IgG antibodies bound to the same three prominent bands at about 70, 40 and 17 kDa in both extracts

(1r; 2r), whereas the IgE-binding patterns using serum from a patient with *A. simplex* allergy were more complex (1p; 2p).

A. simplex protein in the spiked cod muscle ELISA control sample was detected by both the IgG and the IgE antibodies with considerable signal strength although with different band patterns (3r; 3p). The naturally contaminated liver control sample produced two less intense bands with IgG and IgE antibodies (4r; 4p) with different molecular weights as for the spiked cod sample. The comparison of a belly flap sample containing *A. simplex* protein (5r; 5p) with uncontaminated loin (6r; 6p) from the same salmon showed one (5r) or two (5p) detectable low-intensity signals in the belly flap, confirming the ELISA result.

Detection of *A. simplex* Protein by LCMSMS with Specific Marker Peptides

LCMSMS analysis of trypsinated *A. simplex* protein extract resulted in the recognition of typical peptides characterised by their typical mass patterns (precursor mass spectra; MS) and mass fragments (product ion spectra; MSMS). Two specific marker peptides of *A. simplex* haemoglobin that had been identified by LCMSMS sequencing in a previous project (Fæste et al. 2014) were chosen in the present study for the detection of anisakid proteins in fish products. From the six

Table 4 Precision (%) in spiked cod muscle and naturally contaminated cod liver and salmon muscle[a]

	Spiked cod muscle		Cod liver		Salmon muscle	
Year	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay
2009	5.4 (7)	16 (7)	–	–	–	–
2010	11 (3)	41 (3)	–	–	–	–
2011	1.7 (3)	18 (12)	–	–	–	–
2012	4.3 (3)	17 (32)	3.3 (3)	36 (21)	1.2 (3)	30 (22)
2013	1.8 (3)	16 (12)	1.8 (3)	39 (8)	6.8 (3)	37 (11)

[a] Values represent coefficients of variation of (n) experiments

Table 5 Recovery (%) from selected blank food matrices spiked with 1, 10 and 100 mg/kg *Anisakis simplex* standard[a]

Blank food	1	10	100
Fish pudding	59±24	70±24	74±13
Pepper mackerel	110±17	115±13	106±7.4

[a] Values represent the average of four spiking experiments and are reported as mean±standard error of the mean (SEM)

haemoglobin peptides known so far (Fig. 2a), HSWTTIGEEFGHEADK (①) and LFAEYLDQK (②) not only produced the most intense MS signals but were specific for *Anisakis* spp., as shown by comparison with haemoglobin from *Anisakis pegreffi* and other nematodes such as *Ascaris suum* and *Pseudoterranova decipiens* (Fig. 2a).

The comparison of nematode *Anisakis* spp. haemoglobin to vertebrate fish haemoglobin (salmon, *Salmo salar*) showed very little homologies (Fig. 2b). Some general amino acid sequence motifs might exist as indicated by the letters on top of the peptides in the figure, but the chance for cross-identification when measuring anisakid haemoglobin in a fish matrix appeared to be small.

LCMSMS Analysis of *A. simplex* Marker Peptides in Fish Matrix

A mass spectrometry method was developed using samples generated by spiking *A. simplex* PBS-extracted standard
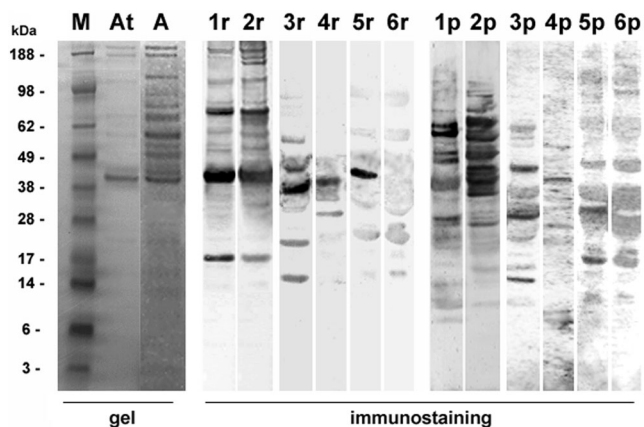

Fig. 1 Gel electrophoresis of *A. simplex* proteins (*left panel*) and immunostaining with polyclonal rabbit antibodies and patient plasma (*right panel*). *M*, molecular weight marker (kDa) (indicated on the *left side* of the gel); *At*, *A. simplex* proteins extracted with Tris-glycine buffer (45 °C) and semi-purified; *A*, *A. simplex* proteins extracted with PBS (room temperature); *1r–6r*, immunostaining with polyclonal anti-*A. simplex* antibodies from rabbit; *1p–6p*, immunostaining with plasma from patient with *A. simplex* allergy. Samples: *1*, At; *2*, A; *3*, spiked cod muscle control (Table 2B); *4*, naturally contaminated cod liver control (Tables 1 and 2B); *5*, contaminated salmon belly flap (Table 2B); *6*, salmon belly flap (Table 2B)

protein into salmon extract. The evaluation of a mass spectrum of a sample containing 2 % (20 ng/μL) *A. simplex* protein (Fig. 2c, top panel) showed that most of the protein load eluted at retention times (RTs) between 17 and 40 min within the 68-min-long LC gradient. The maximum signal intensity of the total ion count (TIC) spectrum was about 1.7×10^9 and of the base peak (BP) spectrum 4.8×10^8, which is referring to the most intense ions in the sample. The haemoglobin marker peptide ① HSWTTIGEEFGHEADK (m/z=615.27) eluted at RT 22.7 min with a signal intensity of 1.1×10^6, and the marker peptide ② LFAEYLDQK (m/z=563.78) eluted at RT=24.9 min with a signal intensity of 5.1×10^5. Marker ② was thus more intense than marker ①.

The LCMSMS analysis of unspiked salmon extract (Fig. 2c, bottom panel) showed the absence (indicated by arrows) of the *A. simplex* haemoglobin marker peptide peaks in the extracted ion spectra at m/z=615.27 and m/z=563.78.

Determination of *A. simplex* Protein by LCMSMS in Naturally Contaminated Fish Samples

The LCMSMS measurement of the cod liver control sample (Table 4) confirmed the presence of *A. simplex* protein (Fig. 3a). Both haemoglobin marker peptides were unambiguously detected with about twice the intensities as for the spiked salmon sample (Fig. 2c). The zoomed display of the respective LC peak and mass pattern of HSWTTIGEEFGHEADK and LFAEYLDQK allowed the determination of typical characteristics of both marker peptides facilitating positive identification in unknown samples.

The analysis of the salmon muscle control sample (Table 4) provided an indication of the LOD of the LCMSMS method (Fig. 3b, top panel). In this low-contaminated sample, only the *A. simplex* marker peptide ②, which was detected with higher sensitivity than marker ①, was positively identified, as shown by the detailed mass pattern.

When two belly flaps from the survey on slaughter-ready salmon (Table 2B) were analysed by LCMSMS for confirmation of the ELISA results, both marker peptides were detected in the sample with a measured content of 9 mg/kg *A. simplex* protein, whereas no signals were registered for a sample with a measured content of <3 mg/kg (Fig. 3b, bottom panel).

Discussion

The presence of *A. simplex* proteins in fish and fish products is a potential health risk for consumers with allergy to the fish parasite or with cross-reacting allergies, e.g. to mites or crustaceans. As with other food allergens, small amounts can be enough to elicit an allergic reaction in sensitive persons (Daschner and Pascual 2005). Recently, reference doses for 11 residues of allergenic foods have been established by an
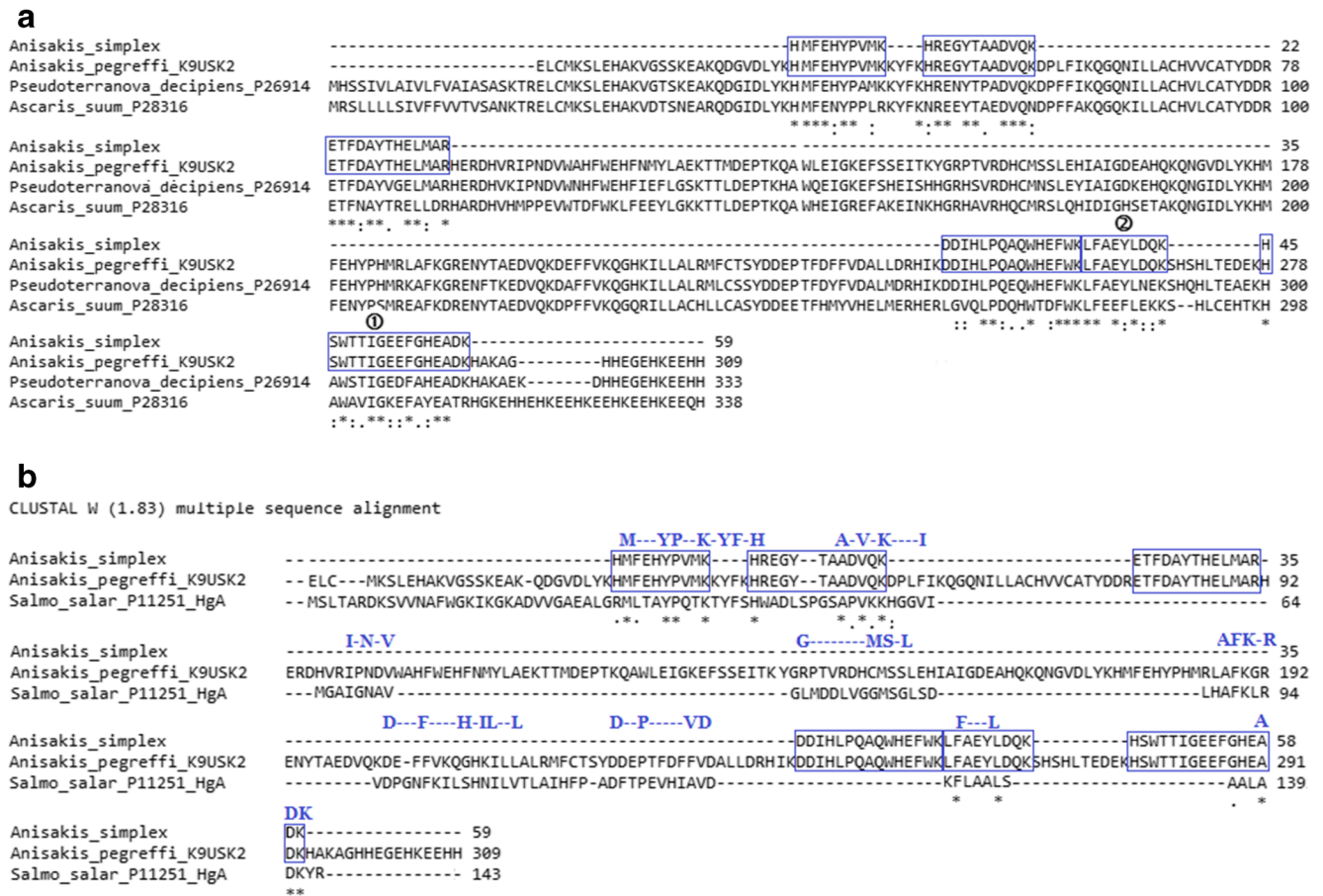
a

```
Anisakis_simplex              -------------------------------------------------HMFEHYPVMK----HREGYTAADVQK----------------------- 22
Anisakis_pegreffi_K9USK2      ---------------------ELCMKSLEHAKVGSSKEAKQDGVDLYKHMFEHYPVMKKYFKHREGYTAADVQKDPLFIKQGQNILLACHVVCATYDDR 78
Pseudoterranova_decipiens_P26914  MHSSIVLAIVLFVAIASASKTRELCMKSLEHAKVGTSKEAKQDGIDLYKHMFEHYPAMKKYFKHRENYTPADVQKDPFFIKQGQNILLACHVLCATYDDR 100
Ascaris_suum_P28316           MRSLLLLSIVFFVVTVSANKTRELCMKSLEHAKVDTSNEARQDGIDLYKHMFENYPPLRKYFKNREEYTAEDVQNDPFFAKQGQKILLACHVLCATYDDR 100
                                                                  ****.**   :   *.** **. ***.
```

```
Anisakis_simplex              ETFDAYTHELMAR----------------------------------------------------------------- 35
Anisakis_pegreffi_K9USK2      ETFDAYTHELMARHERDHVRIPNDVWAHFWEHFNMYLAEKTTMDEPTKQAWLEIGKEFSSEITKYGRPTVRDHCMSSLEHIAIGDEAHQKQNGVDLYKHM 178
Pseudoterranova_decipiens_P26914  ETFDAYVGELMARHERDHVKIPNDVWNHFWEHFIEFLGSKTTLDEPTKHAWQEIGKEFSHEISHHGRHSVRDHCMNSLEYIAIGDKEHQKQNGIDLYKHM 200
Ascaris_suum_P28316           ETFNAYTRELLDRHARDHVHMPPEVWTDFWKLFEEYLGKKTTLDEPTKQAWHEIGREFAKEINKHGRHAVRHQCMRSLQHIDIGHSETAKQNGIDLYKHM 200
                              ***:.**. **: *                                                                              ②
```

```
Anisakis_simplex              -----------------------------------------------------DDIHLPQAQWHEFWKLFAEYLDQK---------H 45
Anisakis_pegreffi_K9USK2      FEHYPHMRLAFKGRENYTAEDVQKDEFFVKQGHKILLALRMFCTSYDDEPTFDFFVDALLDRHIKDDIHLPQAQWHEFWKLFAEYLDQKSHSHLTEDEKH 278
Pseudoterranova_decipiens_P26914  FEHYPHMRKAFKGRENFTKEDVQKDAFFVKQGHKILLALRMLCSSYDDEPTFDYFVDALMDRHIKDDIHLPQEQWHEFWKLFAEYLNEKSHQHLTEAEKH 300
Ascaris_suum_P28316           FENYPSMREAFKDRENYTAEDVQKDPFFVKQGQRILLACHLLCASYDDEETFHMYVHELMERHERLGVQLPDQHWTDFWKLFEEFLEKKS--HLCEHTKH 298
                                                                  ①          :: **:..*  :***** *:*::*            *
```

```
Anisakis_simplex              SWTTIGEEFGHEADK------------------------ 59
Anisakis_pegreffi_K9USK2      SWTTIGEEFGHEADKHAKAG---------HHEGEHKEEHH 309
Pseudoterranova_decipiens_P26914  AWSTIGEDFAHEADKHAKAEK-------DHHEGEHKEEHH 333
Ascaris_suum_P28316           AWAVIGKEFAYEATRHGKEHHEHKEEHKEEHKEEQH 338
                              :*:.**::*.:**
```

b

CLUSTAL W (1.83) multiple sequence alignment

```
                                                 M---YP--K-YF-H       A-V-K----I
Anisakis_simplex              -------------------------------------HMFEHYPVMK---HREGY--TAADVQK-----------------------ETFDAYTHELMAR- 35
Anisakis_pegreffi_K9USK2      --ELC---MKSLEHAKVGSSKEAK-QDGVDLYKHMFEHYPVMKKYFKHREGY--TAADVQKDPLFIKQGQNILLACHVVCATYDDRETFDAYTHELMARH 92
Salmo_salar_P11251_HgA        --MSLTARDKSVVNAFWGKIKGKADVVGAEALGRMLTAYPQTKTYFSHWADLSPGSAPVKKHGGVI------------------------------- 64
                                                     .*. **  *      *     *.*.*:
```

```
                                   I-N-V                                    G--------MS-L                          AFK-R
Anisakis_simplex              ERDHVRIPNDVWAHFWEHFNMYLAEKTTMDEPTKQAWLEIGKEFSSEITK-YGRPTVRDHCMSSLEHIAIGDEAHQKQNGVDLYKHMFEHYPHMRLAFKGR 35
Anisakis_pegreffi_K9USK2      ERDHVRIPNDVWAHFWEHFNMYLAEKTTMDEPTKQAWLEIGKEFSSEITK-YGRPTVRDHCMSSLEHIAIGDEAHQKQNGVDLYKHMFEHYPHMRLAFKGR 192
Salmo_salar_P11251_HgA        ---MGAIGNAV-----------------------------------------GLMDDLVGGMSGLSD--------------------------LHAFKLR 94
```

```
                                 D---F----H-IL--L        D--P-----VD                    F---L                         A
Anisakis_simplex              -------------------------------------------DDIHLPQAQWHEFWKLFAEYLDQK---------HSWTTIGEEFGHEA 58
Anisakis_pegreffi_K9USK2      ENYTAEDVQKDE-FFVKQGHKILLALRMFCTSYDDEPTFDFFVDALLDRHIKDDIHLPQAQWHEFWKLFAEYLDQKSHSHLTEDEKHSWTTIGEEFGHEA 291
Salmo_salar_P11251_HgA        --------------VDPGNFKILSHNILVTLAIHFP-ADFTPEVHIAVD---------------------KFLAALS---------------------AALA 139
                                                                                      *      * .  *
```

```
                              DK
Anisakis_simplex              DK---------------- 59
Anisakis_pegreffi_K9USK2      DKHAKAGHHEGEHKEEHH 309
Salmo_salar_P11251_HgA        DKYR------------- 143
                              **
```

Fig. 2 **a** Comparison of six *A. simplex* haemoglobin peptides identified by LCMSMS to other nematode haemoglobins from *Anisakis pegreffi* (UniProt database accession number: K9USK2), *Pseudoterranova decipiens* (P26914) and *Ascaris suum* (P28316). **b** Comparison of *Anisakis* spp. haemoglobin to salmon (*Salmo salar*) haemoglobin A (P11251). **c** LCMSMS mass spectrum of 20 ng/mL *A. simplex* spiked into salmon matrix with detection of two haemoglobin marker peptides. *Top panel*: total ion count spectrum (RT 0–68 min), BP, detection of *m/z* 615.28 (peptide: HSWTTIGEEFGHEADK) and detection of *m/z* 563.79 (peptide LFAEYLDQK). *Bottom panel*: total ion count spectrum (RT 0–68 min) and BP of unspiked salmon matrix

international expert panel (Taylor et al. 2014). Based on patient data from clinical challenge studies using statistical methods, reference doses ranging from 0.03 mg for egg proteins to 10 mg for shrimp proteins were defined, illustrating the need for sensitive methods for the detection of food allergens. However, no immediate reactions were observed when patients with a clinical history of *A. simplex* allergy were exposed with up to 100 lyophilised larvae containing approximately 45 mg protein in a double-blind placebo-controlled oral challenge study, indicating that the effect dose for *A. simplex* is comparatively high (Sastre et al. 2000). Thus, analytical methods detecting anisakid proteins in the lower milligram range appear to be sufficiently sensitive for the determination of levels that might represent a risk for allergic consumers.

In the present study, Norwegian farmed salmon and fish products were analysed for contents of *A. simplex* protein using a previously developed polyclonal ELISA (Werner et al. 2011). The performance of the *A. simplex* ELISA has been monitored over 5 years showing that the standard curve was stable according to the intra-assay and inter-assay precision assessment, even if it showed a tendency to flatten over time. Because of the observed increase of absorbances at the lowest concentrations, the LLA of the assay was established at 3 mg/kg. The LLA, as defined in best practice documents by an international reference group for food allergen methodologies, represents a level of confidence below which the assay may not give a definite result, e.g. due to matrix interferences (Abbott et al. 2010). Polyclonal assays are often preferred for food surveillance purposes because they are robust and have the advantage of recognising a large variety of protein species and fragments also in processed foods (Abbott et al. 2010).

The precision data for the spiked cod muscle control sample, as well as for the naturally contaminated cod liver and salmon muscle control samples, were acceptable according to best practice guidelines (Abbott et al. 2010). As expected, precision was slightly better for the artificially spiked than for the naturally contaminated samples, not least because the concentration of the latter was considerably lower.
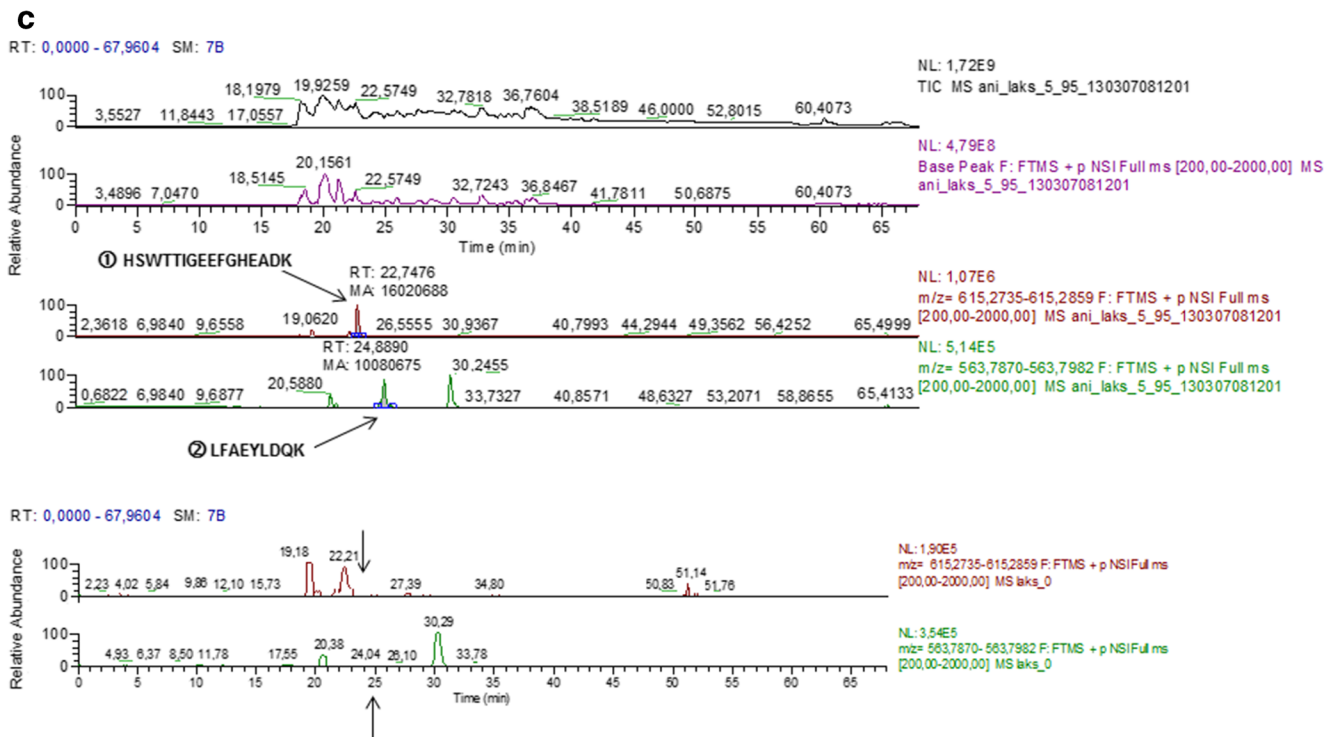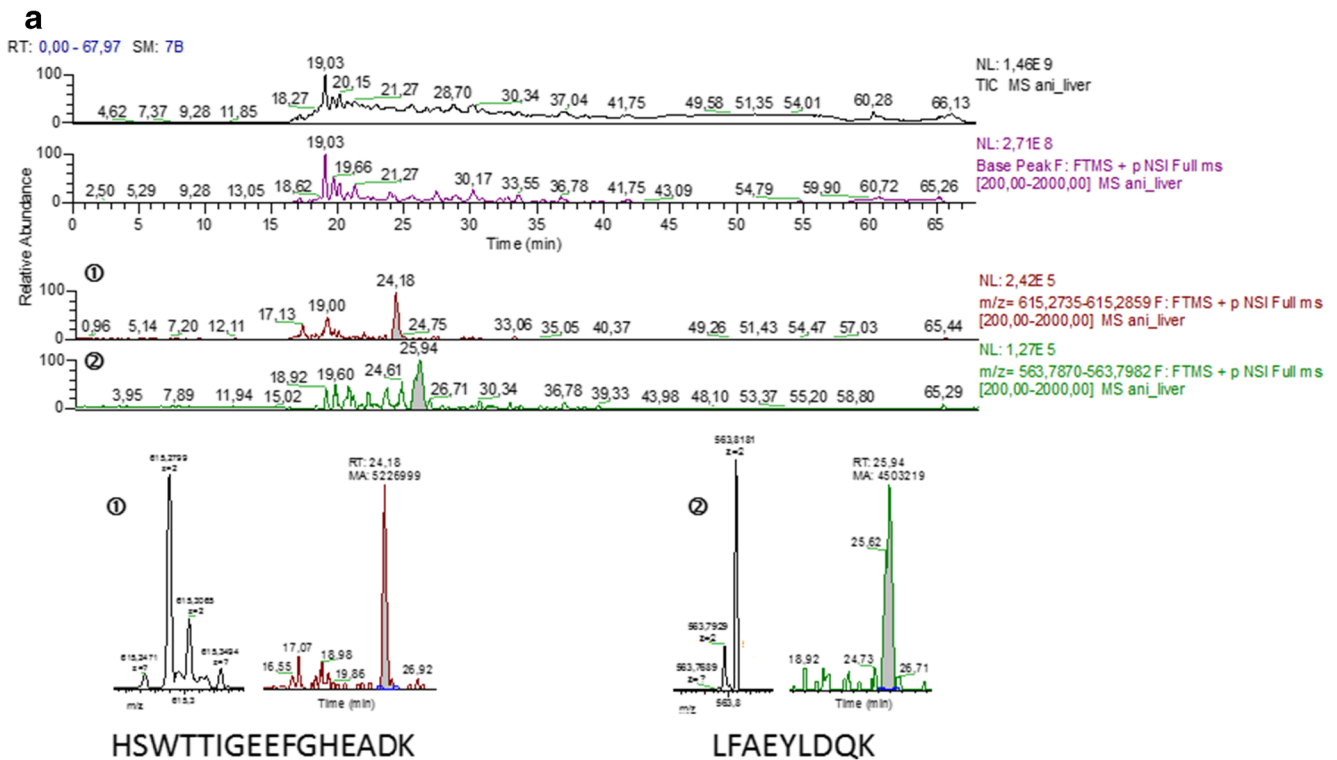
Fig. 2 (continued)

Fig. 3 **a** LCMSMS mass spectrum of naturally contaminated cod liver control with detection of two haemoglobin marker peptides. *Top panel* total ion count spectrum (RT 0–68 min), BP, detection of *m/z* 615.28 (peptide: HSWTTIGEEFGHEADK) and detection of *m/z* 563.79 (peptide LFAEYLDQK). *Bottom panel*: detailed MS spectrum and isotope peaks of HSWTTIGEEFGHEADK (*left side*) and LFAEYLDQK (*right side*). **b** LCMSMS mass spectrum of naturally contaminated salmon with detection of two haemoglobin marker peptides. *Top panel*: detection of LFAEYLDQK and isotope peak pattern in the salmon control sample (Table 4). *Bottom panel*: detection of HSWTTIGEEFGHEADK and LFAEYLDQK in contaminated salmon belly flap (*left side*) and uncontaminated belly flap (*right side*)
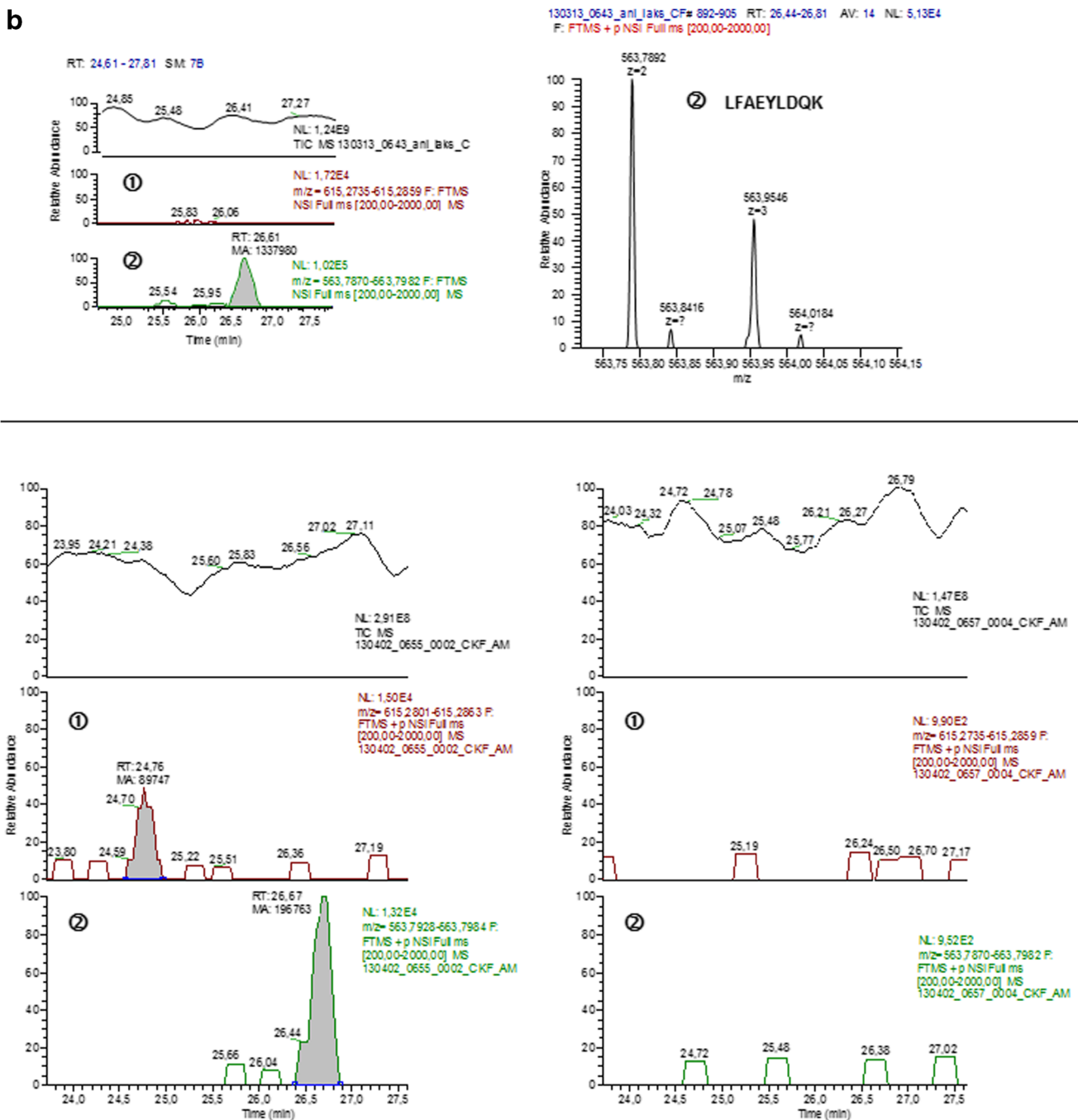
Fig. 3 (continued)

Furthermore, extractability from incurred material such as the naturally contaminated samples could be lower due to stronger matrix binding of the analyte, as compared to generally weaker interactions in spiked material. Thus, the inclusion of incurred reference material in validation studies is highly recommended in the allergen methodology guidelines (Abbott et al. 2010).

Recovery in the ELISA for *A. simplex* protein from typical matrices at three concentration levels was nearly complete. However, extraction from the more homogenous mackerel matrix was apparently more efficient than from the complex fish pudding matrix that contained not only haddock fillet but also potato starch, egg white and milk. The long-term performance of the ELISA regarding sensitivity, stability, precision and recovery was considered acceptable according to guidelines, and the assay appeared applicable for the detection of *A. simplex* protein in fish and fish products.

The survey on fish products from the Norwegian market revealed that the contamination with *A. simplex* was low. Anisakid protein levels above 3 mg/kg were mainly detected in little processed products from fish species such as mackerel, herring and cod that are known hosts for the larvae (Levsen and Lunestad 2010; Klimpel and Palm 2011). Not surprisingly, the highest contamination level was detected in cod liver since the parasite lives mainly in the viscera of infected fish (Karl et al. 2011). However, a study on Scottish herring has shown a large-scale time-dependent migration of larvae into the flesh of ungutted fish after capture and while stored on ice (Smith and Wootten 1975). The mandatory freezing routines for fish required by food safety authorities are thus important to observe to avoid cases of human anisakiasis (European Commission 2004; FDA 2011). In contrast, the risk for consumers with allergy to *A. simplex* protein appeared to be low considering the detected levels in the present study and comparing them to the clinical data (Sastre et al. 2000). All analysed products, even the cod liver, contained only trace amounts of anisakid proteins.

The occurrence of *A. simplex* proteins in salmon and a few other fish species sampled from sushi restaurants in Norway by the food authority was low. Concentrations slightly above the LLA were detected in only two samples (4 %) illustrating the low degree of contamination. Additionally, trace amounts (>LOD) were found in about 10 % of the samples. This very low incidence was in fact expected since farmed salmon receive an industrially formulated diet and are not feeding on nematode larvae-infested small fish and crustaceans as wild salmon. The infection prevalence for wild salmon caught on the northwestern US Coast and in the Norwegian Sea was as high as 100 % (Deardorff and Kent 1989; Bristow and Berland 1991; Marty 2008). In a study on more than 1,000 farmed salmon from all salmon-producing counties in Norway using acidified pepsin degradation for nematode detection, none of the samples contained intact larvae (Lunestad 2003), confirming previous findings obtained by the candling method (Angot and Brasseur 1993). In a comparable Canadian study analysing about 900 farmed salmon by histopathology, one single larva was found in a fish viscera leading to the conclusion that it was almost 600 times safer to eat farmed than wild salmon (Marty 2008). The one affected Canadian salmon was 40 % smaller than the mean of the other fish in the same rearing pen but not emaciated. It might have contracted the parasite by feeding on crustaceans or small fish that are able to pass through the meshes of the pen.

At this point, it can only be speculated about the origin of the anisakid protein traces that were found in some of the farmed salmon samples in the present study. So-called runt fish from Norwegian salmon farms have been found to carry *A. simplex* larvae, which they might have contracted from intermediate hosts that happened to be passing by (Mo et al. 2014). For sushi, however, only healthy fully grown fish are used, and these have repeatedly been shown to be free of nematodes (Bristow and

Berland 1991; Lunestad 2003; Mo et al. 2014). Thus, it could be assumed that the anisakid proteins might originate from the fish feed and have migrated into edible salmon parts in a similar way as assumed in a chicken study (Armentia et al. 2006). Since several *A. simplex* proteins are relatively resistant to digestion and heat treatment (Moneo et al. 2005; Caballero and Moneo 2004), fragments of sufficient length might be present in the fish feed and able to progress into the fish. The analysis of commercial fish feed used in salmon farms with the polyclonal *A. simplex* ELISA has confirmed anisakid proteins (unpublished data), but the migration hypothesis could only be clarified by a controlled feeding experiment.

The third survey of the present study analysing belly flaps and loins from farmed Atlantic salmon delivered similar results as the sushi survey. Whereas traces of *A. simplex* proteins were not found in the loins, several belly flaps contained very low amounts. This uneven distribution could be interpreted as an argument against migration with blood as the carrier because in that case, a more equal distribution would be expected. The slight contamination of the belly flaps could also have occurred by carry-over from the viscera, when the fish were opened after slaughter.

The use of confirmative analytical methods is advantageous, especially when low-level concentrations of potentially hazardous contaminants are measured. Immunostaining and LCMSMS have both the advantage to detect directly the target protein, comparable to ELISA methods and in contrast to DNA-based PCR. The antibodies applied in the immunostaining experiments in the present study were the same polyclonal rabbit anti-*A. simplex* antibodies that were used in the ELISA. Since they had been raised against the Tris-glycine-extracted semi-purified *A. simplex* standard protein fraction, which contained one dominant protein band with a molecular weight of about 40 kDa, the major IgG-binding signals in the immunostaining of *A. simplex*-containing samples were of the same size. Signals below 10 kDa were not detected because small proteins had been removed from the At-protein by dialysis. Immunostaining with patient serum was confirmative detecting *A. simplex* proteins in the standard protein preparations, the control samples and one salmon belly flap sample, whereas only background interference was found in the loin of the same salmon. The results agreed well with the outcome of the ELISA, and the observed different binding intensities appeared to correlate with the anisakid protein concentration ratios measured by ELISA.

LCMSMS was used as the second confirmative method. The specific detection of *A. simplex* protein in fish was based >on the measurement of two typical marker peptides from anisakid haemoglobin, which had been characterised in a previous project (Fæste et al. 2014). The marker peptides were unique for haemoglobin from *Anisakis* spp. and thus allowed the differentiation from other nematodes and from fish. Sample extracts were filtered, digested directly without a

time-consuming gel electrophoresis step and separated by reversed-phase liquid chromatography with a relatively flat gradient. Fish matrix with and without content of *A. simplex* protein was clearly differentiable through detection of the haemoglobin marker peptides, even against the background of a high protein load from the matrix. The signal intensity of the LFAEYLDQK peptide was two to three times as strong as that of the HSWTTIGEEFGHEADK peptide, which could be observed in samples spiked with standard protein and in the cod liver control. Thus, in samples with low *A. simplex* protein content such as the salmon muscle control, only the stronger peptide was unambiguously identified.

The results obtained by LCMSMS confirmed the findings from the ELISA measurements. Trace amounts of *A. simplex* protein were detected in some samples from the sushi survey and the survey on slaughter-ready salmon. It could be of interest to find the cause for these contaminations, especially in view of the EFSA evaluation (EFSA-BIOHAZ 2010; Wootten et al. 2010). The detected anisakid protein levels were, however, low and probably do not represent a health risk for consumers with allergy to *A. simplex*.

Conflict of Interest Christiane Kruse Fæste declares that she has no conflict of interest. Christin Plassen declares that she has no conflict of interest. Kjersti Eriksen Løvberg declares that she has no conflict of interest. Anders Moen declares that he has no conflict of interest. Eliann Egaas declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

Ethics Requirements The polyclonal antibodies were produced in a previous project (for details, see Werner et al. 2011) in rabbits in accordance with the Norwegian guidelines for the care and use of laboratory animals.

Human serum was obtained and used by informed consent in a previous study (for details, see Fæste et al. 2014) in accordance with the ethical standards of the Hospital Universitario de La Princesa in Madrid, Spain.

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