

ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

Variability of allergens in commercial fish extracts for skin prick testing

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Abstract

Background: Commercial allergen extracts for allergy skin prick testing (SPT) are widely used for diagnosing fish allergy. However, there is currently no regulatory requirement for standardization of protein and allergen content, potentially impacting the diagnostic reliability of SPTs. We therefore sought to analyse commercial fish extracts for the presence and concentration of fish proteins and in vitro IgE reactivity using serum from fish-allergic patients.

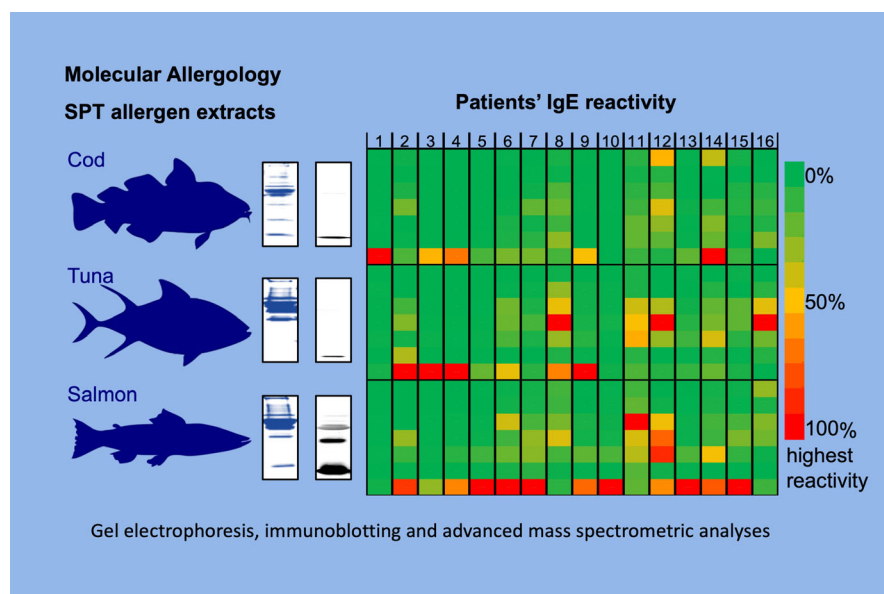
Methods: Twenty-six commercial fish extracts from five different manufacturers were examined. The protein concentrations were determined, protein compositions analysed by mass spectrometry, followed by SDS-PAGE and subsequent immunoblotting with antibodies detecting 4 fish allergens (parvalbumin, tropomyosin, aldolase and collagen). IgE-reactive proteins were identified using serum from 16 children with confirmed IgE-mediated fish allergy, with focus on cod, tuna and salmon extracts.

Results: The total protein, allergen concentration and IgE reactivity of the commercial extracts varied over 10-fold between different manufacturers and fish species. The major fish allergen parvalbumin was not detected by immunoblotting in 6/26 extracts. In 7/12 extracts, five known fish allergens were detected by mass spectrometry. For cod and tuna, almost 70% of patients demonstrated the strongest IgE reactivity to collagen, tropomyosin, aldolase A or β -enolase but not parvalbumin.

Conclusions: Commercial fish extracts often contain insufficient amounts of important allergens including parvalbumin and collagen, resulting in low IgE reactivity. A comprehensive proteomic approach for the evaluation of SPT extracts for their utility in allergy diagnostics is presented. There is an urgent need for standardized allergen extracts, which will improve the diagnosis and management of fish allergy.

KEYWORDS

allergy diagnostics, fish allergy, IgE reactivity, parvalbumin, skin prick test



GRAPHICAL ABSTRACT

Commercial skin prick test (SPT) extracts often contain insufficient amounts of important fish allergens, resulting in low IgE reactivity. Collagen, tropomyosin and aldolase A are of under-recognized importance for the diagnosis of fish allergy. Understanding the molecular allergology of SPT allergen extracts is essential for best diagnostics.

1 | BACKGROUND

IgE-mediated fish allergy is typically a lifelong disease,¹ with sensitization rates of up to 3% in the general population² and up to 8% among fish processing workers.³ The diagnosis of fish allergy is often confirmed by in vivo allergy skin prick tests (SPT),^{4,5} a relatively non-invasive point of care testing, providing immediate results. Commercial fish extracts for SPT for approximately 30 (mostly European species) are available, while over 1000 different fish species are consumed worldwide. Allergic sensitization to fish seems to be region- and species-specific, leading to study bias when utilizing SPT extracts from European species in other regions⁶. Furthermore, commercially available extracts do not appear to be standardized regarding protein and allergen content, thereby impacting the reproducibility and diagnostic value of SPTs.

Previous studies have demonstrated significant variation in allergen content of commercial SPT extracts for birch⁷ and grass pollen,⁸ mould^{9,10} and occupational allergens.¹¹⁻¹³ A large heterogeneity in

in vivo and in vitro reactivity and allergen content among five commercial crustacean extracts was recently demonstrated.¹⁴ Some commercial fish extracts have previously been analysed for their protein concentration and parvalbumin (PV) content.¹⁵ The current study aimed to examine a more complete fish allergen repertoire including PV,¹⁶ tropomyosin,^{17,18} aldolase A,¹⁹ β -enolase,¹⁹ vitellogenin²⁰ and collagen²¹ in a large range of commercially available fish extracts for SPT as well as the molecular IgE reactivity using serum from fish-allergic children in a Southern hemisphere setting.

2 | METHODS

2.1 | Skin prick test and in-house extracts

Twenty-six commercial extracts for SPT from five different manufacturers, covering 11 fish families/groups including mixes of species, were obtained (details are listed in Table S1). Extracts from different manufacturers for the same species were assigned a number

TABLE 1 Clinical characteristics and relevant sIgE profiles of 16 fish-allergic patients recruited for the study

Patients			ImmunoCAP results						Implicated fish*	Symptoms
#	Sex	Age (y)	Cod		Tuna		Salmon			
			(kU/L)	Class	(kU/L)	Class	(kU/L)	Class		
1	M	11	88.50	5	4.83	3	25.30	4	Tuna and white fish	AE, GIS, OAS, U
2	F	1	5.47	3	5.14	3	5.58	3	Asian sea bass	AE, RD, U
3	M	11	92.10	5	39.50	4	66.10	5	Salmon	AE
4	F	2	90.40	5	31.00	4	73.40	5	Cod fish	AE, U
5	M	8	75.00	5	21.00	4	70.50	5	White fish	GIS, RD, U
6	F	7	2.87	2	1.19	2	3.08	2	White fish	U
7	M	4	25.50	4	17.30	3	21.00	4	White fish	U, Abdominal pain
8	F	4	1.54	2	0.71	2	0.47	1	Basa fish	AE, E, U
9	F	12	55.00	5	14.90	3	38.90	4	Flathead	AE
10	M	12	7.01	3	3.90	3	31.80	4	Salmon, tuna	E, U
11	M	14	0.10	1	0.28	1	0.21	1	Salmon	AE
12	F	2	0.14	1	0.78	2	0.60	1	Salmon	AE, U
13	M	8	11.50	3	4.67	3	9.80	3	Salmon	OAS
14	M	14	1.42	2	0.71	2	1.60	2	Salmon	OAS, U
15	M	18	3.36	2	1.06	2	3.21	2	Tuna	RD, U
16	M	1	<0.01	0	<0.01	0	<0.01	0	Tinned salmon	AE, U

Note: AE, angioedema (lip swelling); E, eczema; GIS, gastrointestinal syndrome (vomiting); OAS, oral allergy syndrome (itchy mouth or swelling throat); RD, respiratory distress; U, urticaria (erythema, hives, rash).

*Fish species which was associated with the clinical reaction upon ingestion.

according to Table S1. In-house protein extracts and purified PVs were generated as controls for this study, using frozen muscle tissue from Atlantic cod (*Gadus morhua*), yellowfin tuna (*Thunnus albacares*) and Atlantic salmon (*Salmo salar*). Tissue was homogenized with a rotor-stator homogenizer (5 minutes at 13 000 rpm on ice) in phosphate-buffered saline (PBS, 10 mmol/L phosphate; pH 7.2; 2 mL/g tissue). After gentle agitation overnight at 4°C, subsequent centrifugation (20 000× g) and filtration, extracts were stored at −20°C until further use, referred as raw protein extracts. For the heated extracts, tissue was heated in PBS (95–100°C) for 20 minutes before homogenization. PV from these three species was purified from heated extracts by ammonium sulphate precipitation with subsequent dialyses against 100 mmol/L ammonium bicarbonate as previously described.⁶

2.2 | Patients

Sera were obtained from 16 Australian children (1–18 years) with allergist confirmed clinical history of IgE-mediated symptoms after ingesting fish (Table 1). The median sIgE levels, determined by ImmunoCAP (Thermo Fisher Scientific), were 7.0 kU/L for cod (F3), 4.7 for tuna (F40) and 9.8 for salmon (F41), with a interquartile range of 1.6–75.0, 0.8–17.3 and 1.6–38.9, respectively (Stata v14). The sIgE level for patient 16 was <0.01 kU/L for all three species and has been excluded from the statistical analyses. The fish implicated in the allergic reaction was in eight

cases white fish/cod, in seven salmon and in three tuna. A pool of serum from three nonatopic fish-tolerant donors was used as a negative control. Parents of all participants gave written informed consent, and patient anonymity was preserved. Ethical approval was obtained from the Sydney Children's Hospitals Network (LNR-14/SCHN/185).

2.3 | Protein concentration and SDS gel-electrophoresis

The protein concentration for all fish extracts was estimated using the Pierce™ 660 nm Protein Assay (Thermo Scientific) with bovine serum albumin as standard. In-house extracts were diluted to 1 mg/mL, while commercial extracts were analysed undiluted. Proteins (2.5 µL) were separated according to their molecular weights using a Criterion™ SDS-PAGE system (Bio-Rad) as described by Laemmli²². The acrylamide content varied between 10% and 16% depending on the molecular weight of the proteins of interest. Proteins were visualized by Coomassie Brilliant Blue R-250 (CBB) staining and identified by subsequent immunoblotting with allergen-specific antibodies or patient serum IgE.

2.4 | Immunoblotting

The separated proteins were transferred onto nitrocellulose membrane. After drying, rehydrating in PBS and blocking for 1 hour with

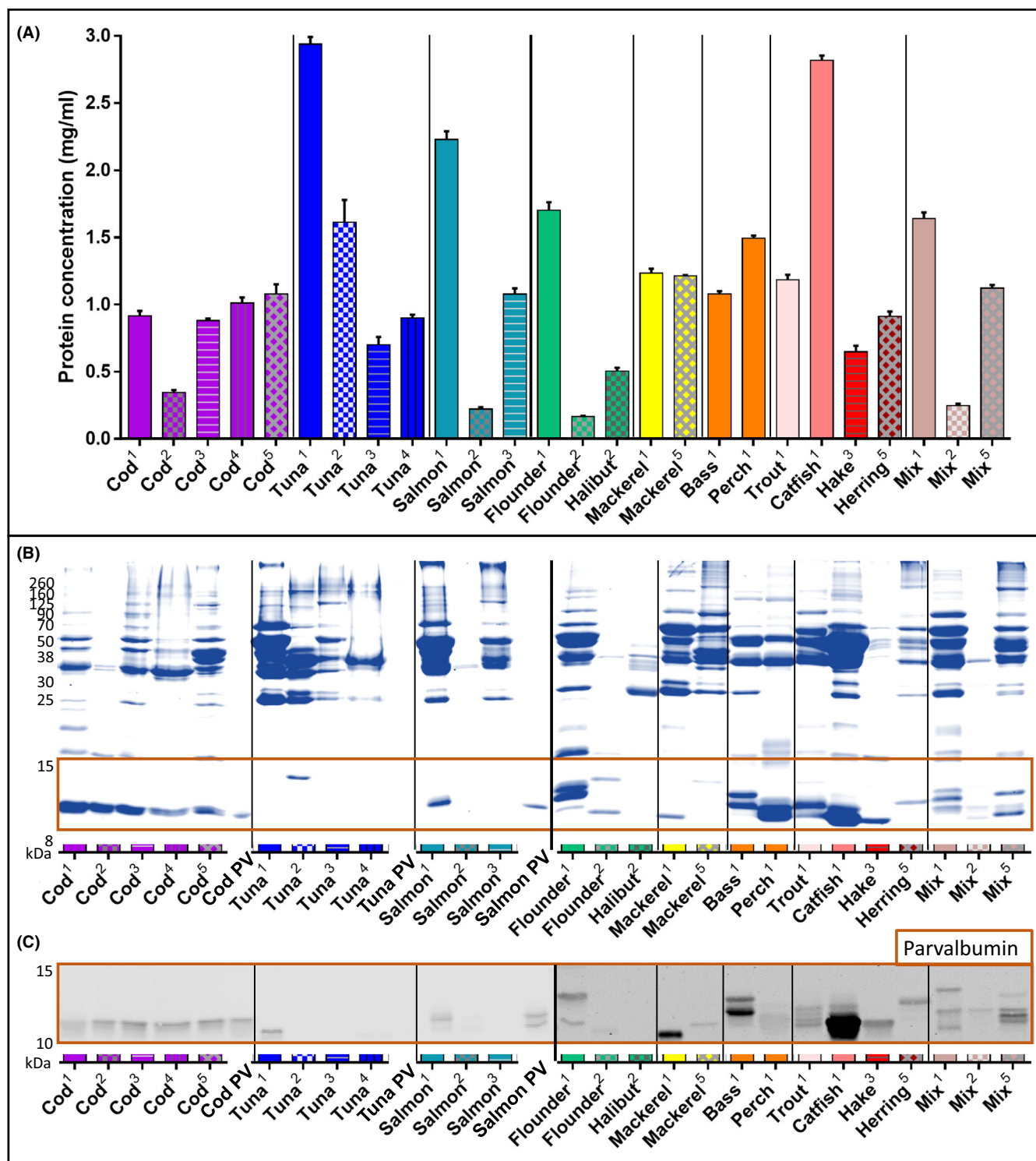


FIGURE 1 Protein concentration, SDS-PAGE profile and PV-specific antibody reactivity of 26 different fish SPT extracts from five different manufacturers. Protein concentrations were determined and the mean values from three replicates with the corresponding standard deviation are shown (A). The extracts were further analysed by SDS-PAGE (B) and immunoblot with anti-PV antibody (C). Purified PV from Atlantic cod, yellowfin tuna and Atlantic salmon was used as positive control for the cod, tuna and salmon SPT extracts. The numbers in superscript on the common species names represent the different manufacturers listed in Table S1

1xCasein (Thermo Scientific) in PBS at room temperature, the membranes were incubated with four antibodies detecting fish allergens for 1 hour at room temperature or overnight with patient serum at

4°C (1:15 with 0.2x Casein in PBS-T (PBS with 0.5% Tween-20®)). The allergens, PV and tropomyosin, were detected using in-house generated polyclonal antibodies raised in rabbits against PV from

Asian sea bass and Atlantic salmon²³ and tropomyosin from prawn.²⁴ Aldolase A and collagen were identified utilizing commercial polyclonal antibodies (goat anti-rabbit aldolase 100-1141 by Rockland Immunochemicals¹⁹ and rabbit anti-tuna collagen ab23730 by Abcam, respectively). No antibody against β -enolase or vitellogenin was available.

After washing with PBS-T, the patient blots were incubated with monoclonal mouse anti-human IgE antibody (sc-53346 by Santa Cruz, 1:1000 with 0.2x Casein in PBS-T for 1 hour at room

temperature) and subsequently washed. All blots were developed with a corresponding IR-labelled antibody (DyLight anti-mouse/rabbit 4xPEG by Thermo Scientific or IR-Dye anti-goat by LI-COR[®]). The Odyssey[®] Imaging Systems (LI-COR[®]) was used for imaging membranes and CBB-stained gels. Densitometric analyses were conducted utilizing Image Studio Version 5.2 (LI-COR[®]) allowing sensitive, semi-quantitative evaluation of signals. The colours presented in heatmaps arise from individual protein bands or the sum of all signal intensities for each separate sample. The

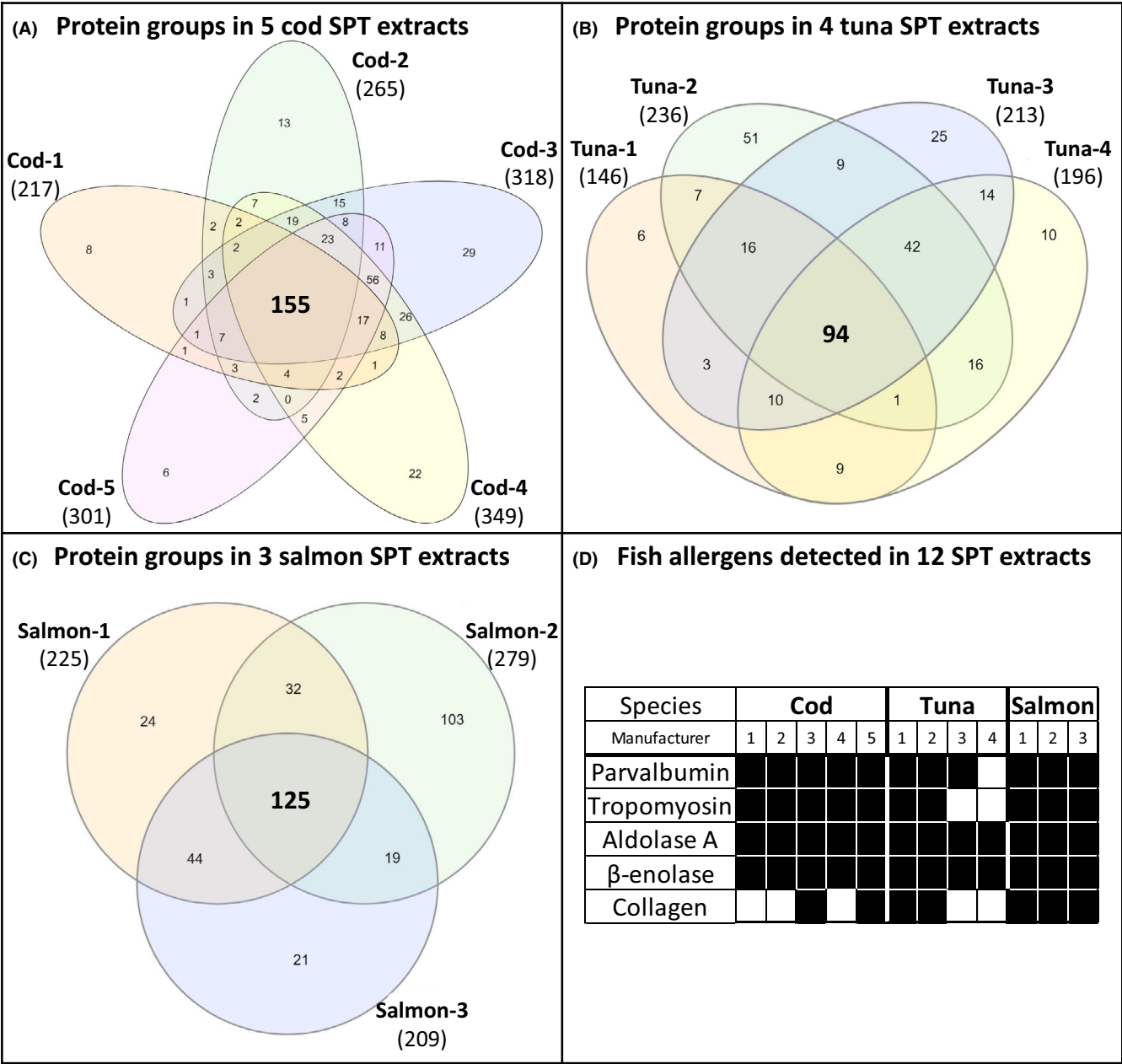


FIGURE 2 Protein groups and fish allergens detected in cod, tuna and salmon SPT extracts. Mass spectrometric analyses were performed after in solution tryptic digestion. Proteins were identified by mascot and groups were defined using Proteome Discoverer (Thermo Scientific). For each SPT extract, the total number of identified protein groups is given in brackets. The Venn diagram (InteractiVenn) shows the number of shared protein groups between the five cod (A), four tuna (B) and three salmon (C) SPT extracts. Detected fish allergens are indicated by solid field (D). The numbers after species name in A-C represent the different manufacturers listed in Table S1

densitometric analyses utilizing this system are independent of background, contrast or other settings used for best visualization of the immunoblot.

2.5 | Mass spectrometry analysis

All commercial cod, tuna and salmon extracts (diluted to 200 µg/mL), as well as all prominent IgE-reactive bands of the respective extracts, were subjected to mass spectrometric analysis after tryptic digestion (see²⁵ for details). In brief, the extracted peptides were analysed using an LTQ Orbitrap Elite (Thermo Scientific) coupled to an Ultimate 3000 RSLC nanosystem (Dionex). The nanoLC system was

equipped with an Acclaim Pepmap nano-trap column and an Acclaim Pepmap analytical column. The peptide mix was loaded onto the trap column before the enrichment column was switched in-line with the analytical column. The LTQ Orbitrap Elite mass spectrometer was operated in the data-dependent mode, spectra acquired first in positive mode at 240 k resolution followed by collision-induced dissociation (CID) fragmentation. Twenty of the most intense peptide ions with charge states ≥ 2 were isolated and fragmented using normalized collision energy of 35 and activation Q of 0.25 (CID). All results were analysed with Mascot search engine and cross-referenced against NCBI protein database for all bony fish (February 2018). Variable modifications of carbamidomethyl-C and N-terminus,

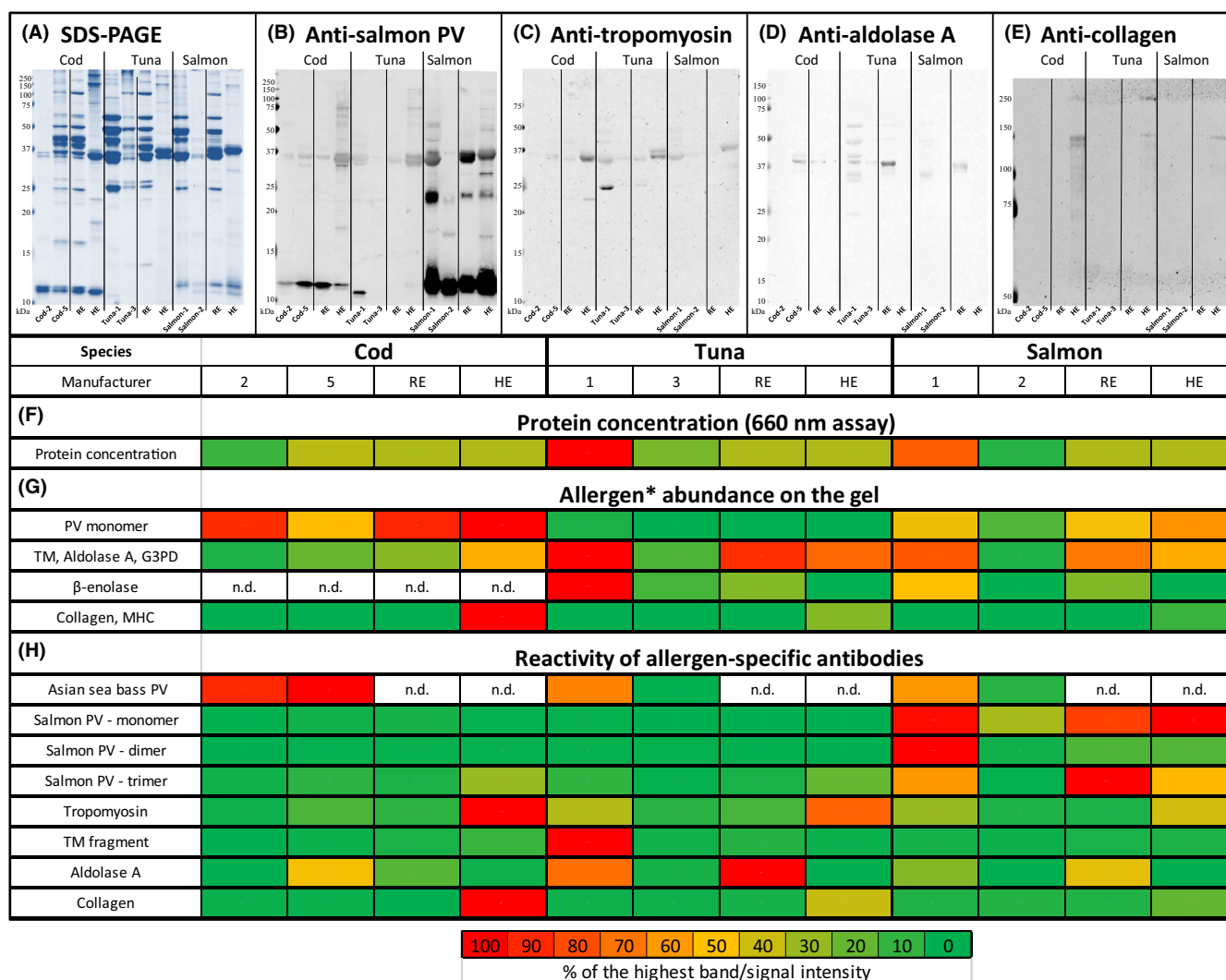


FIGURE 3 Protein profile and allergen-specific reactivity of selected cod, tuna and salmon extracts. The two SPT extracts with the lowest protein concentration (cod-2, tuna-3, salmon-2) and the highest (cod-5, tuna-1, salmon-1) were compared with in-house prepared raw (RE) and heated (HE) protein extracts from the corresponding species. Proteins were separated by SDS-PAGE (A) and allergens identified using specific antibodies for PV (B), tropomyosin (C), aldolase A (D) and collagen (E). Heatmaps were generated for the protein concentration of each SPT extracts (F), the allergen abundance on the gel (G) and reactivity of the allergen-specific antibodies (H). *The identity of proteins extracted from bands was confirmed by mass spectrometric analyses. The numbers after the species name represent the different manufacturers listed in Table S1. Note: The colouring of the boxes represents intensity, with red being the highest, orange 50% and green 0% of the highest band/signal intensity. The colour scheme is valid for each row only and not comparable with shading in other figures of the manuscript. G3PD, glyceraldehyde-3-phosphate dehydrogenase; MHC, myosin heavy chain; n.d., not determined; PV, parvalbumin; TM, tropomyosin

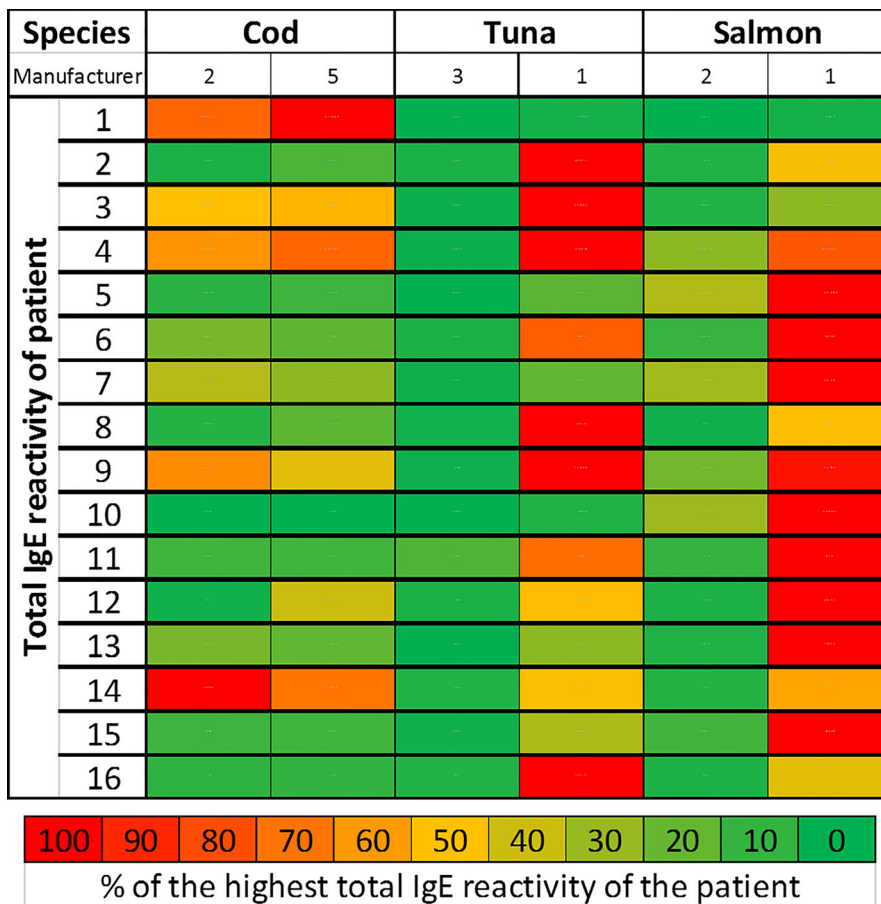


FIGURE 4 Patients' total IgE reactivity to proteins of selected cod, tuna and salmon SPT extracts. The IgE reactivity of 16 patients to the two SPT extracts with the lowest protein concentration (cod-2, tuna-3, salmon-2) and the highest (cod-5, tuna-1, salmon-1) was compared. The numbers after the species name represent the different manufacturers listed in Table S1. The patient number corresponds to Table 1. Note: The colouring of the boxes represents intensity, with red being the highest, orange 50% and green 0% of the highest total IgE reactivity of the patient. The colour scheme is valid for each row only and not comparable with shading in other figures of the manuscript

deamidation N, deamidation Q and oxidation of M were selected. The search results were further processed using Proteome Discoverer (Thermo Scientific) for false discovery rate control (1% at peptide spectral match, peptide and protein level) and protein grouping. A protein group was considered to be identified with at least one significant peptide. Perseus was used for generation of the Venn diagram.²⁶

3 | RESULTS

3.1 | Protein contents of extracts

The total protein content varied between the 26 commercial fish extracts more than 17-fold, ranging from 0.17 to 2.94 mg/mL (Figure 1A).

3.2 | SDS-PAGE and parvalbumin detection

The protein composition showed large differences in number and abundance of proteins of different molecular weight in all analysed commercial fish extracts (Figure 1B). In 29% of the commercial fish extracts, no protein bands were visible in the 10–15 kDa range, the molecular weight of the major allergen, PV.

Utilizing a PV-specific antibody, PV was detected with similar intensities in all commercial cod extracts (Figure 1C). Among the commercial tuna extracts, PV was only detected in tuna-1. For salmon-1, the signal

for PV was seven times higher compared to salmon-2, while no PV was detected in salmon-3. In all other commercial extracts, except flounder-2 and halibut-2, PV was identified. The highest PV-specific antibody reactivity was observed for the catfish-1 extract, while the reactivity was very weak for extracts mackerel-5, perch-1 and mix-2.

3.3 | Protein composition evaluated by mass spectrometric analyses

All analysed commercial cod, tuna and salmon extracts revealed large differences in the presence of fish proteins and allergens. Across all 12 extracts, an average of 251 protein groups were identified, ranging from 217 to 381 for cod, 146 to 236 for tuna and 209 to 279 for salmon (Figure 2A–C). In all commercial cod extracts, 155 common protein groups were identified, while only 94 and 125 were found in all tuna and salmon extracts, respectively. The cod-3, tuna-2 and salmon-2 extracts differed the most from other extracts of the same species with 29, 51 and 103 unique proteins identified, respectively.

Four fish proteins registered with the WHO/IUIS, as well as allergenic collagen, were investigated for all five cod, four tuna and three salmon commercial extracts (Figure 2D). PV, tropomyosin, aldolase A, β -enolase and collagen were detected in 2/5 cod (cod-3 and cod-5), 2/4 tuna (tuna-1 and tuna-2) and all 3 salmon extracts. In the other three cod extracts, no collagen was detected but the other allergens. No detectable traces of tropomyosin or collagen were

found in the other two tuna extracts, as well as no PV was detected in tuna-4. The fish allergen vitellogenin was not detected in any of the commercial fish extracts.

3.4 | Allergen profiles of commercial cod, tuna and salmon extracts by SDS-PAGE and mass spectrometric analyses

The subsequent analysis focused on the two commercial extracts with the lowest and highest protein concentrations each for cod, tuna and salmon—cod-2 (0.3 mg/mL) and cod-5 (1.1 mg/mL), tuna-3 (0.7 mg/mL) and tuna-1 (2.9 mg/mL), and salmon-2 (0.2 mg/mL) and salmon-1 (2.2 mg/mL) (Figure 3F). The selected extracts were compared to the respective in-house generated raw and heated extract by SDS-PAGE and subsequent immunoblotting utilizing allergen-specific antibodies (Figure 3).

The protein profiles (Figure 3A) of cod-5, tuna-1, tuna-3 and salmon-1 were similar to the corresponding raw extract, while cod-2 and salmon-2 showed fewer protein bands than the corresponding heated extract. Salmon raw and heated extract, as well as salmon-2, contained

two prominent bands in the molecular weight region of PV monomers (10–15 kDa), but only one was observed in salmon-1.

Bands identified as allergens by mass spectrometric analyses were quantified by densitometric analyses (Figure 3G). The abundance of the PV monomer varied maximal twofold between all cod extracts, salmon-1, and salmon raw and heated extract. In salmon-1, the PV monomer was three times more abundant as compared to salmon-2, while among all tuna extracts only in tuna-1 a PV monomer could be identified. The fish allergens tropomyosin and aldolase A have a similar molecular weight as many other allergenic proteins such as glyceraldehyde-3-phosphate dehydrogenase, making a clear distinction not possible. Notable differences between the extracts were found in the abundance of proteins in this molecular weight range (35–40 kDa).

In cod-5, tuna-1 and salmon-1, a 4, 6 and 13 times higher abundance was found as compared to cod-2, tuna-3 and salmon-2, respectively. The heat-sensitive allergen β -enolase could not be identified in any of the commercial cod extracts or heated extract of any of the three species. The highest abundance of β -enolase was detected in tuna-1, being seven and four times more abundant than in tuna-3 and raw tuna extract, respectively. In salmon-1, β -enolase was twice as

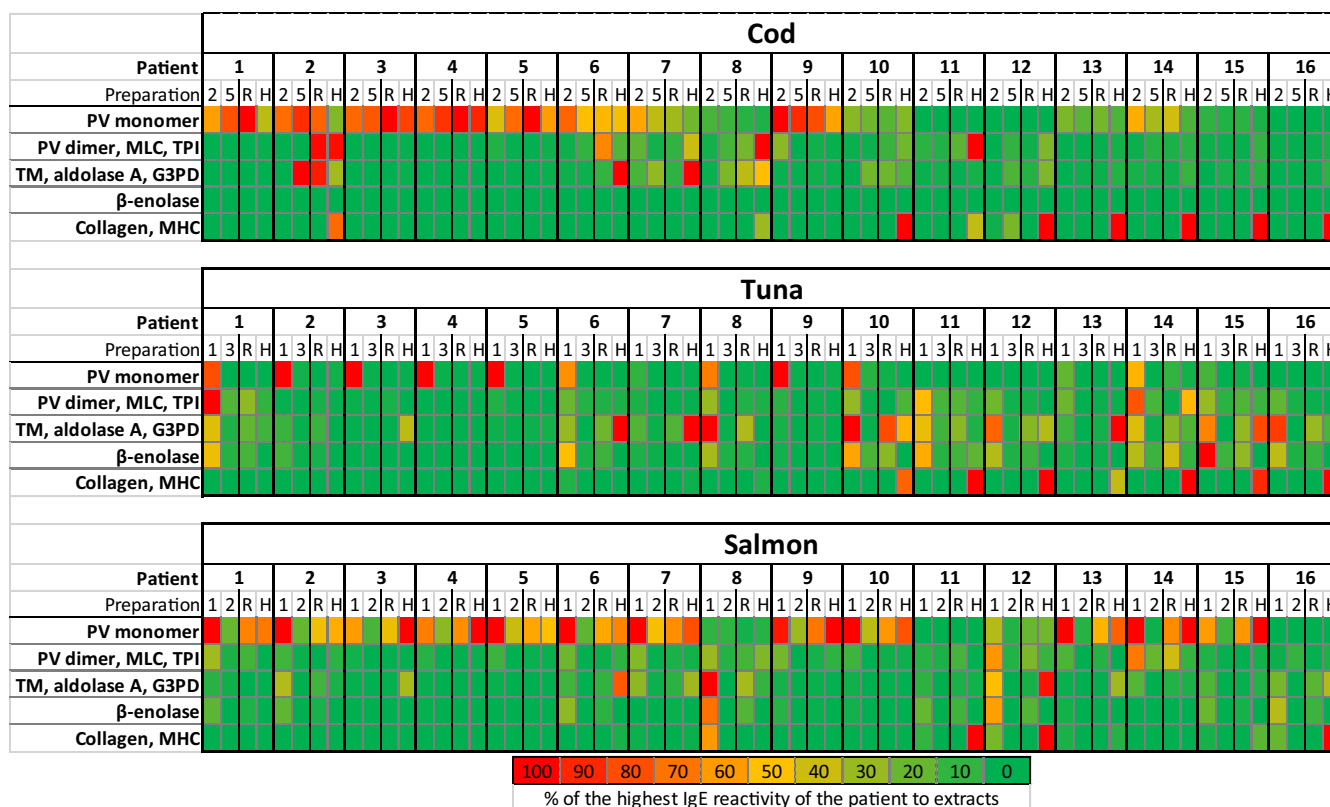


FIGURE 5 Allergogram analysis of patients' IgE reactivity to allergens in different cod, tuna and salmon extracts. The IgE reactivity of 16 patients to the two SPT extracts with the lowest protein concentration (cod-2, tuna-3, salmon-2) and the highest (cod-5, tuna-1, salmon-1) was compared with in-house prepared raw (R) and heated (H) protein extracts from the corresponding species. The identity of proteins extracted from IgE-reactive bands (Figure S1) was confirmed by mass spectrometric analyses. The numbers after the species name represent the different manufacturers listed in Table S1. The patient number corresponds to Table 1. Note: The colouring of the boxes represents intensity, with red being the highest, orange 50% and green 0% of the highest IgE reactivity of the patient to extracts. The colour scheme is valid for each patient and species only and not comparable with shading in other figures of the manuscript. PV, parvalbumin; MLC, myosin light chain; TPI, triosephosphate isomerase; TM, tropomyosin; G3PD, glyceraldehyde-3-phosphate dehydrogenase; MHC, myosin heavy chain

abundant as compared to the raw salmon extract. No β -enolase was identified in salmon-2. Collagen could only be identified in the heated extract from all three species; however, myosin heavy chain was also identified in addition to collagen in the high molecular weight region. In cod heated extract, collagen was four times and nine times more abundant compared to tuna and salmon heated extract, respectively.

3.5 | Allergen profiles of commercial cod, tuna and salmon extracts using specific antibodies

The presence and relative abundance of the four fish allergens PV, tropomyosin, aldolase A and collagen was further evaluated by immunoblotting (Figure 3B-E) with subsequent comparative densitometric analyses (Figure 3H). Using the anti-salmon PV antibody, PV monomers (11–13 kDa) were detected in all cod and salmon extracts, but in only 1 tuna extract (tuna-1). The antibody reactivity was five and three times stronger for cod-5 and salmon-1 as compared to cod-2 and salmon-2, respectively. A PV dimer (22–26 kDa) was identified with a strong signal in salmon-1, while the corresponding signal was six times weaker in raw and heated extract and 44 times weaker for salmon-3. In cod and tuna extracts, no PV dimers were detected. In the molecular weight range of PV trimers (33–39 kDa), faint signals were observed in all extracts; however, their intensity varied greatly.

The signal for the tropomyosin monomer (35–40 kDa) was 11, six and seven times stronger for the heated compared to raw extract from cod, tuna and salmon, respectively, and stronger than the signal for any of the corresponding commercial extracts. Cod-5, tuna-1 and salmon-1 showed a 4–6 times stronger signal intensity as compared to cod-2, tuna-3 and salmon-2. Faint signals below 35 kDa were observed in all extracts when using the anti-tropomyosin antibody. However, only for tuna-1 a very strong signal was observed at 25 kDa, indicating a fragment of this allergen as recently documented.²⁷

Aldolase A was detected in all raw but none of the heated extracts, and neither in cod-2, tuna-3 nor salmon-2. The signal for cod-3 was three times stronger as for the respective raw extract, while the signal for tuna-1 and salmon-1 was about twofold weaker as for the respective raw extract.

Collagen was detected in all heated extracts, but none of the raw extracts or commercial extracts. The signal for cod heated extract was 3 and 5 times stronger than for the tuna and salmon heated extract, respectively.

3.6 | Patient IgE reactivity to specific allergens

The 12 extracts were further analysed for IgE reactivity using serum from 16 patients with confirmed IgE-mediated fish allergy (Figure S1) and further evaluated for the total IgE reactivity (Figure 4) and reactivity to specific allergens (Figure 5). Subsequent densitometric analyses demonstrated large differences in total IgE reactivity among tuna and salmon, but less between commercial cod extracts (Figure 4). A stronger IgE reactivity was observed, using densitometric analysis, for cod-5 compared to cod-2 for 11/16 patients, while the IgE reactivity differed in average 1.4 times. Salmon-2 showed a weak

to moderate IgE reactivity with 8/16 patients, while tuna-3 showed a relatively weak IgE reactivity only with three patients (3, 4 and 11). In average, tuna-1 and salmon-1 showed a 22 and eight times stronger IgE reactivity, respectively, with an up to 65-fold difference between the two commercial salmon extracts. For 11 and three patients, the IgE reactivity was more than 10-fold different between the two commercial tuna and salmon extracts, respectively.

Among the six analysed commercial SPT extracts, cod demonstrated the lowest IgE reactivity, while 2/16 patients showed the strongest IgE reactivity to cod, whereas six and eight patients showed the strongest reactivity to tuna or salmon, respectively.

The most prominent IgE-reactive bands were further analysed by mass spectrometry and the signal intensities compared between all patients (Figure 5). IgE from all, but two patients (11 and 16), detected a PV monomer (11–13 kDa) in at least one of the 12 extracts. However, the PV monomer was the most and exclusive IgE-reactive band for only 5/16 patients for cod and tuna, but 12/16 patients for salmon. Most patients with no or little IgE reactivity to the PV monomer as well as a low sIgE level for the respective fish species identified collagen (100–250 kDa) as the most prominent IgE-reactive protein. IgE-reactive collagen was detected in all heated, but none of the raw extracts. Collagen was the most IgE-reactive protein in cod, tuna and salmon extracts for 6/16, 4/16 and 3/16 patients, respectively.

Tropomyosin and aldolase A were identified in the 35–40 kDa region, while PV and triosephosphate isomerase were in the 20–30 kDa region. Additional proteins detected in these regions include glyceraldehyde-3-phosphate dehydrogenase and myosin light chain. Patients with weak or no IgE reactivity to the PV monomer and collagen showed the strongest binding to one of these two regions, applicable for 5/16, 6/16 and 1/16 patients for cod, tuna and salmon, respectively. Half of the patients showed weak to moderate IgE reactivity to β -enolase (50 kDa) from tuna (10/16) and salmon (8/16) extracts. No β -enolase band was detected in any of the cod extracts.

4 | DISCUSSION

This study demonstrates a considerably high heterogeneity in protein and allergen content in SPT extracts used in routine diagnosis of fish allergy. Twenty-six commercial fish extracts were evaluated through a comprehensive biochemical and immunological analysis of the complete protein and allergen content, using immunoblotting and advanced mass spectrometric analysis. We found the total protein concentration between 26 fish extracts varying up to 17-fold, from the lowest in flounder-2 (0.17 mg/mL) to the highest in tuna-1 (2.94 mg/mL). In contrast, comparable small variations in total protein content (up to 7-fold) were previously reported for 14 commercial fish SPT extracts.¹⁵ Subsequently using a PV-specific antibody, PV was detected in all 4 analysed salmon extracts and none of three tuna extracts.¹⁵ In our study, using an in-house antibody, PV was detected in most of the 26 extracts except in 3 of 4 tuna, 1 of 3 salmon and 1 of 3 flounder/halibut extracts.

The difference in PV content and antibody reactivity among tuna extracts is likely explained by the different species utilized, as none of the manufacturers provided the details of the exact species. A comparative analysis of in-house raw and heated extracts from yellowfin tuna (*Thunnus albacares*) and albacore tuna (*T. alalunga*) demonstrated over 100 times stronger antibody reactivity for the latter (unpublished data by T. Ruethers). We therefore conclude that the analysed extracts are likely derived from very different tunas, highlighting the importance of choosing the appropriate region-specific species when preparing SPT extracts or performing prick-to-prick tests using fresh fish.

Subsequent analysis of all soluble proteins by mass spectrometry demonstrated that protein diversity varied greatly between the extracts. PV, tropomyosin and/or collagen were not detected in 3/5 cod and 2/4 tuna extracts, while aldolase A and β -enolase were detected in all 12 commercial cod, tuna and salmon extracts. For example, 155 protein groups were identified in five cod extracts, while the total number of identified proteins groups varied from 217 to 349, indicating very different methods of generating the commercial extracts between manufacturers.

A recent study by Asero et al¹⁴ demonstrated the unsuitability of three commercial prawn extracts, using SDS-PAGE and antibody-based approaches, due to the absence of important shellfish allergens. In our study, we present for the first time a comprehensive proteomic approach using mass spectrometry for a fast and reliable evaluation of commercial SPT extracts for their utility in allergy diagnostics. We demonstrate that 5/9 commercial cod and tuna extracts, examined by mass spectrometry, lack important fish allergens.

Among the six cod, tuna and salmon extracts analysed in detail, using four antibodies detecting fish allergens, we confirmed the large heterogeneity in allergen content. Heat-labile aldolase A was detected in all in-house raw extracts as well as the three commercial extracts with a high total protein concentration. The same three extracts also showed a comparable high PV and tropomyosin content. In contrast, collagen, poorly extracted without heat treatment, was only detected in in-house heated extracts, but not in the raw or any SPT extracts. Therefore, we can assume that these six extracts were not heat-treated.

The total in vitro IgE reactivity to commercial fish extracts depends on antigen content and differed between patients and fish species. In the Asia-Pacific region, clinicians often consider reactivity to cod as a marker for allergy to any white fish. According to our data, even the cod SPT extract with the highest antigen amount showed less reactivity than salmon and tuna extracts with higher antigen content. This study highlights region- and species-specific IgE reactivity to fish, leading possible to false-negative diagnostics.⁶ Species-specific sensitization was also demonstrated in other studies for salmonids, tilapia, catfish and angler.²⁸⁻³⁰ However, the patient- and fish species-dependant reactivity requires further investigations with a larger patient cohort and region-specific species.

Comparing the IgE reactivity of serum from patients with clinically confirmed fish allergy to cod and tuna, 11/16 patients showed the strongest IgE reactivity to collagen, tropomyosin, aldolase A and

β -enolase and 5/16 to PV. In contrast, for salmon PV was the most IgE-reactive in 12/16 (75%) patients. Several studies have previously indicated that the PV content is species-specific affecting allergenicity; however, no additional fish allergens were investigated.^{15,31-33} While it is generally accepted that PV is the major fish allergen accounting for 70%-95% of allergic reactions,⁴ here we demonstrate the importance of additional fish allergens in different fish species.

Analysing the IgE reactivity for in-house heated extracts, collagen was the second most IgE-reactive fish protein after PV, with up to 9/16 Australian fish-allergic patients showing strong reactivity to this allergen. Initial protein analysis confirmed the differential abundance of collagen, which was four and nine times higher in cod as compared to tuna and salmon, respectively. However, in all analysed commercial extracts collagen was underrepresented, resulting in low IgE reactivity in serum from patients sensitized to the fish allergen collagen. In a recent study, prick-to-prick testing with raw fish resulted in false-negative results due to lack of collagen.³⁴ Several studies from Japan confirmed the importance of collagen as a fish allergen and highlighted the high collagen content in the skin.^{21,35} According to the ImmunoCAP results, one patient in our cohort was not sensitized to fish, but showed strong IgE reactivity to only collagen. The next generation of improved SPT extracts should consider the utilization of fish skin and heat treatment. The possible introduction of purified collagens in in vitro fish allergy diagnostics should also be evaluated in future studies.

In conclusion, many commercial fish extracts contain insufficient amounts of allergens such as PV and collagen, resulting in low IgE reactivity and limited suitability for reliable in vivo allergy testing. We recommend either standardization and regulation of protein concentration and profiles in commercial allergen extracts or the use of improved in vitro diagnostics implementing a large variety of fish species and purified allergens. Further research must be conducted to meet the urgent need of region- and species-specific diagnostics, allowing improved management of this life-threatening disease.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

TR performed the study and wrote the manuscript. AL led the study. TR, AT, SK, SM, DC and AL designed the study. SM and DC recruited

the patients and acquired the commercial fish extracts. TR, RN, TC, MK, SN, SO and NW contributed significantly to the generation of the data. All co-authors were substantially involved in the analyses and interpretation of all data and critically reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1: Details of analysed 26 Skin Prick Test fish extracts from 5 different manufacturers.

		Manufacturer				
		1 - Greer USA	2 - ALK-Abello USA	3 - Immunotek	4 - Alyostal	5 - ALK-Abello Spain
Family/Tribe	Gadidae	Codfish <i>Gadus spp.</i>	Atlantic cod fish <i>Gadus morhua</i>	Cod	Cod	Atlantic cod <i>Gadus morhua</i>
	Thunnini	Tuna <i>Thunnus spp.</i>	Tuna <i>Thunnus spp.</i>	Tuna	Tuna	
	Salmonidae	Salmon <i>Salmo salar</i>	Atlantic salmon <i>Salmo salar</i>	Salmon		
	Pleuronectidae	Flounder <i>Platichthys sp.</i>	Flounder <i>Pleuronectes spp.</i>	Atlantic halibut <i>Hippoglossus hippoglossus</i>		
	Scombrini	Mackerel <i>S. cavalla</i>				Atlantic mackerel <i>Scomber scombrus</i>
	Scomberomorini					
	Salmonidae	Trout <i>O. mykiss</i>				
	Merlucciinae			Hake		
	Clupeidae					Atlantic herring <i>Clupea harengus</i>
	Ictaluridae	Catfish <i>Ictalurus spp.</i>				
	Serranidae	Bass <i>Centropomus striata</i>	Perch <i>Serranus scriba</i>			
		Fish mix Cod, flounder, halibut, mackerel, tuna	Fish, mixed Flounder, cod, halibut			Fish mix II Cod, sole, sea bass, hake

Note: Common and scientific names of fish species are listed according to manufacturer's information where available. Classification of family/tribe is according to the UniProt database (www.uniprot.org).

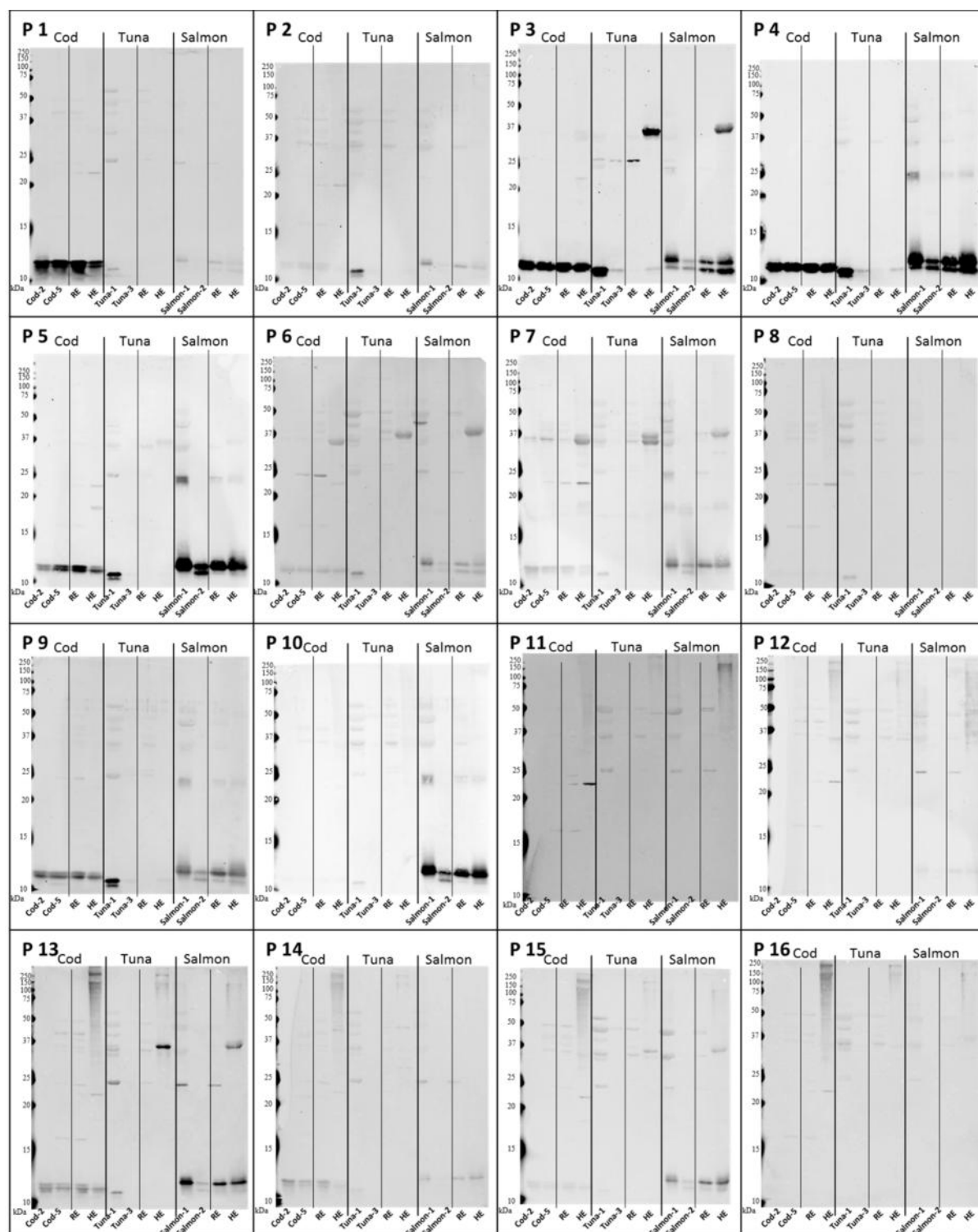


Figure S1: IgE reactivity of 16 fish-allergic patients to cod, tuna, and salmon extracts.

The two SPT extracts with the lowest protein concentration (cod-2, tuna-3, salmon-2) and the highest (cod-5, tuna-1, salmon-1) were compared with in-house prepared raw (RE) and heated (HE) protein extracts from the corresponding species. The numbers in superscript on the common species names represent the different manufacturers listed in Table S1. The patient number corresponds to Table 1. Note: P, Patient.