STSM Report

STSM title:

Detection of Anisakidae allergens

Reference:

ECOST-STSM-FA1408-110416-072893

STSM dates:

from 11-04-2016 to 15-04-2016

Aims and objectives of the STSM:

A lot of *Anisakis simplex* proteins are allergenic for humans. So far, 13 allergens of *A. simplex* were detected. *A. simplex* allergens are considered to be one of the most common hidden allergens in food. Allergic reactions in patients caused by *Anisakis* may cause the development of urticaria, asthma, conjunctivitis, gingivitis, stomatitis and even anaphylactic shock. Probably allergies caused by the *A. simplex* proteins can be induced by allergens, both living and dead *Anisakis* larvae. Furthermore human allergic reactions were noted due to the consumption of highly processed fish products, eg. canned fish. This is due to the resistance many *A. simplex* allergens to high temperature, even the sterilization. Probably the meat from animals fed with fishmeal containing *A. simplex* allergens can cause allergic reactions in people too.

Basic methods for detection of *A. simplex* in fish are based on macroscopic examination, pressing with white or UV-light or on pepsin digestion. However, these methods may be unsuitable for the examination of the processed products, when the cuticula is broken. For the detection of *Anisakis* occurrence PCR methods are also used. But, these methods do not allow the detection of allergens that may be present in the products for human or animal consumption. Therefore, the main aim of STSM was training on Anisakidae allergens detection in fish and fishery products.

Description of work undertaken:

The training contained four subtasks with were the following:

1. To observe one example of inactivation of fishery product. Heating was chosen since it allowed to performing the experiments in a short time. Along with the preparation of samples, the actual set up of the experiment as well as one of the detection methods (Visualization under UV light) was chosen
2. To learn the protocol used for the extraction of allergens in parasitized fish by the method used by QUALIFISH and FIBHULP groups.
3. To get training on the quantification of Ani s 1, Ani s 4 and parasite antigens by dot blot analysis
4. Examination of the allergenic response to fish muscle allergens by Western blot with human sera
5. To learn about the posibilities of a method to discriminate between fresh and frozen/thawed fish which could be used as a tool in order to verify that the legislation about freezing fish destined to be eaten raw or lightly cooked is followed. Low Field NMR relaxometry was used.

I. Materials and methods

1. Inactivation of *Anisakis* in fishery products

Hake (*Merluccius merluccius*) fillets were obtained from a local fishmonger. The first stage of work was fish fillets preparation. Two portions of fillets were prepared in a sandwich form (Figure 1), and were spiked with a known number of L3 *Anisakis simplex*. They were left in 4˚C for 12 hours to allow migration of the larvae into the flesh. After this time, sandwiches were introduced in thermoresistant plastic bags, and heat treated in an oven (200°C, 15 min). The temperature profiles of the fillets during cooking and of the oven were recorded by a portable temperature acquisition sensor. Then, after cooling, samples were observed under UV light to detect the heat treated larvae, and all added parasites were collected.

Figure 1. Preparation of fillets in sandwich form

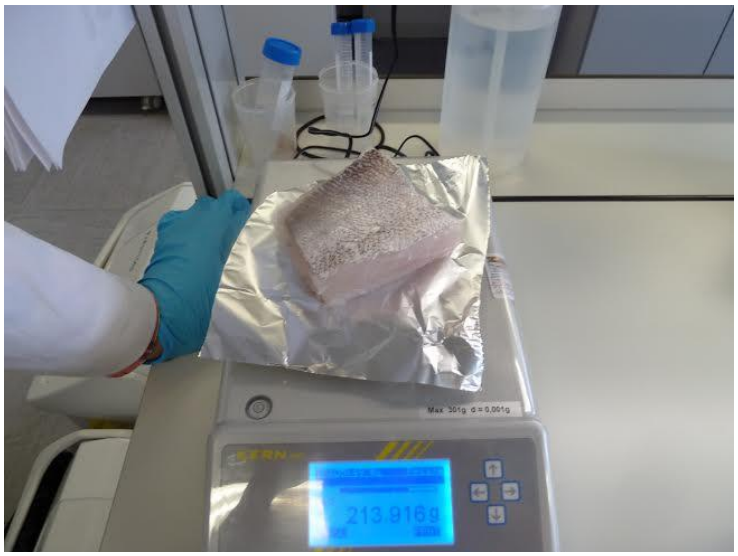


Figure 2. Tubes with samples, during *Anisakis* allergens extraction

2. Extraction of allergens in artificially parasitized fish

10 g of prepared fish muscle with 30 ml Tris-buffered saline was mechanically homogenised using an Ultra-Turax T25 (IKA) (Figure 2). The mixture was sonicated (17W, 30 sec.) in a Microson ultrasonic cell disruptor XL (Misonix) and incubated on the rotator (40 rpm, 15 min.) at room temperature. Next, mixture was centrifuged (5000 g, 30 min.). An aliquot of the supernatant (5 ml) was acidified to pH around 1 with 125 µl 5N HCl solution, and incubated 15 min at room temperature. Mixture was neutralized to pH around 7 with 125 µl 5N NaOH, and centrifuged (5000 g, 15 min.). The pellet was discarded and supernatant was analysed.

3. Dot blot analysis

Samples (Table 1) were analysed by dot-blot method. Three microliter of samples was dotted in duplicate to nitrocellulose membranes and dried in room temperature. Membranes were hydrated with PBS for 10 min. with shaking. Next, membranes were blocked with NP-40 3% in PBS, at room temperature, with shaking for 30 min. After blocking membranes were washed three times with Tris-buffered saline (0.5 M NaCl, 2 mM Tris-HCl, 0.1% Tween 20; pH 7.4) for 5 min. each time. Membranes were incubated with anti-Anis 1 antibody (dilution 1:1000), anti-Anis 4 antibody (1:10000) or anti-crude extract antibody of *A. simplex* (1:10000) diluted in diluent (10mM Tris-HCl, 5% foetal calf serum, 1% Tween 20; pH 7.4). After incubation membranes were washed three times with Tris-buffered saline for 5 min. each time. Next membranes were incubated with anti-rabbit IgG antibody conjugated with alkaline phosphatase (in dilution 1:20000) for 1 h with shaking. Next membranes were washed three times with Tris-buffered saline for 5 min. each time. Washed membranes were incubated with substrate BCIP-NBT: for 15 min. – membrane incubated earlier with anti-crude extract antibody of *A. simplex*; 40 min. - membrane incubated with anti-Anis 4 antibody; 45 min. – with anti-Anis 1 antibody. Reaction was stopped by washing membranes with the PBS. Reference curve was calculated in duplicate dotting 3 µl of Anis-1, Anis-4, *Anisakis* crude extract with known concentration of protein. Membranes were scanned and measured by densitometry using Quantity One software (Biorad).

Table 1. Types of samples examined by dot-blot (AD – dorsal anterior part of fish, AV – ventral anterior part, PD- dorsal posterior part, PV – ventral posterior part)

Washing effect (a – 1 washing, b – 2, c -3)	Natural parasitism
Tc 151 min. (final T^A -10°C):	Small fish:
33.2a	CG TALLA<600 IND1 AD
33.2b	CG TALLA<600 IND1 AV
33.2c	CG TALLA<600 IND1 PD
Tc 155 min. (final T^A -15°C):	CG TALLA<600 IND1 PV
36.1a	Big fish:
36.1b	CG TALLA>2500 IND5 AD
36.1c	CG TALLA>2500 IND5 AV
Tc 145 min. (final T^A -15°C):	CG TALLA>2500 IND5 PD
4.1a	CG TALLA>2500 IND5 PV
4.1b	Canning effect:
	Canned L3
4.1c	

4. Western blot with human sera

Examination of human serum for detection of anti-*Anisakis* IgE antibody was performed. SDS-Page electrophoresis of recombinant Ani s 1, Ani s 4, Ani s 5 and Ani s 11-like protein and *Anisakis* crude extract was performed. For each well of 4-20% Tris-glycine gel: 20 µg of crude extract of *A. simplex*, 5 µg of rAni s 1, 5 µg of rAni s 4, 10 µg of rAni s 5, 5 µg of rAni s 11 were used. SDS-Page electrophoresis was conducted in standard conditions (120V, 1h). After electrophoresis proteins

were transferred to nitrocellulose membrane by Trans-blot Turbo (Biorad) (2.5 A, 7 min.). Next, membrane was blocked with NP-40 3% in PBS at room temperature with shaking for 30 min. After blocking, membrane was washed three times with Tris-buffered saline (0.5 M NaCl, 2 mM Tris-HCl, 0.1% Tween 20; pH 7.4) for 5 min. each time, with shaking. Membrane was incubated with human serum in dilution 1:20 (diluent: 10mM Tris-HCl, 5% fetal calf serum, 1% Tween 20; pH 7.4), overnight at room temperature, with shaking. In the next step, membrane was incubated with monoclonal anti-human IgE (dilution 1:1000) for 2 h, at room temperature, with shaking. After incubation, membrane was washed three times with Tris-buffered saline, for 5 min. each time, with shaking. Membrane was incubated with anti-mouse IgG antibody conjugated with alkaline phosphatase (dilution 1:30000) for 1h at room temperature, with shaking. After incubation, membrane was washed three times with Tris-buffered saline, for 5 min. each time, with shaking. Colour developing with substrate BCIP-NBT was performed for 30 min at room temperature, with shaking. Reaction was stopped by washing membrane with the PBS

5. Low field NMR relaxometry

Low field NMR relaxometry was used in hake samples for differentiating fresh from frozen/thawed. To the experiment hake fillets frozen in – 30°C and then thawed were used.

Samples were cut in 1x2x1cm pieces, placed in NMR tubes, and kept in ice until use. They were then placed into LF-NMR spectrometer (Bruker Minispec NMR mq Series) with a magnetic field strength of 0.47 T corresponding to a proton resonance frequency of 20 MHz. Transverse relaxation data (T_2) were measured using the Carr-

Purcell-Meiboom-Gill pulse sequence (CPMG) with a τ-value of 150 μs. For each sample 16 scans were acquired at a 2 s interval with a total of 3000 echoes. At least three measurements per fish were performed. The data was analyzed using the CONTIN regularized algorithm resulting in the corresponding distributions of the relaxation times from the decay curve. This software was provided with the equipment (CONTIN- the minispec- v 1.2).

II. Results and discussion

Inactivation of Anisakis in fishery products. Results of temperature profiles (Figure 3) during heat treating of hake sandwiches, showed as expected, that the higher temperature of fish sandwich was on the edge of fillets. The temperature in the centre and edge of fillets had increased for about 17 minutes. Next temperature obtained maximum and after 20 minutes of heat treated had decreased. Under these heating conditions all larvae emitted fluorescence under UV light (figure 4)

and none of them were found viable.

Figure 3. Temperature profiles during heat treating of fillets

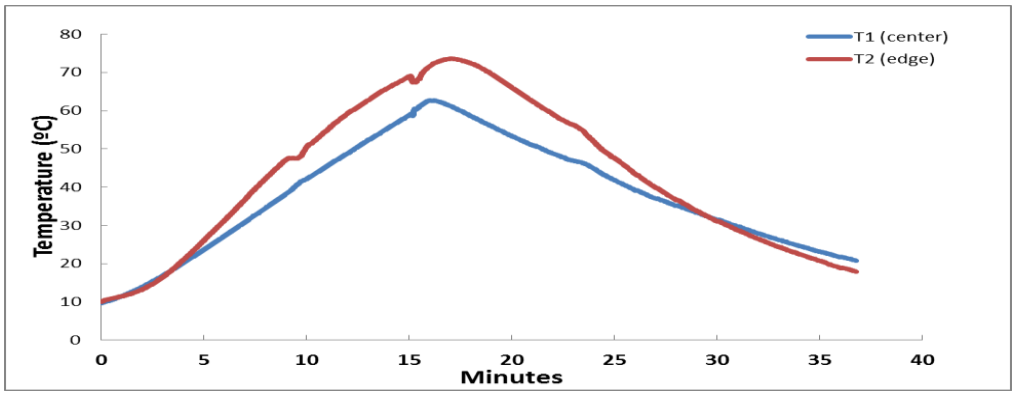
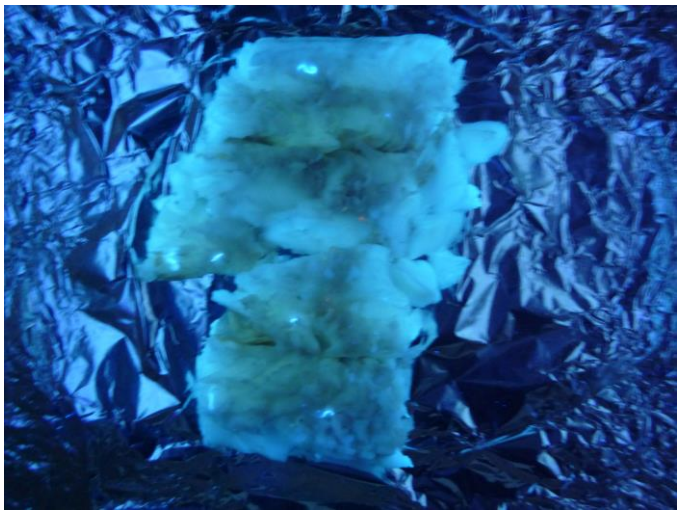


Figure 4. Cooked sample with *Anisakis* larvae under UV-light

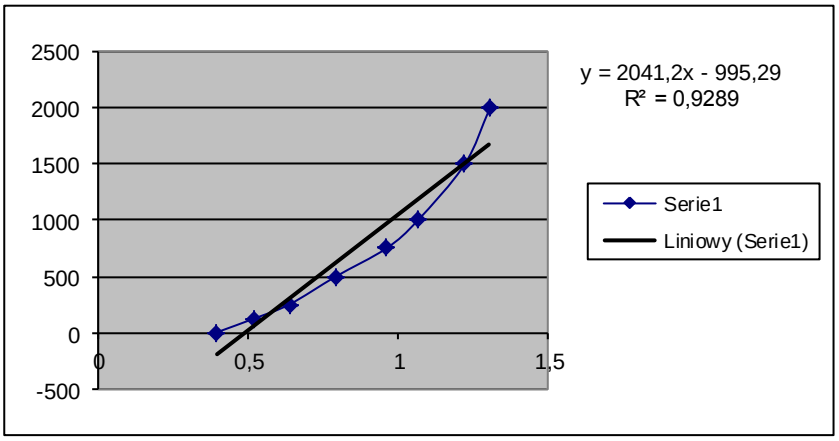


Extraction and detection of allergens. Obtained results of samples after following washing (Table 2.) showed that concentration of allergens had decreased. The highest *Anisakis* allergens concentration was after first washing process, lower after the second, and the lowest after third one. Moreover, concentration of *Anisakis* allergens was the highest in ventral anterior part (AV) (Table 2.) for both small and big fish (reaction with antibody anti-crude extract of *A. simplex*). Reactions of washed and naturally parasitized samples with antibody anti-Ani s1 and anti-Ani s4 were negative. While allergens in canned larvae were detected with use all types of antibody. Furthermore, high correlation coefficient (R^2) (Figure 5.) confirmed good linearity for results of *Anisakis* allergens quantified by dot-blot method.

Table 2. Results of samples examined by dot-blot

Samples	reaction with antibody anti-crude extract of *A. simplex*, average concentration (µg/mL)	reaction with antibody anti-Ani s1, average concentration (µg/mL)	reaction with antibody anti-Ani s4, average concentration (µg/mL)
33.2a	11,60643297	7,65117729	0,056634767
33.2b	2,842633659	1,006128289	0,012070951
33.2c	0,680155662	0,553181353	0,008276476
36.1a	5,066385837	5,703845279	0,053789554
36.1b	0,586464736	1,143873511	0,006932286
36.1c	0,213731322	0,553134622	0,002658801
4.1a	12,6997313	6,191351059	0,153537118
4.1b	0,725261295	1,242576529	0,027368103
4.1c	0,02998863	0,281088011	0,008616395
SF AD	0,020148722	- (not detected)	-
SF AV	0,340282192	-	-
SF PD	0,048650134	-	-
SF PV	0,148190073	-	-
BF AD	0,070366576	-	-
BF AV	0,643790784	-	-
BF PD	0,173431577	-	-
BF PV	0,11162181	-	-
canned L3	19,65569793	0,475259087	0,034054487

Figure 5. Determination of linear range in quantitative dot-blot using a standard proteins.



Low Field NMR relaxometry of hake muscle. Figure 6 and 7 showed results of low field NMR relaxometry which was used in hake samples for differentiating fresh from frozen/thawed. For fresh hake sample LF NMR peak was high. While, for frozen/thawed hake sample peak was lower, and additionally second very low peak was obtained. Based on LF NMR spectra differentiation fresh from frozen/thawed fish is possible.

Figure 6. T_2 relaxation times of average fresh hake analysed in the LF NMR

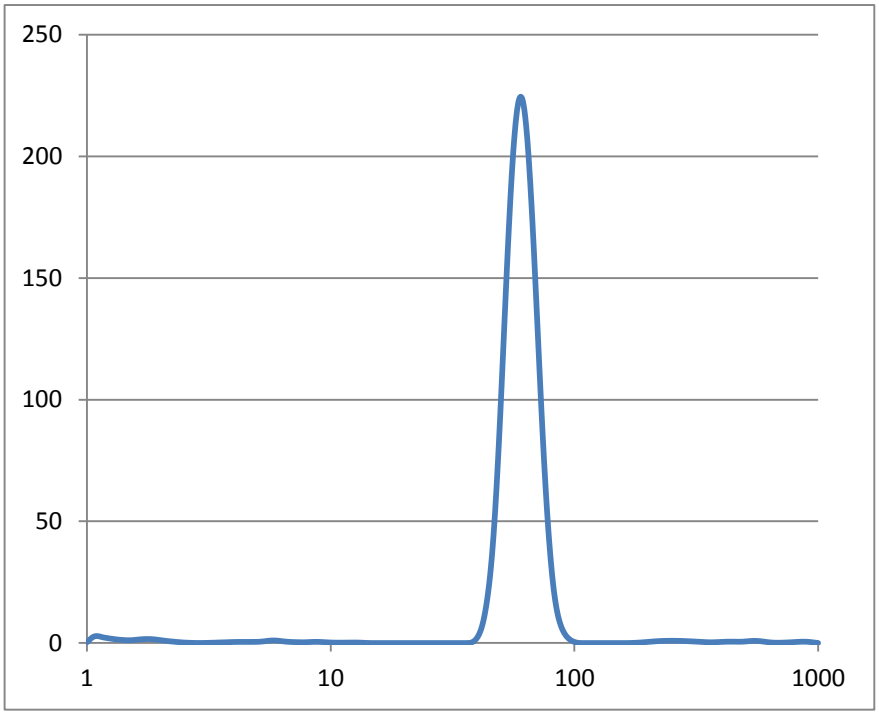
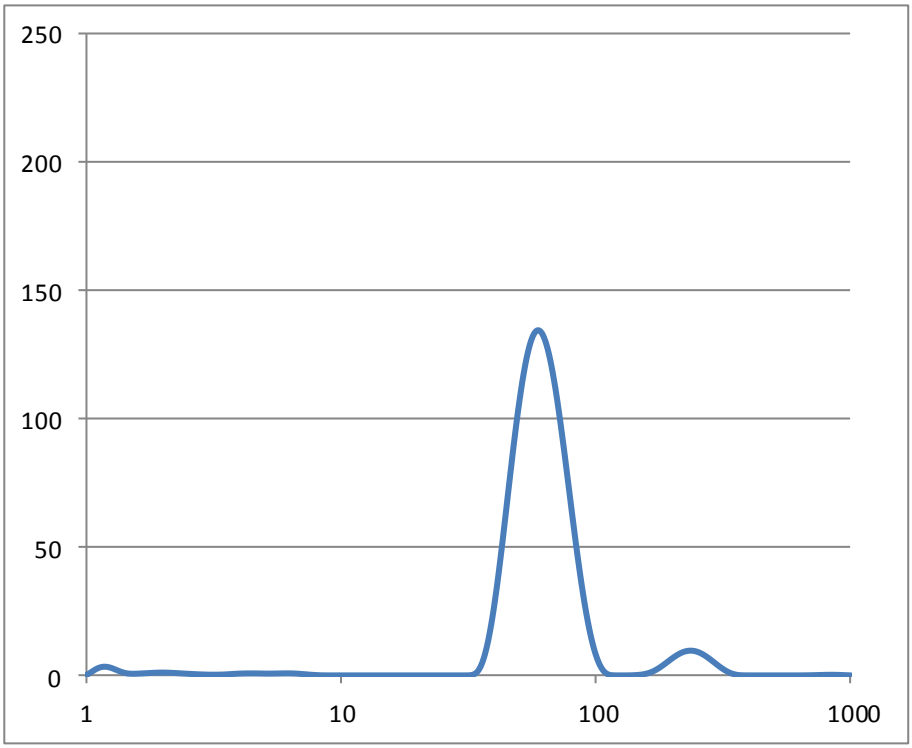


Figure 7. T2 relaxation times of frozen/thawed hake analysed in the LF NMR



STSM effect:

A result of the training was get knowledge about Anisakidae allergens. Moreover, detection skills of *A. simplex* allergens in fish products were obtained. Also, detection method of anti-*Anisakis* IgE antibody was trained. Conducted examinations allow for sensitive and specific detection of fish and fishery product in Poland. It will be first analyse of fish and fishery product Anisakidae allergens contamination in Poland. Moreover, risk assessment of Anisakidae allergens for Polish consumers will be

developed. Furthermore, basic knowledge of low field NMR relaxometry application to differentiation fresh from frozen/thawed fish was obtained.

Future collaboration possibilities with the host institution:

There are many opportunities for collaboration between our institutes. The collaboration is related particularly with Anisakidae investigations. It is possible to conduct collaborative research on *Anisakis* detection in fish and fish products, and humans allergic to *Anisakis* in Poland. Moreover it is possible to collaborate in the subject of distinguish between fresh and frozen products. This subject is also important since the EU regulations require proper freezing of fish because of *Anisakis* risk.

Future plans, including potential future publications:

Anisakis allergen detection method will be introduced for routine diagnostic in our laboratory. That allow, for examination of fish products from Polish markets for the presence of *Anisakis simplex* allergens. This research will rise the food safety in Poland. Obtained result will allow to prepare the publication on the presence of *Anisakis* allergens in fish products in Poland.

In conclusion, the training was successful in terms of established goals. The STSM visit has successfully contributed to know-how detection of Anisakidae allergens and improving collaboration between NVRI, ICTAN and FIBHULP on *Anisakis* research. It has to be underlined commitment of the management of ICTAN and FIBHULP. The friendly atmosphere leads to good cooperation that will turns into the fruitful future cooperation.