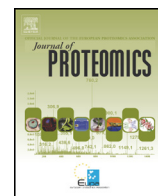




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Protein biomarker discovery and fast monitoring for the identification and detection of Anisakids by parallel reaction monitoring (PRM) mass spectrometry

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ABSTRACT

Anisakids are fish-borne parasites that are responsible for a large number of human infections and allergic reactions around the world. World health organizations and food safety authorities aim to control and prevent this emerging health problem. In the present work, a new method for the fast monitoring of these parasites is described. The strategy is divided in three steps: (i) purification of thermostable proteins from fish-borne parasites (Anisakids), (ii) in-solution HIFU trypsin digestion and (iii) monitoring of several peptide markers by parallel reaction monitoring (PRM) mass spectrometry. This methodology allows the fast detection of Anisakids in <2 h. An affordable assay utilizing this methodology will facilitate testing for regulatory and safety applications.

Significance: The work describes for the first time, the Protein Biomarker Discovery and the Fast Monitoring for the identification and detection of Anisakids in fishery products. The strategy is based on the purification of thermostable proteins, the use of accelerated in-solution trypsin digestions under an ultrasonic field provided by High-Intensity Focused Ultrasound (HIFU) and the monitoring of several peptide biomarkers by Parallel Reaction Monitoring (PRM) Mass Spectrometry in a linear ion trap mass spectrometer. The workflow allows the unequivocal detection of Anisakids, in <2 h. The present strategy constitutes the fastest method for Anisakids detection, whose application in the food quality control area, could provide to the authorities an effective and rapid method to guarantee the safety to the consumers.

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1. Introduction

Anisakis simplex, *Pseudoterranova krabbei* and *Pseudoterranova decipiens* are the main species of fish-borne parasites responsible for registered episodes of human Anisakidosis and allergic reactions around the world [1]. Currently, 2000–3000 cases are reported annually; >90% of these cases are from Japan, and other countries with certain prevalence rates include the Netherlands, Germany and Spain [1,2].

Anisakidosis is a fish-borne parasitic disease caused by the ingestion of undercooked or raw fishes that are parasitized with third-stage larvae (L3) [3,4]. The larvae can elicit a parasitic infection of the digestive tract and can also occasionally affect other organs, causing erosive and hemorrhagic lesions, ascites, and perforations leading to granulomas. The European Food Safety Authority (EFSA) recommends that fish products should be frozen at -20°C for 24 h or at -35°C for not <15 h or heating at temperatures $>60^{\circ}\text{C}$ for 1 min to kill any live nematodes in the product [5]. To date, the only effective treatment for Anisakidosis is symptomatic treatment and endoscopic removal of live larvae. Educational programs are the best method for preventing Anisakidosis. Even

after freezing and thoroughly cooking, Anisakids are responsible for allergic reactions (type-I allergy) in some sensitive patients after fish ingestion [6,7]. The symptoms include urticaria, asthma, gastrointestinal problems and even anaphylactic shocks. Currently, 20 different allergens of *A. simplex* have been described [8]. Many of these allergens are thermostable proteins that are resistant to gastrointestinal enzymes. In these cases, following a fish-free diet is the only treatment recommended.

World health organizations and food safety authorities claim to control and prevent this emerging major public health problem. To protect the consumers from food-borne illnesses, food parasitism and allergy legislations have been promulgated [9]. In accordance with the current EU legislation, fresh fish products must be visually inspected to detect the possible presence of fish-borne parasites. However, these visual inspection procedures have demonstrated a lack of sufficient accuracy and sensitivity [10].

The first molecular techniques developed for the detection of Anisakids larvae in fish products were based on immunological methods [11,12]. Several polyclonal antibodies were developed against specific parasites [11]. However, the problems of these methods are the absence of a universal antibody to cover different species and the cross-reactivity problems. DNA techniques have been as well developed [13]. However, these techniques are laborious and time-consuming methods

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that are unsuitable for use on highly perishable fish products. Therefore, it is necessary the establishment of a quicker, sensitive and specific detection method that permits the direct recognition of fish-borne parasites.

In the last years, targeted proteomics methods have gained more acceptances among the food proteomics community. Selective/Multiple reaction monitoring (SRM/MRM) methods mainly on triple-quadrupole (QqQ) mass spectrometers are the common strategies that have been applied for the identification and detection of bioactive peptides, traceability peptide markers, microorganisms proteins and allergens in foodstuffs [14–16]. However, in a SRM/MRM assay the MS/MS spectra that corroborate the sequence of the peptides targeted are not registered.

Parallel Reaction Monitoring (PRM) [17], also named and previously described as Selected MS/MS Ion Monitoring (SMIM) in ion trap mass spectrometers [18], is a targeting MS mode that performs the parallel acquisition of all transitions of the target peptides. The complete acquisition of MS/MS spectra allows confirming the identity of the peptides monitored. In the context of food science, the PRM/SMIM scanning mode have been applied in several studies, as for the monitoring of species-specific peptide biomarkers to authenticate fish and shellfish species [18–21] and to detect in the foodstuffs the presence of the major fish allergen (parvalbumin) [22]. However, the applicability of PRM to the direct identification and detection of fish-borne parasites still has not been explored.

Recently, label-free semi-quantitative nLC-nESI-Orbitrap-MS/MS (MS1) and absolute-quantitative (AQUA) LC-TripleQ-MS/MS (MS2) experiments have been applied successfully for the detection of *A. simplex* in fish matrix [23]. However, these methodologies were not explored to detect other important and prevalent Anisakids parasites belonging to the genera *Pseudoterranova* and were laborious and time-consuming strategies (> 12 h).

In this sense, the fast detection of the most prevalent marine fish-borne parasites, including *A. simplex*, *P. krabbei* and *P. decipiens* is described in this work. The methodology is based on the following: (a) purification of parasite thermostable proteins, (b) HIFU-assisted trypsin digestion, and (c) monitoring of peptide biomarkers by PRM in a linear ion trap mass spectrometer. The present strategy allows the fast detection of the presence of Anisakids in fishery products in <2 h. This new affordable assay could be very useful for sanitation inspection authorities to protect consumers and guarantee their safety.

2. Materials and methods

2.1. Anisakids

A. simplex and *P. krabbei* specimens were collected from the Biobank platform implemented for the PARASITE project (www.parasite-project.eu) at IIM-CSIC, Spain. Additionally, *P. decipiens* specimens were obtained from the MNCN-CSIC, Spain. All these species were taxonomically identified using conventional PCR to amplify the ITS region and Cox2 gene [24,25].

2.2. Protein extraction

A total of 5–10 specimens for each parasite species and biological replicate were collected and crushed manually with a pestle in a mortar. Then, the extraction of protein was performed by homogenizing 0.25 g of sample in 4.5 mL of lysis buffer (60 mM Tris-HCl pH 7.5, 1% lauryl-maltoside, 5 mM PMFS and 1% DTT) on ice for 10 cycles of 5 s pulses in a sonicator device (Werke, Germany). Thermostable proteins were also purified by heating the protein samples at high temperatures for 5 min (70 °C, 90 °C, 110 °C) and centrifuged for 20 min at 40,000 g in a J221-M centrifuge (Beckman, CA, US). The supernatant proteins were quantified using the bicinchoninic acid method (Sigma Chemical Co., US). All analyses were performed in triplicate.

For the PRM experiments in fish samples, European hake protein extracts were prepared as described previously Carrera et al., 2011 [20].

2.3. Tryptic digestion by HIFU

Protein supernatants (20 µg) were ultrafast digested with trypsin (Promega, WI, US) applying at the same time high-intensity focused ultrasound (HIFU) [26]. The trypsin digestion was carried out as described Carrera et al. 2011 [20].

2.4. Shotgun LC-MS/MS analysis

Peptides were acidified with formic acid, cleaned on a C₁₈ MicroSpin™ column (The Nest Group, South-borough, MA) and analyzed by LC-MS/MS using a Proxeon EASY-nLC II liquid chromatography system (Thermo Fisher Scientific, San Jose, CA, US) coupled to a LTQ-Orbitrap Elite (Thermo Fisher Scientific). Peptide separation (2 µg) was done on a RP column (EASY-Spray column, 50 cm × 75 µm ID, PepMap C18, 2 µm particles, 100 Å pore size, Thermo Fisher Scientific) with a 10 mm pre-column (Accucore XL C18, Thermo Fisher Scientific) using 0.1% formic acid (mobile phase A) and 98% ACN with 0.1% formic acid (mobile phase B). A 240 min of linear gradient from 5 to 35% B, at a flow of 300 nL/min was used. A spray voltage of 1.95 kV and a capillary temperature of 230 °C were used for ionization. The peptides were analyzed in positive mode (1 µscan; 400–1600 amu), followed by 10 data-dependent CID MS/MS scans (1 µscans), using a normalized collision energy of 35% and an isolation width of 3 amu. Dynamic exclusion for 30 s after the second fragmentation event was applied and unassigned charged ions were excluded from the analysis.

2.5. PRM mass spectrometry

PRM analysis was performed using a Dionex UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) coupled to a LTQ Velos-Pro mass spectrometer (Thermo Fisher Scientific). The separation of the peptides (1 µg) was done on an Acclaim PepMap100 Nano Trap Column, C18, 5 µm, 100 Å, 100 µm × 1 cm (Thermo Fisher Scientific) coupled to an RP column Acclaim PepMap RSLC 75 µm × 150 mm, C18, 2 µm, 100 Å (Thermo Fisher Scientific), using 0.1% formic acid (mobile phase A) and 98% ACN with 0.1% formic acid (mobile phase B). A linear gradient of 45 min from 5 to 35% B, at a flow rate of 300 nL/min was used. For ionization, a spray voltage of 2.10 kV and a capillary temperature of 200 °C were used. The peptides were monitored using the PRM acquisition mode performing MS/MS scans (3 µscans) of the doubly-charged precursor ions for the all peptide markers along the complete chromatographic run [15,21]. The normalized collision energy was fixed to 35%, and a mass window of 1 amu was used to fragment the selected precursor ions.

2.6. Processing of the mass spectrometry data

All the MS/MS spectra acquired were analyzed using SEQUEST-HT (Proteome Discoverer 1.4 package, Thermo Scientific) against the Nematoda UniProt/TrEMBL database (release 2014_10; 440.821 entries). The following restrictions were used: semi-tryptic cleavage with up to 2 missed cleavage sites and tolerances of 1.2 Da for parent ions and 0.6 Da for MS/MS fragment ions. The permissible variable modifications were: carbamidomethylation of Cys (C*), methionine oxidation (Mox), acetylation of the N-terminus of the protein (N-Acyl) and heavy C-terminus of lysine and arginine (K*, R*) for the synthetic heavy peptide biomarker experiments. The results were subjected to statistical analysis with the Percolator algorithm [27] to keep a FDR below 1%. For the PRM mode, Skyline software [28] (version 3.5.0.9319) was used for the selection of the suitable *m/z* precursor ion → *m/z* fragment ion transition for the selected candidate peptide biomarkers. Peptides with potential modifications such as tryptophan oxidation (W*) were not

considered. Virtual chromatogram traces of the extracted transitions chromatograms were plotted using QualBrowser program (Thermo Fisher Scientific). In addition, MS/MS spectra collected in the PRM mode were used to validate the peptide identities using SEQUEST-HT as described before.

2.7. Stable-isotope labeled peptide biomarkers

Stable-isotope peptide biomarkers from the Anis 9 protein were selected and synthesized as isotopically labeled C-terminus Arg U-13C6;U-15N4 or Lys U-13C6;U-15N2 heavy peptides (SpikeTides™, JPT Peptide Technologies GmbH, Volmerstrasse, Berlin, Germany). The lyophilized heavy peptides were individually reconstituted with purified water/5% formic acid (v/v) producing a 56 pmol/μL of working solution, which was stored at −80 °C.

A dilution series of stable-isotope peptide biomarkers in 5% formic acid were prepared in concentrations ranging 0 fmol/μL to 500 fmol/μL. Calibration curves, were also constructed by spiking dilution series of the standard heavy peptide biomarkers into original European hake protein extracts (1 μg/μL) [20]. All samples were heat-treated, HIFU digested and PRM analyzed as described before. Standard curves were performed by measuring transition intensities for the reference peptide biomarkers. All analyses were performed in triplicate, and the mean of intensities for each peptide marker was represented.

3. Results and discussion

3.1. Scheme for the direct identification and detection of Anisakids

The study integrates two consecutive steps: (a) Discovery Phase and (b) Targeted-driven Phase (Fig. 1).

In the first step (Discovery Phase), a shotgun proteomics approach was used to create a reference proteome dataset for the different fish-borne parasite species (*A. simplex*, *P. krabbei* and *P. decipiens*). To perform the direct identification and detection of the presence of parasites in any fish product, a simple protein purification step was performed considering their thermostability. This property was selected to reduce the complexity of the sample and to choose potential biomarkers that survive cooking and other food processing treatments. Subsequently, thermostable protein biomarkers were identified and selected.

In a second step (Targeted-driven Phase), potential tryptic peptide biomarkers from the previous thermostable candidate protein biomarkers were characterized and selected for Anisakids detection purposes. The accelerated tryptic digestions using HIFU were compared, and several species-specific peptide biomarkers were selected. The monitoring of these peptide markers by PRM in a linear ion trap mass spectrometer allowed the fast identification and detection of these Anisakids species in fishery products.

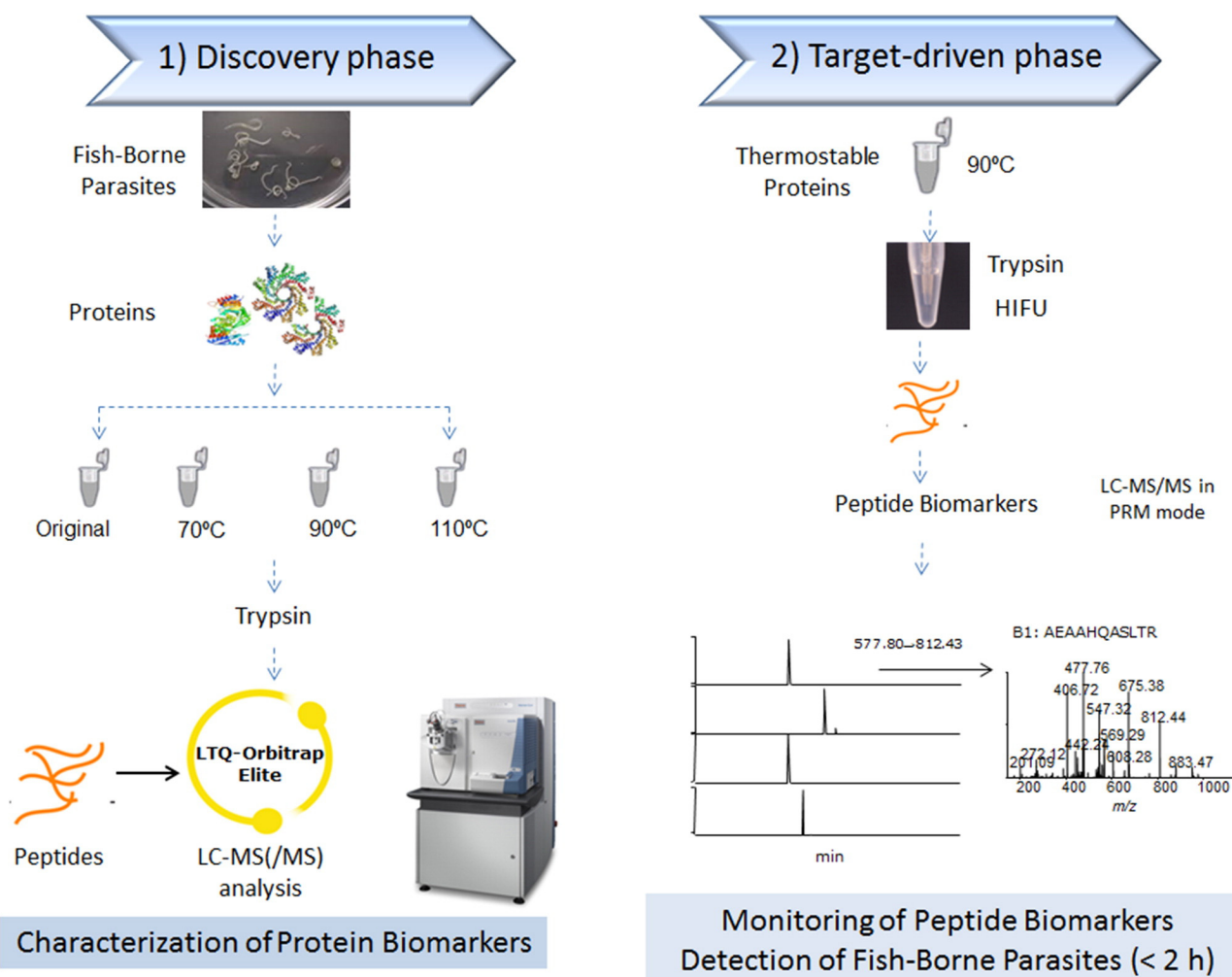


Fig. 1. Analytical scheme for fast detection of Anisakids that is proposed in this work.

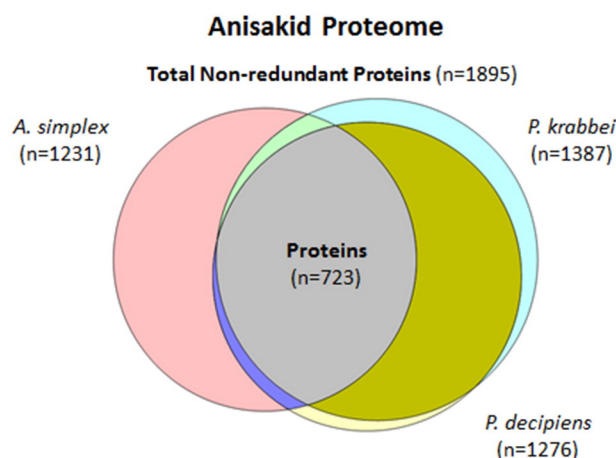


Fig. 2. The Anisakid proteome.

3.2. Discovery phase

3.2.1. Anisakid proteome

The first shotgun Anisakid Proteome for the *A. simplex*, *P. krabbei* and *P. decipiens* species is presented in this work (Supporting Data 1). This discovery phase was based on the LC-MS/MS analysis and SEQUEST-HT search, of the tryptic digestions for the global protein extracts from each fish-borne parasite. Using this shotgun proteomics approach, a total of 3151 non-redundant peptides corresponding to 1895 different proteins were identified. As expected, the results showed a high degree of overlap (723 different proteins) among the *A. simplex*, *P. krabbei* and *P. decipiens* species (Fig. 2).

To our knowledge, this is the most comprehensive dataset of peptides and proteins for marine fish-borne parasites identified to date. This valuable protein repository will add new and significant information to the universal public protein databases and will be very useful for further investigations.

3.2.2. Protein biomarkers

To select the best protein biomarker for the direct identification and detection of parasites present in any food product, we selected those proteins based on their thermostability. Extracts of proteins for each parasite species were heated during 5 min at different temperatures (70 °C, 90 °C, 110 °C), and after centrifugation, the supernatants

containing the thermostable proteins were analyzed by shotgun proteomics. After the SEQUEST-HT search the identified thermostable proteins/peptides are presented in Supplemental Data 1. Fig. 3 shows a summary of heat-resistant sub-proteomes.

Considering the Nematoda protein sequence entries in the UniProtKB database, numerous peptides identified after heat treatment corresponded to proteins belonging to the SXP/RAL-2 protein family. This family of proteins is specific to nematodes, including animal and plant parasitic nematodes [29]. Ani s 5, Ani s 8 and Ani s 9 are the registered protein entries (15 kDa) of this family for fish-borne parasites. Although this family is characterized by immunologically active proteins, nothing is known about their concrete function. The DUF148 domain (Pfam-PF02520) is present in their sequences, which contains two conserved motifs (SXP1, SXP2) [30]. Although the function is unknown, recent research has found similarities between the motifs of the allergenic Ani s 5 protein and the patterns found for the EF-hand motifs [31]. These latter motifs are characteristics of the sequence of major fish allergens, parvalbumin (PRVB) [20]. Because homologs of these proteins have not been identified outside of Nematoda, they may be appropriate targets for the implementation of novel control strategies.

Ani s 9 protein (B2XCP1_ANISI) belongs to the SXP/RAL-2 family [29]. This protein was identified in all of the parasite species that were tested (*A. simplex*, *P. krabbei* and *P. decipiens*), even in the highly heat-resistant sub-proteomes (90 °C–110 °C). Ani s 9 is an allergen protein consisting of 147 amino acids that shares 100% of the homology sequence with the Ani s 9 from *Anisakis pegreffii* (L7VOL4_9BILA). Other allergens of *Anisakis* displaying heat stability and lower sequence homology are Ani s 4, Ani s 5 and Ani s 8. However, we do not have enough information on the presence of these allergens in other parasites to consider them to be protein biomarkers. Additionally, Ani s 9 is present in the secretory products of these fish-borne parasites, as reported previously [32]. Moreover, because Ani s 9 is related to the onset of allergic episodes involving well-cooked or canned fish products [33], their detection may also be highly relevant for additional allergic and clinical diagnostic biomarkers.

For these reasons, Ani s 9 was selected as the best candidate protein biomarker for the following step of the present strategy (Target-driven phase).

3.3. Target-driven phase

3.3.1. Selection of peptide biomarkers

In a second step (Target-driven Phase) (Fig. 1), potential tryptic peptide biomarkers from the candidate protein biomarker (Ani s 9) were characterized and selected for authentication purposes.

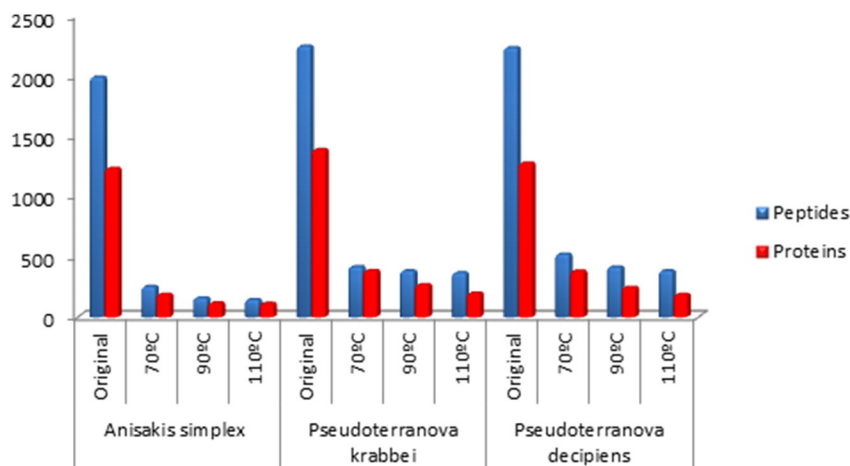


Fig. 3. Heat-resistant peptides and proteins for Anisakids.

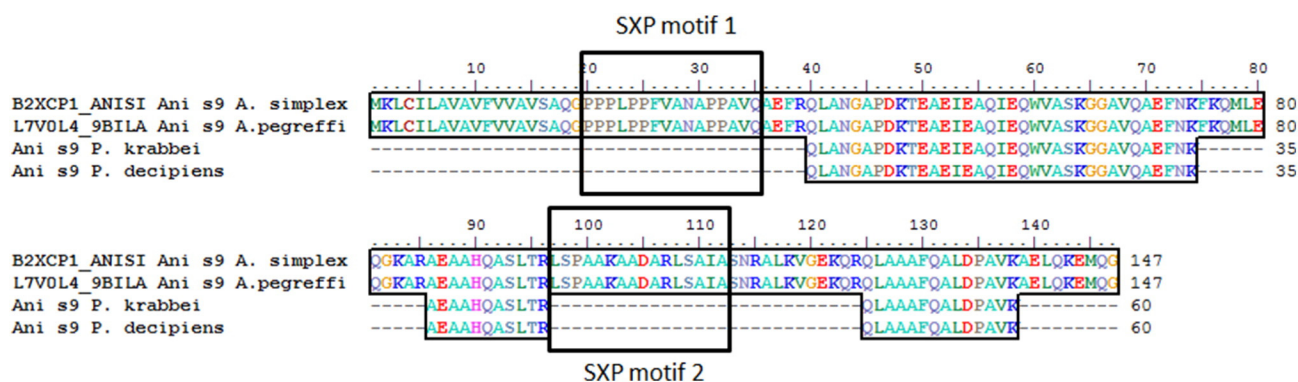


Fig. 4. Alignment of the protein sequence (Ani s 9) for Anisakids.

The Ani s 9 protein sequences for *A. simplex*, *A. pegreffi* and the identified peptides by MS/MS for *P. krabbei* and *P. decipiens* were aligned by ClustalW (Fig. 4). All the sequences present 147 amino acid residues; however, the first 17 residues correspond to the signal peptide, and the mature protein consists of 130 amino acids [34]. The calculated molecular mass for the mature protein is 13.7 kDa, and the isoelectric point is 9.0.

PeptideMass program [35] was used to predict the potential fully tryptic peptides for these proteins. However, unique peptides from the most conserved regions corresponding to the motifs SXP1 and SXP2 were not identified by MS/MS. These motifs are composed of several residues of proline that are most likely responsible for providing the enzymatic and heat resistance properties that these allergen proteins exhibit. In addition, peptide candidates with potential amino acid modifications during sample preparation, such as tryptophan oxidation (W^*), were not considered. For that, after the specificity parameter rendered by BLAST, only four sequences were selected as peptide markers for the detection of these fish-borne parasites (Table 1a).

3.3.2. Rapid detection of fish-borne parasites using PRM

For each parasite species (*A. simplex*, *P. krabbei* and *P. decipiens*), the proteins were heated at 90 °C and subjected to accelerated tryptic digestions using HIFU. Thus, the combination of a fast and easy protein purification procedure (Time: 45 min) with the use of HIFU for protein digestion (Time: 2 min), considerably simplified and reduced the time

needed for sample preparation, which was reflected in the overall time required for monitoring.

The peptide results were analyzed by PRM in a linear ion trap mass spectrometer, centering the MS/MS scans on the parent ions of the four peptide biomarkers (Fig. 5). For each precursor ion the m/z value selected corresponded to the major charge state ($z = 2$) (Table 1). Once the MS/MS data were acquired, virtual chromatogram traces for all the different daughter ions could be represented and peptide sequence information to validate the corresponding peptide biomarker could be obtained after the SEQUEST-HT searching. The transitions in each case were chosen using the fragments with the major intensity, which were y -ions for all of them. Thus, the use of simple peptide mixtures together with highly sensitive transitions (precursor $m/z \rightarrow$ fragment m/z) (Table 1), enables the representation of specific transitions with a remarkable high S/N ratio. By tracing these transitions for every of peptide markers presented in Table 1a, it was possible to unequivocally detect the existence of fish-borne parasites in <2 h (Fig. 5).

Therefore, monitoring only four peptide biomarkers from Ani s 9 by PRM in a linear ion trap mass spectrometer allows the fast identification and detection of Anisakids in <2 h.

3.3.3. PRM-targeted detection of Anisakids peptide biomarkers in fish extracts

Stable-isotope peptide biomarkers synthesized as isotopically labeled C-terminus Arg U-13C6;U-15N4 or Lys U-13C6;U-15N2 heavy peptides (Table 1b) were used to validate the proposed strategy. Thus, dilution series of the stable-isotope peptide biomarkers were analyzed in concentrations ranging from 0 fmol/ μ L to 500 fmol/ μ L. The chromatography results for 60 min on a C18 pre- and analytical column resulted in peaks tailed with widths less than one minute and with elution times between 11.57 and 32.32 min (Supplemental Data 2). Accordingly, measuring a standard curve by intensity resulted in linearity ranging from 0 fmol/ μ L to 500 fmol/ μ L (Fig. 6). The regression curves of the four peptides ran in parallel with a higher sensitivity for the B2 and B1 peptide biomarkers.

Analysis of the reference peptide biomarkers that spiked in hake protein extracts (Fig. 7) showed that the proportional relationships remained the same as that with the buffer-diluted samples, which corroborate the validation of the PRM method in real fish samples.

4. Conclusions

The rapid detection of Anisakids in fishery products is described in this work. The strategy is following: (i) purification of thermostable proteins from Anisakids by heat treatment, (ii) in-solution HIFU tryptic digestion and (iii) monitoring of several peptide biomarkers from the Ani s 9 protein by PRM. The strategy achieves the detection of the presence of zoonotic Anisakids in <2 h. This strategy will facilitate testing for

Table 1

(a) Anisakids peptide biomarkers, (b) reference synthetic heavy peptide biomarkers and the corresponding specific transitions for the identification and detection of Anisakids.

a)			
Biomarker code	Peptide biomarker sequence	PRM transition m/z precursor ion (z) $\rightarrow$ m/z fragment ion	Retention time (min)
B1	AEAAHQASLTR	577.80 (2+) $\rightarrow$ 812.43 (y''_7)	11.57 $\pm$ 0.21
B2	GGAVQAEFNK	510.76 (2+) $\rightarrow$ 736.36 (y''_6)	16.75 $\pm$ 0.23
B3	QLAAAFQALDPAVK	721.90 (2+) $\rightarrow$ 988.54 (y''_8)	32.13 $\pm$ 0.28
B4	QLANGAPDK	457.24 (2+) $\rightarrow$ 672.33 (y''_7)	12.19 $\pm$ 0.31
b)			
Biomarker code	Heavy peptide biomarker	PRM transition m/z precursor ion (z) $\rightarrow$ m/z fragment ion	Retention time (min)
B1*	AEAAHQASLTR*	582.80 (2+) $\rightarrow$ 822.44 (y''_7)	11.72 $\pm$ 0.230
B2*	GGAVQAEFNK*	514.76 (2+) $\rightarrow$ 744.37 (y''_6)	16.915 $\pm$ 0.235
B3*	QLAAAFQALDPAVK*	725.91 (2+) $\rightarrow$ 996.56 (y''_8)	32.32 $\pm$ 0.27
B4*	QLANGAPDK*	461.25 (2+) $\rightarrow$ 680.34 (y''_7)	12.055 $\pm$ 0.185

* synthetic heavy peptide biomarkers.

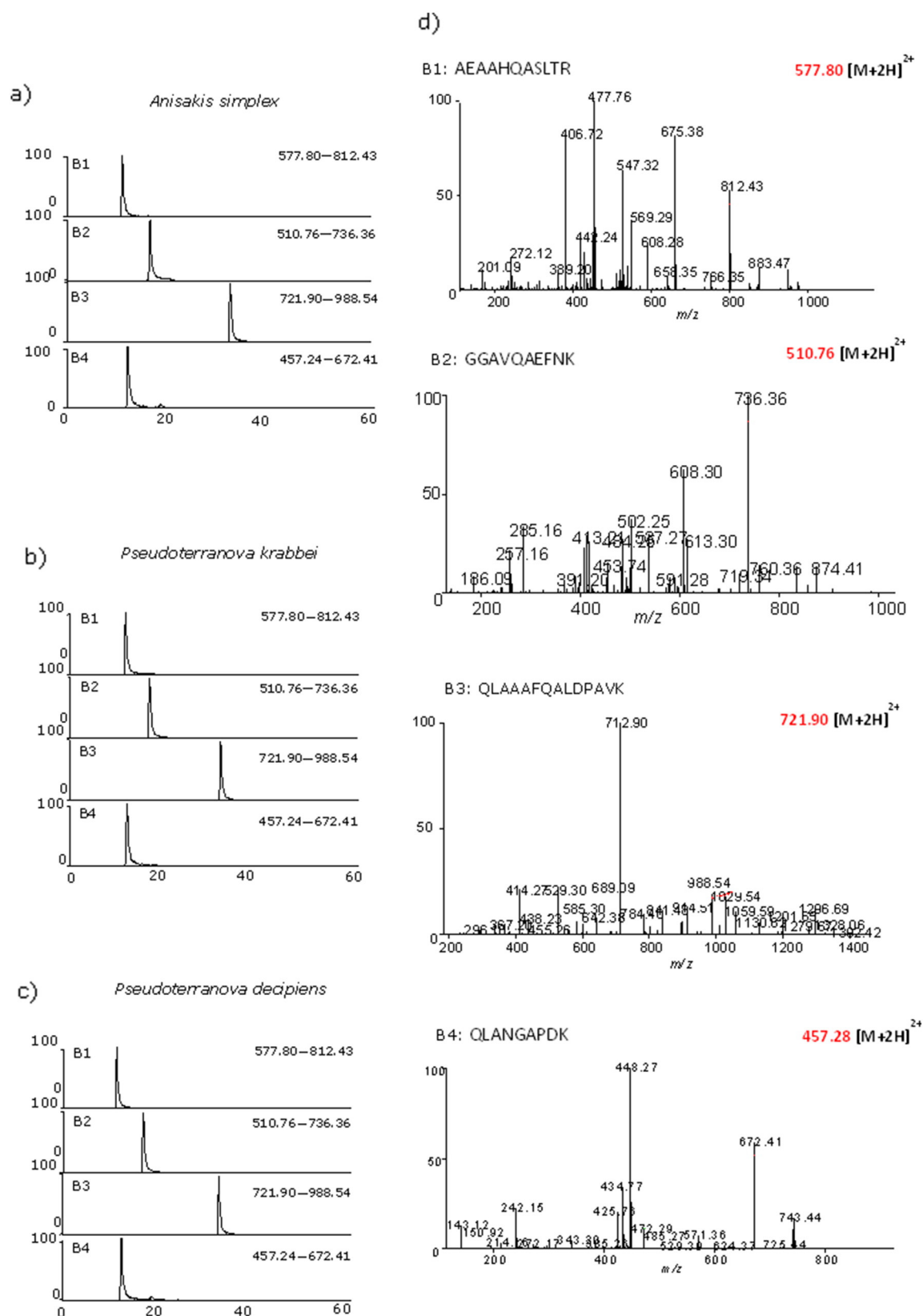


Fig. 5. a, b, c) PRM traces for each of the Anisakid species, plotting the corresponding canonical transition for each peptide biomarker derived from Anis 9, d) MS/MS spectrum for each of the four doubly-charged peptide biomarkers.

regulatory and safety applications. Moreover, the potential utility of this strategy could be applied to other parasite contexts.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2016.05.012>.

Transparency document

The Transparency document associated with this article can be found, in online version.

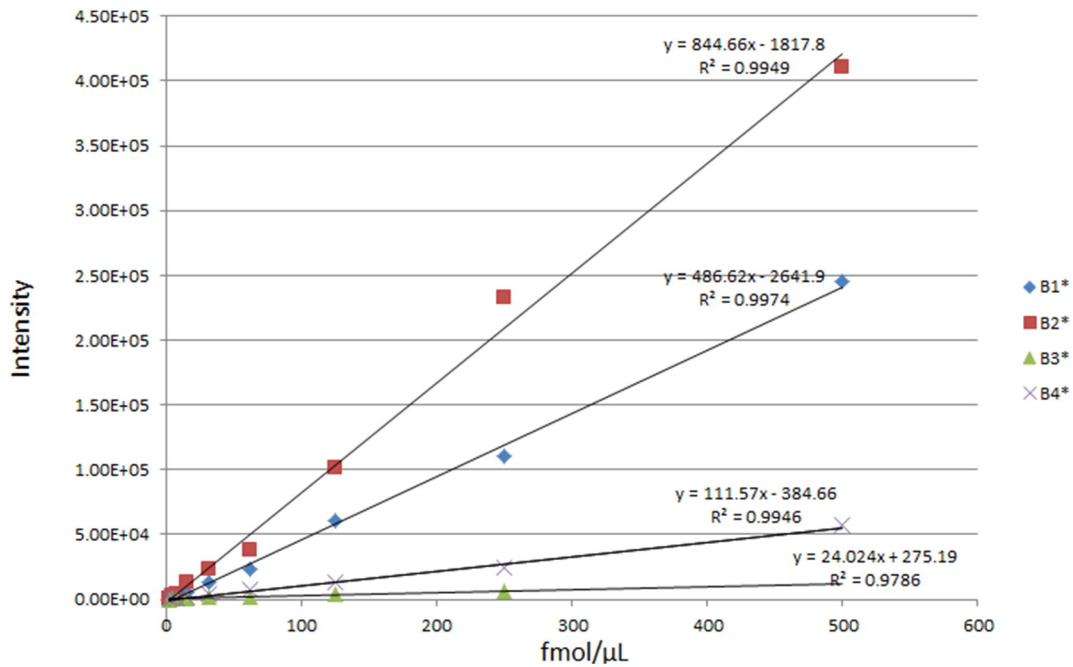


Fig. 6. Standard curves for the PRM analysis of 0–500 fmol/μL of the four selected heavy peptide biomarkers and linearity expressed by regression coefficients (R^2).

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Synthetic heavy peptide Biomarkers (B*) in Hake Protein Extracts

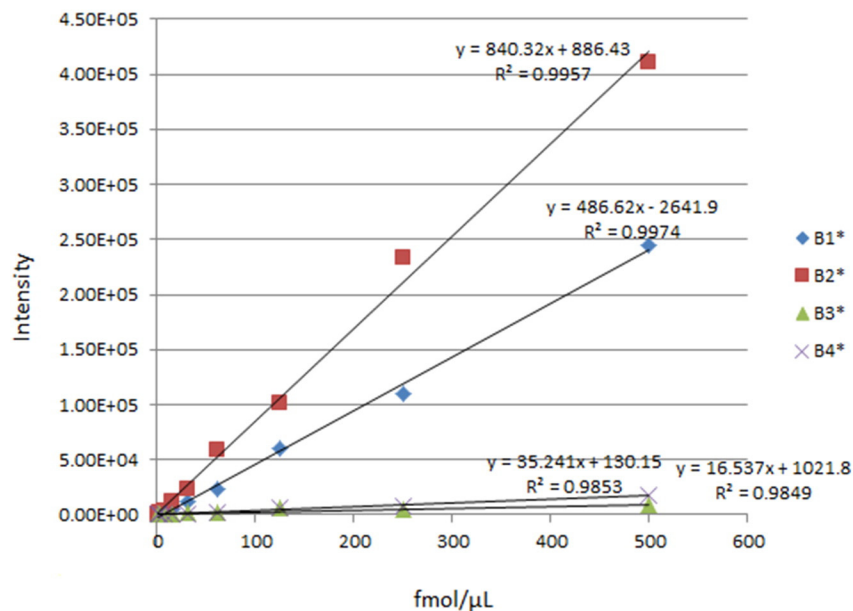
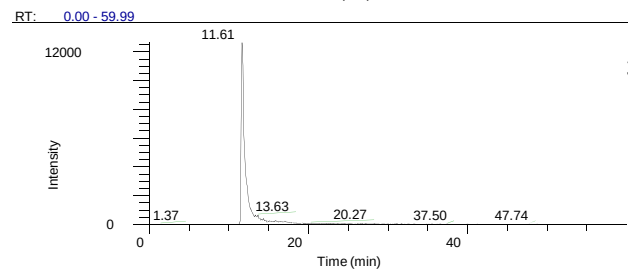
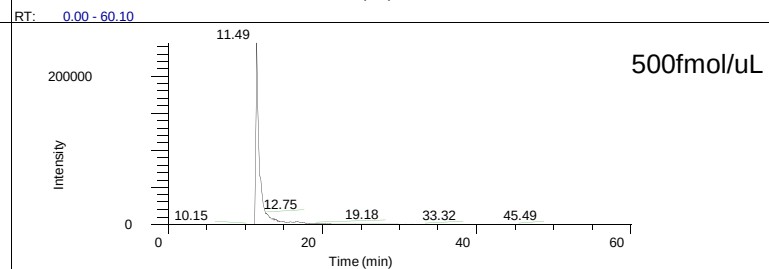
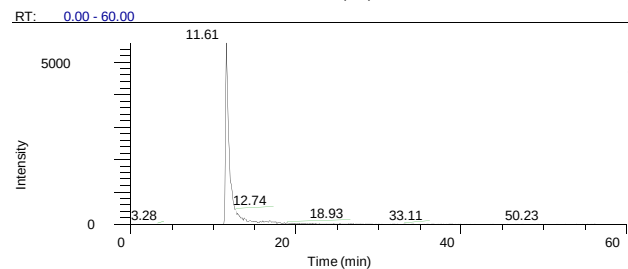
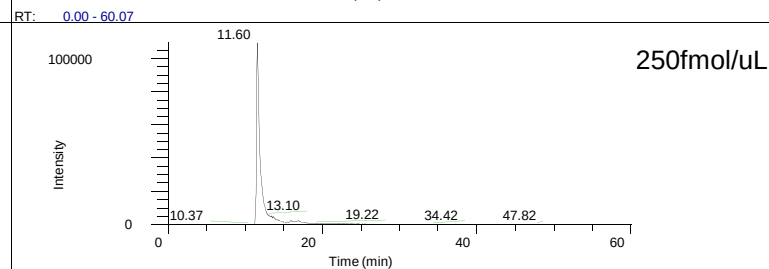
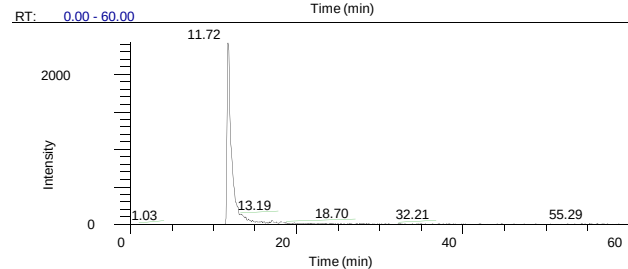
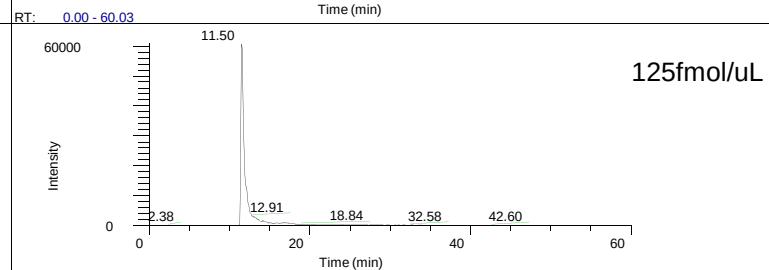
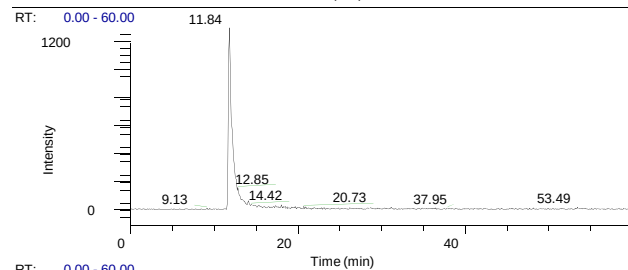
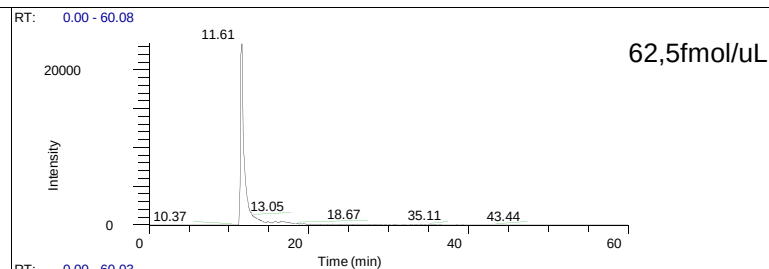
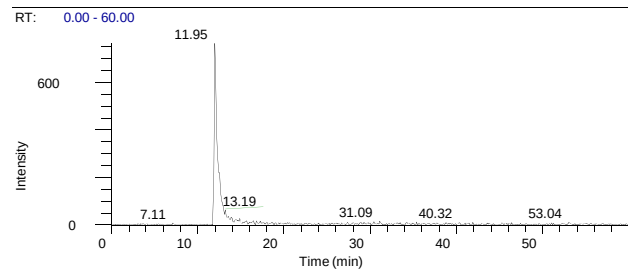


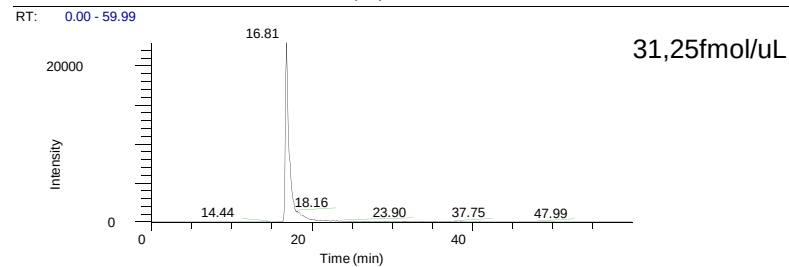
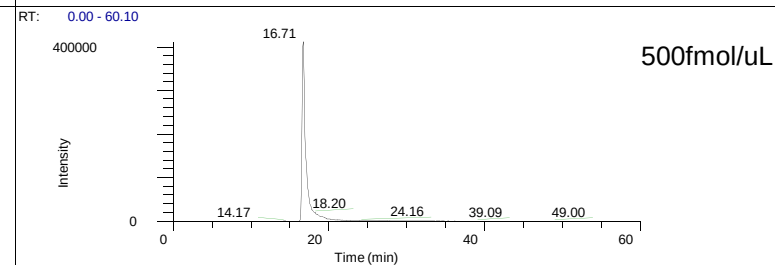
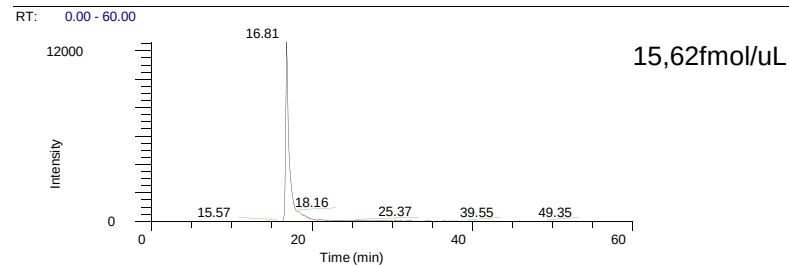
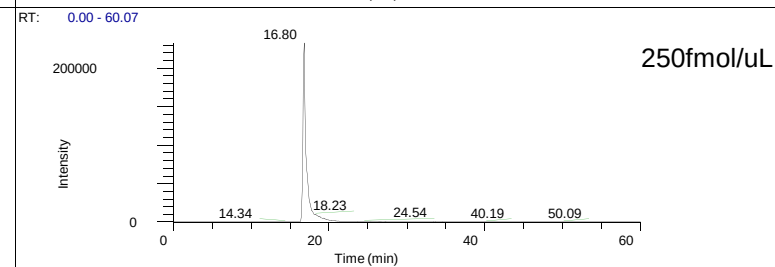
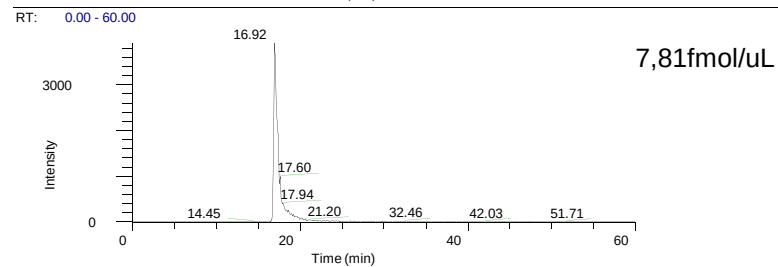
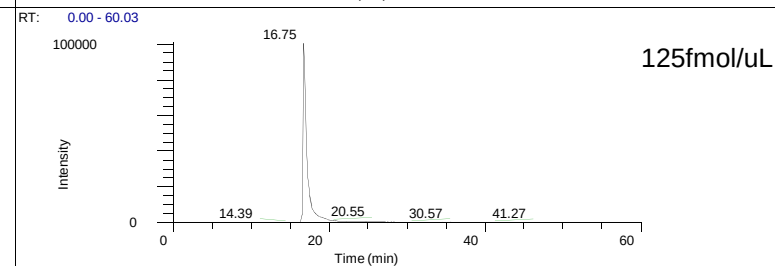
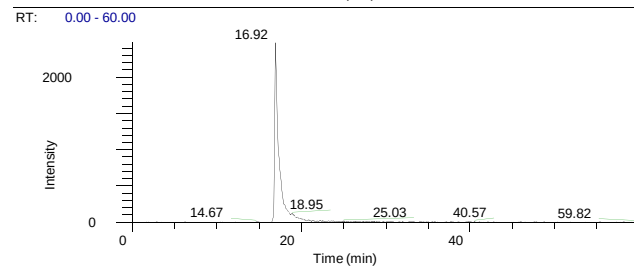
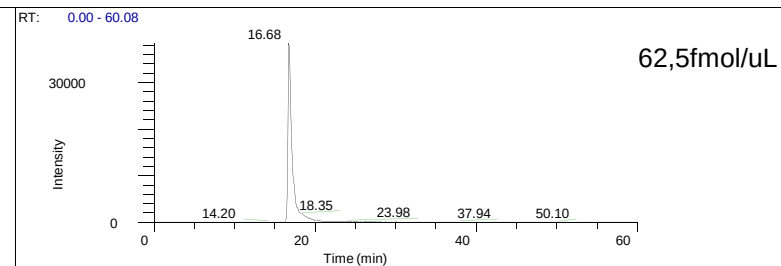
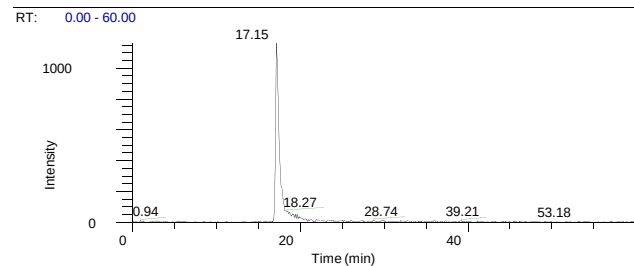
Fig. 7. Standard curves for the PRM analysis of 0–500 fmol/μL of the four selected heavy peptide biomarkers in hake muscle protein extract. Linearity expressed by regression coefficients (R^2).

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PRM (1.95-500 fmol/ μ L) in buffer B1* AEAHQASLTR* 582.80 (2+) $\rightarrow$ 822.44 (γ'' 7+)

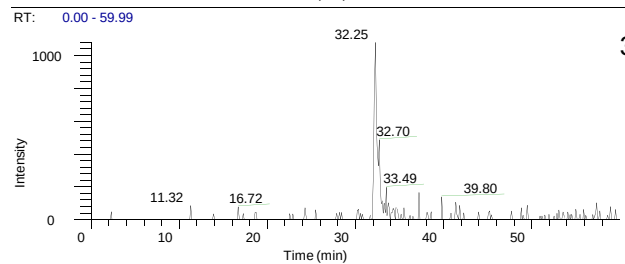
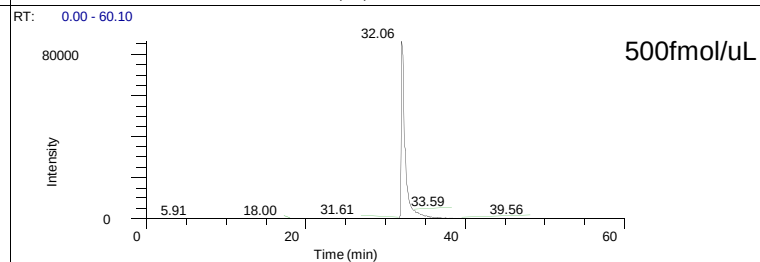
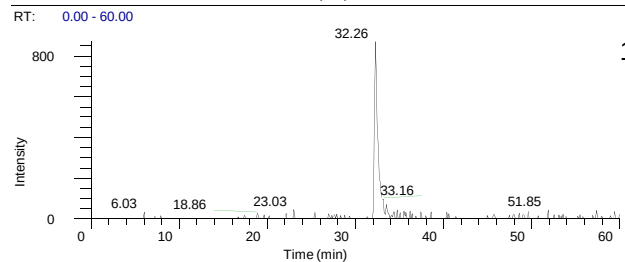
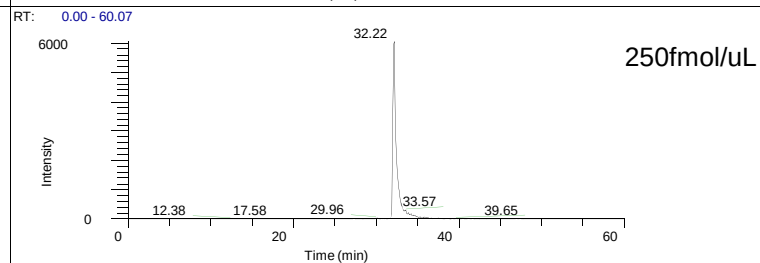
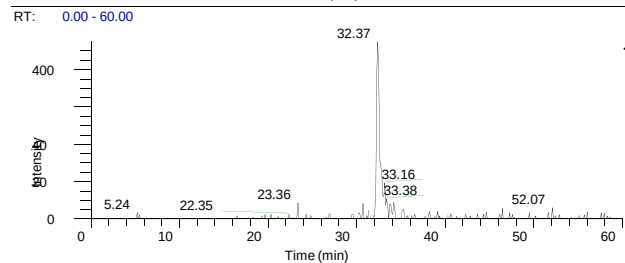
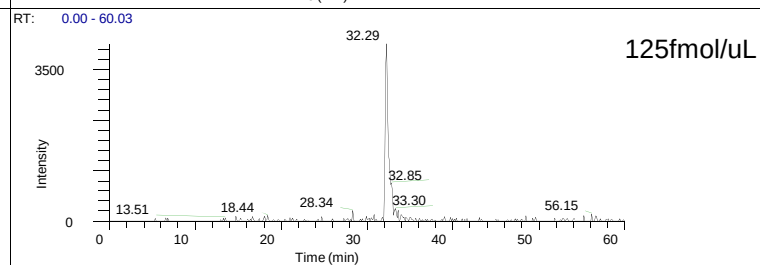
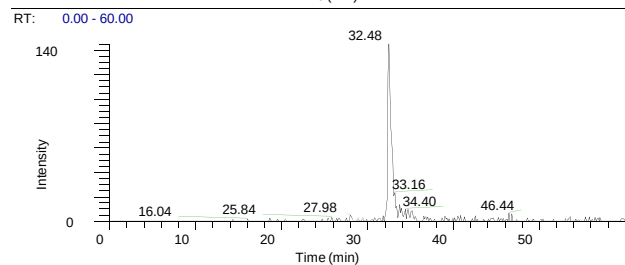
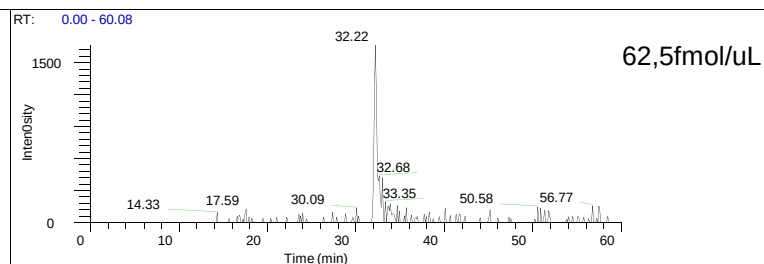
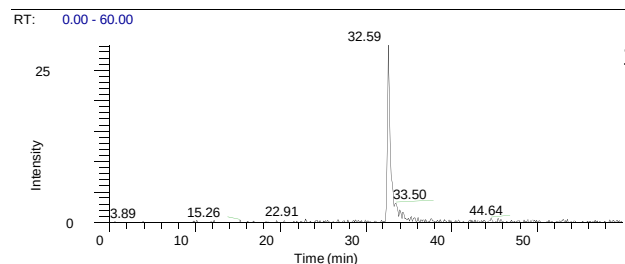


PRM (1.95-500 fmol/ μ L) in buffer B2* GGAVQAEFNK* 514.76 (2+) $\rightarrow$ 744.37 (γ "6+)



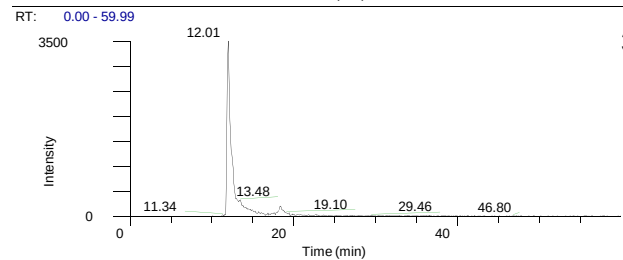
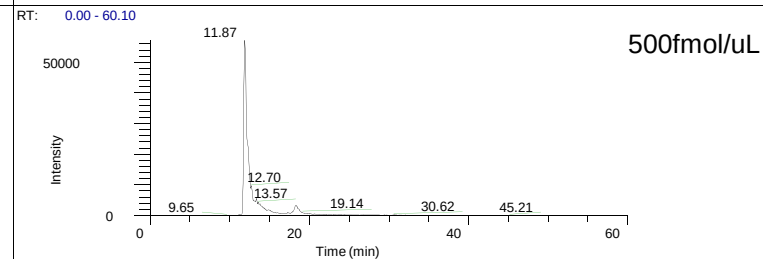
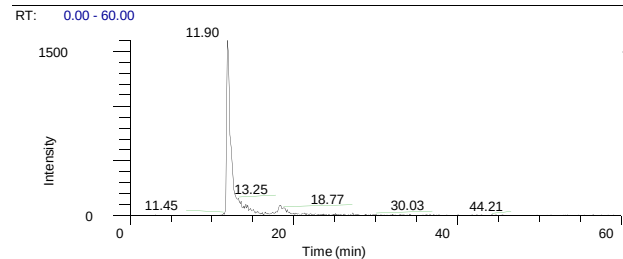
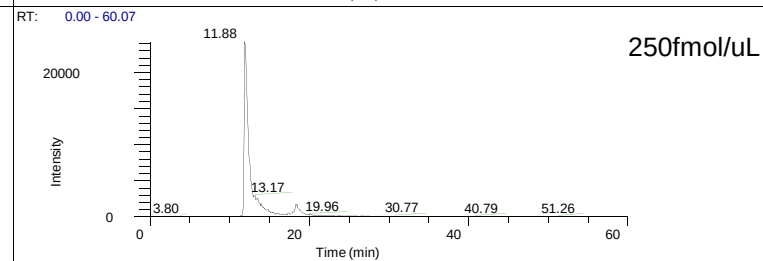
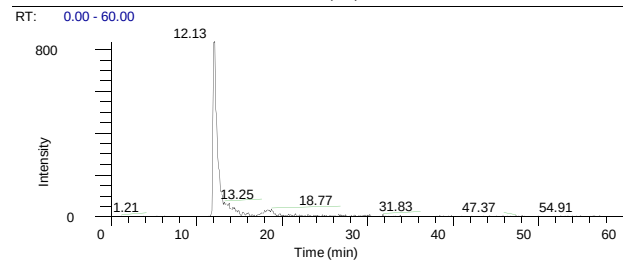
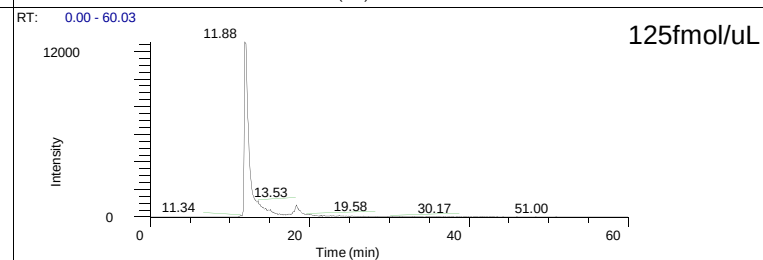
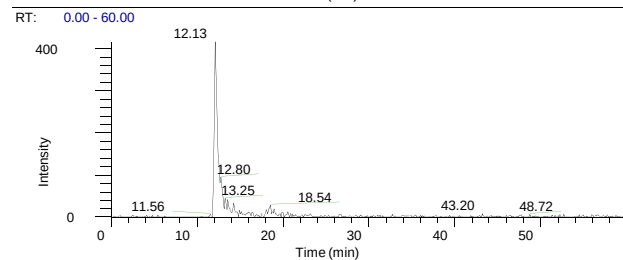
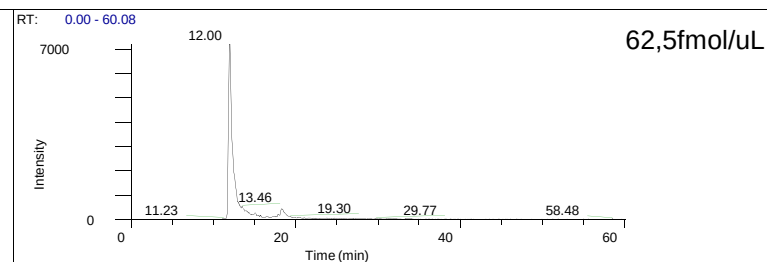
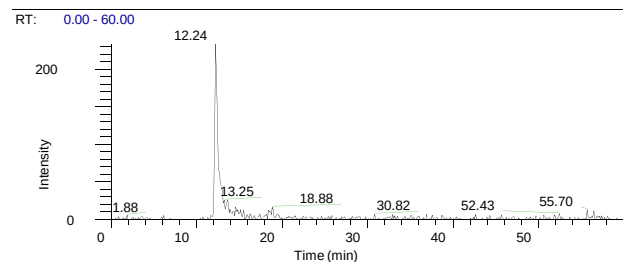
PRM (1.95-500 fmol/ μ L) in buffer

B3* QLAAAFQALDPAVK* 725.91 (2+) $\rightarrow$ 996.56 (γ "6+)



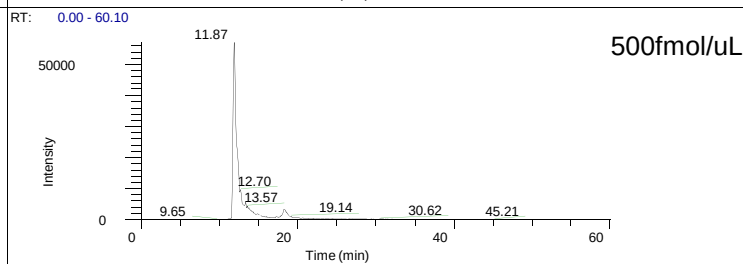
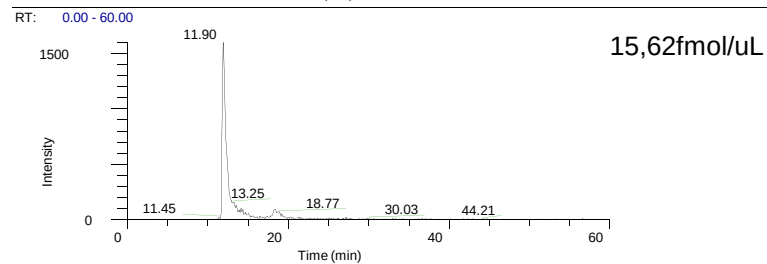
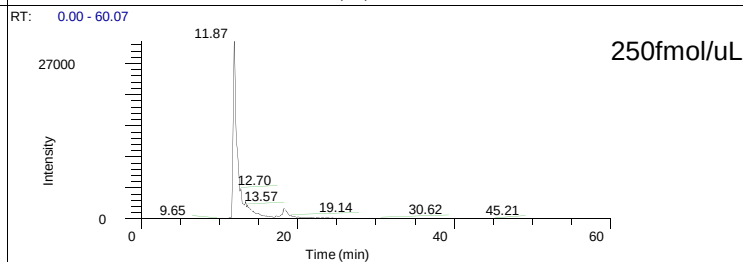
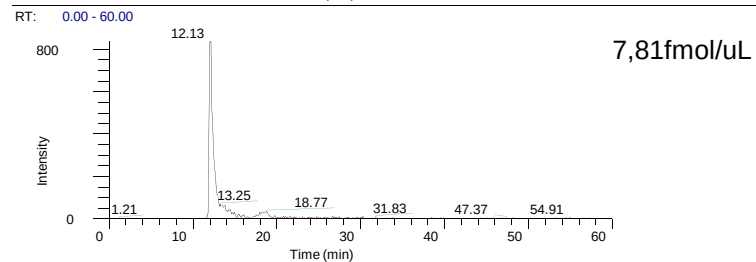
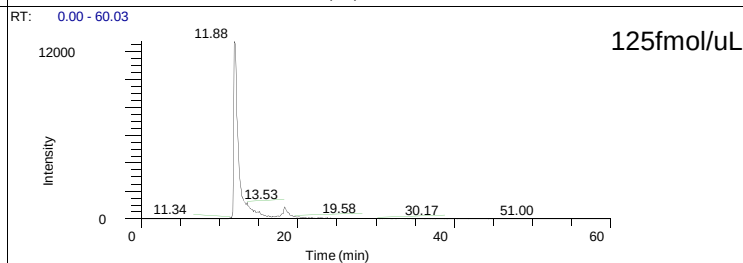
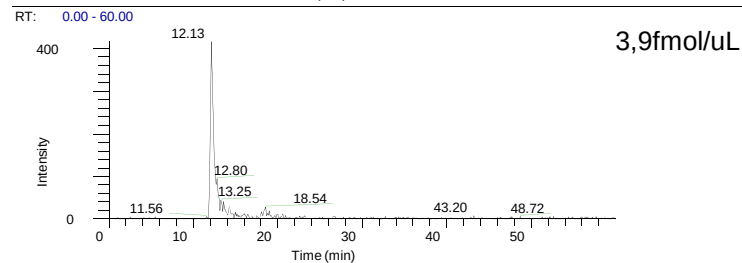
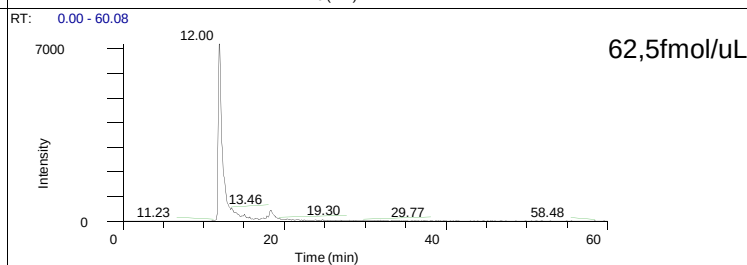
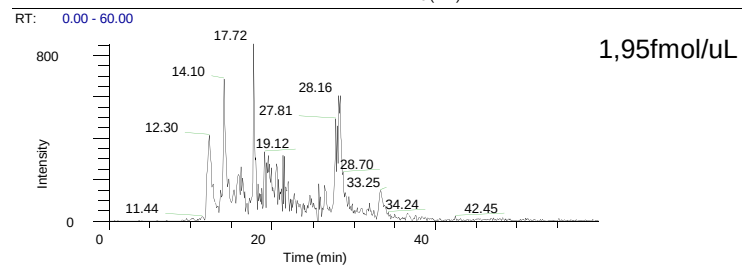
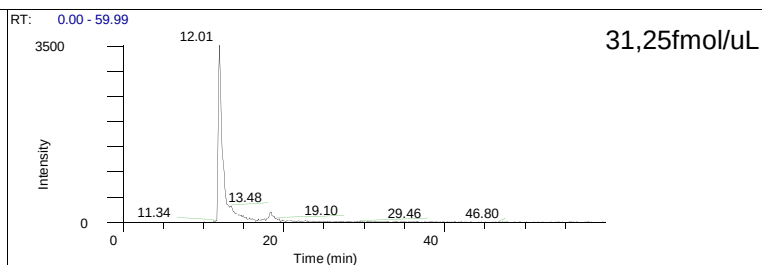
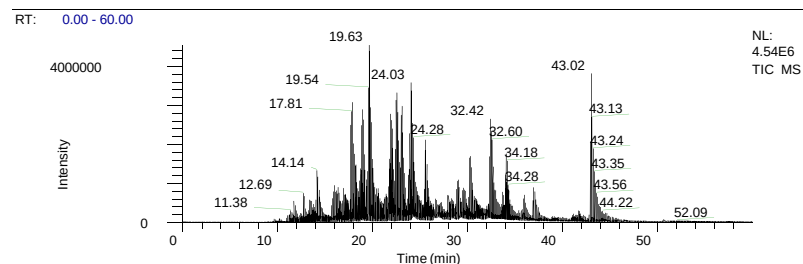
PRM (1.95-500 fmol/ μ L) in buffer

B4* QLANGAPDK* 461.25 (2+) $\rightarrow$ 680.34 (γ "7+)



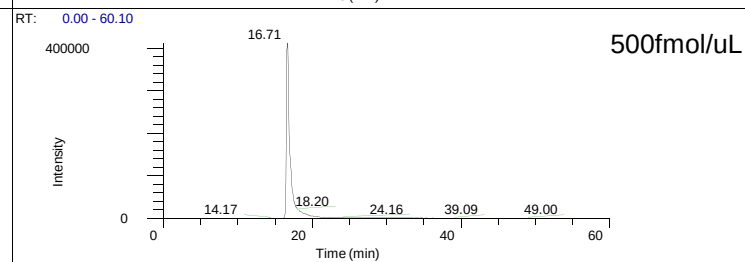
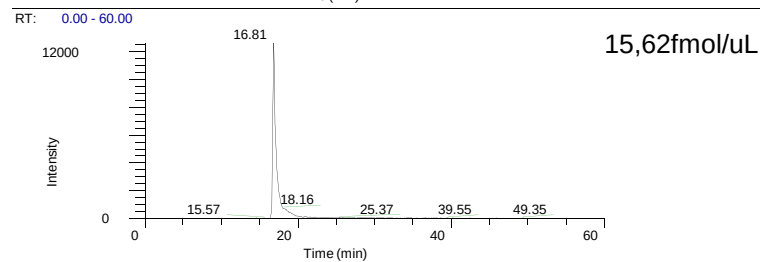
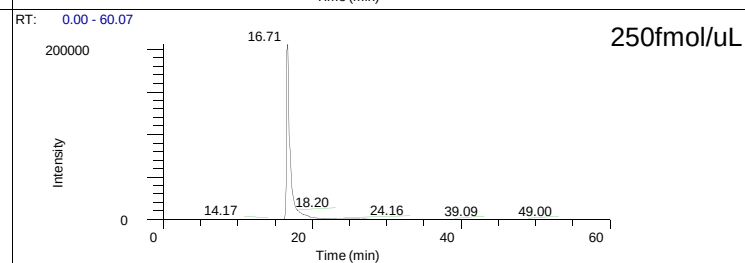
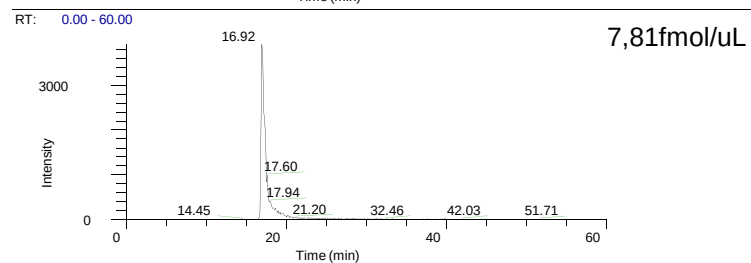
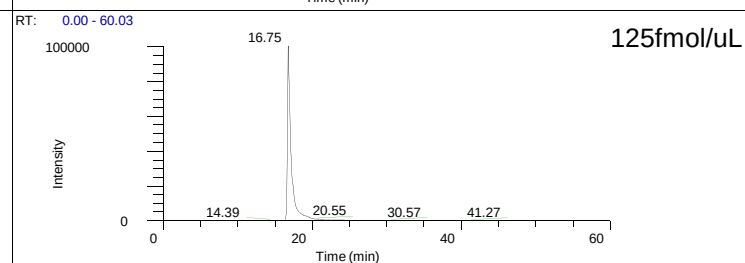
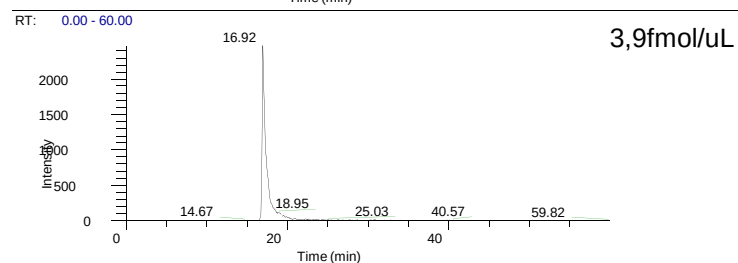
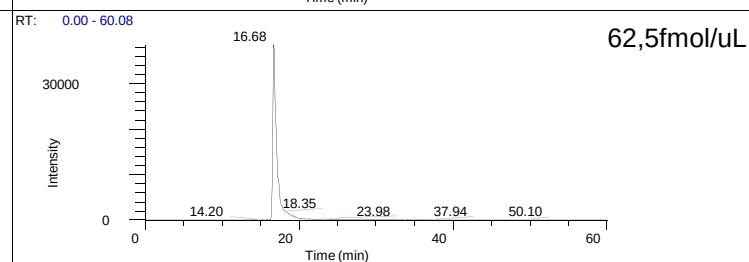
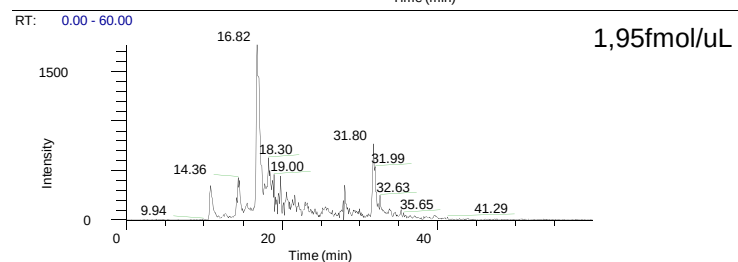
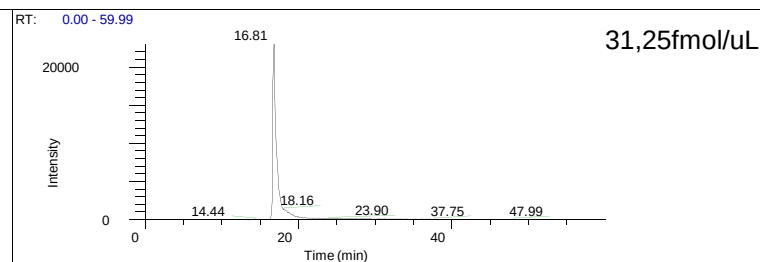
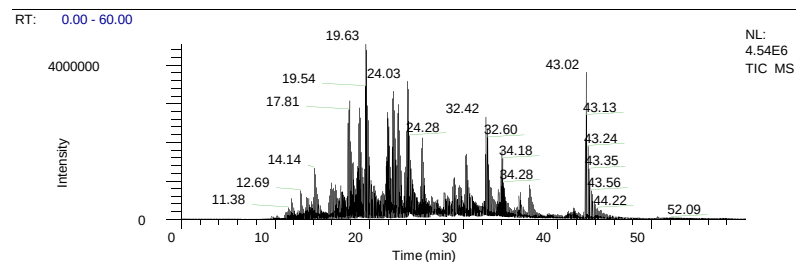
PRM (1.95-500 fmol/ μ L) in Hake extracts

B1* AEAHQASLTR* 582.80 (2+) $\rightarrow$ 685.38 (γ'' 6+)

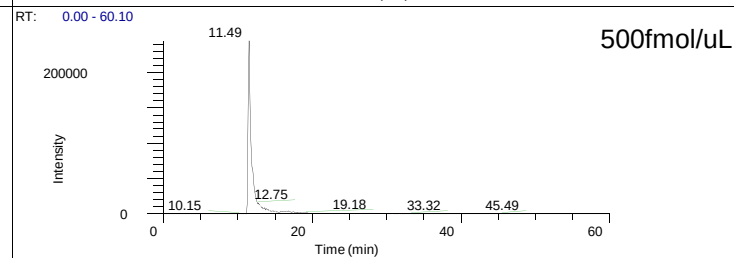
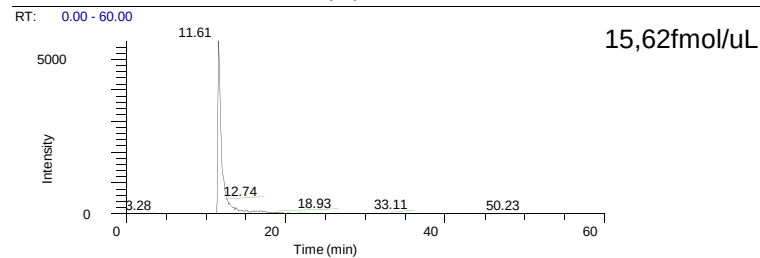
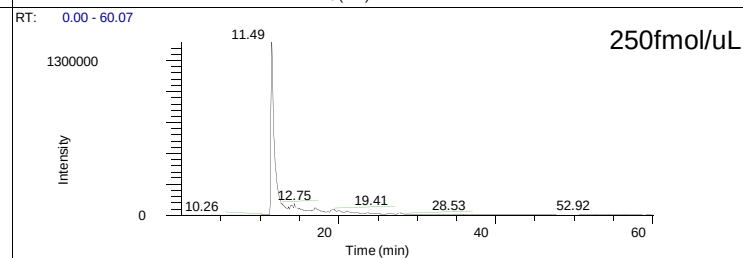
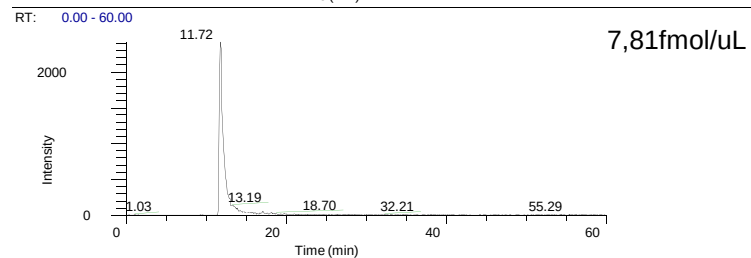
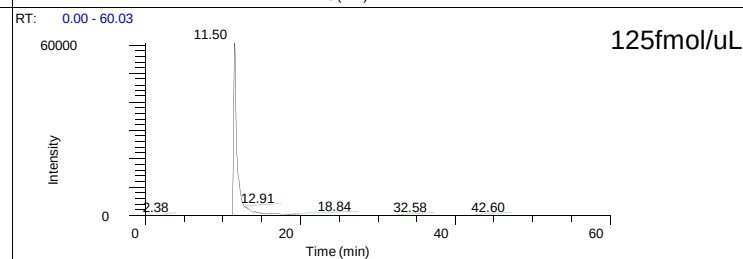
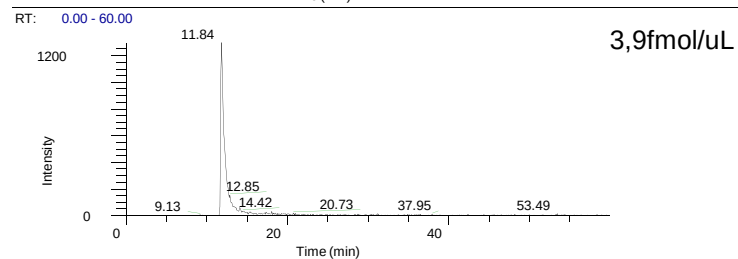
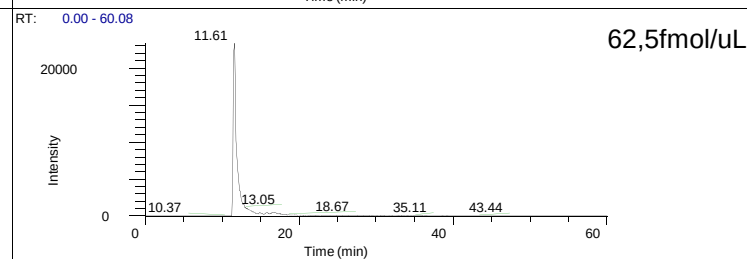
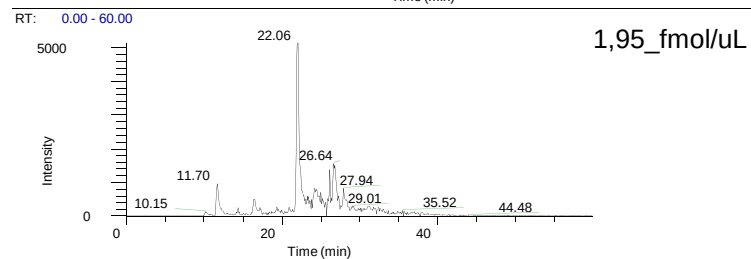
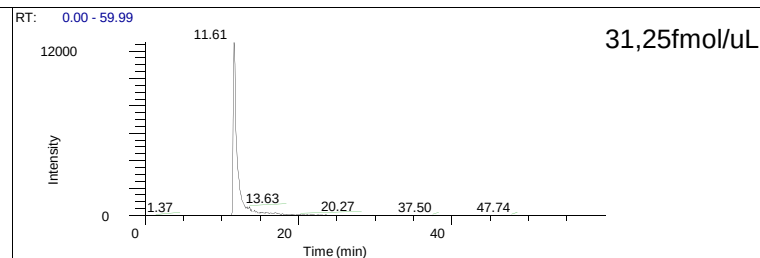
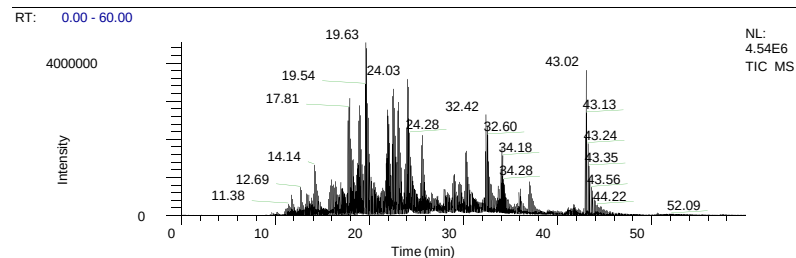


PRM (1.95-500 fmol/ μ L) in Hake extracts

B2* GGAVQAEFNK* 514.76 (2+) $\rightarrow$ 744.37 (γ "6+)



PRM (1.95-500 fmol/ μ L) in Hake extracts B3* QLAAAFQALDPAVK* 725.91 (2+) $\rightarrow$ 996.56 (γ "6+)



PRM (1.95-500 fmol/ μ L) in Hake extracts B4* QLANGAPDK* 461.25 (2+) $\rightarrow$ 680.34 (γ "7+)

