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# Identification of an albumin-like protein in plasma of Atlantic cod (*Gadus morhua*) and its biomarker potential for PAH contamination

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## Abstract

Increased research efforts are currently focusing on Atlantic cod (*Gadus morhua*) and its significance for monitoring the contaminant situation in marine environments. Polycyclic aromatic hydrocarbons (PAHs) are well known toxic and carcinogenic compounds, thus continuous monitoring is required to ensure ecosystem sustainability and human food safety. A sensitive biomarker of PAH exposure in humans is the detection of PAH metabolites bound to albumin in blood. The potential of a similar PAH-albumin biomarker in Atlantic cod was therefore investigated by a desktop bioinformatic study followed by liquid chromatography mass spectrometry/mass spectrometry analysis of plasma from 16 fish. For the first time, an albumin-like protein in plasma of Atlantic cod is described, and the biomarker potential based on PAH-albumin adduct detection is discussed. Due to the detected low abundance of the albumin-like protein, it was found unlikely to be applicable as a new biomarker tool for evaluation of PAH exposure.

Keywords: Bioinformatics, Toxicology, Environmental science, Biological sciences

## 1. Introduction

Increased research efforts are directed towards Atlantic cod (*Gadus morhua*) and its role as a key species in environmental investigations focusing on ecosystem sustainability and human food safety [1, 2, 3, 4, 5]. One focus area is the environmental risks stemming from the constant flux of oil-related polycyclic aromatic hydrocarbons (PAHs) into the habitats and spawning grounds of Atlantic cod [2, 6, 7]. PAHs can cause serious adverse effect, such as developmental defects and cancer [8], and as PAHs transcend tropic levels, it also becomes an issue of food safety [2]. It is therefore of high significance to ensure continuous evaluation of the extent of PAH contamination in the marine environment. Presently, large efforts are being made to decode the Atlantic cod toxicology systems to find novel tools applicable for environmental monitoring and risk assessment [3, 4, 5]. A sensitive proteomic marker for PAH exposure is the detection of PAHs covalently bound to albumin (i.e. PAH-albumin adduct) in human blood [9, 10, 11]. The use of a similar PAH-albumin biomarker in Atlantic cod could therefore be of high value for the evaluation of the marine PAH contamination and associated environmental risks.

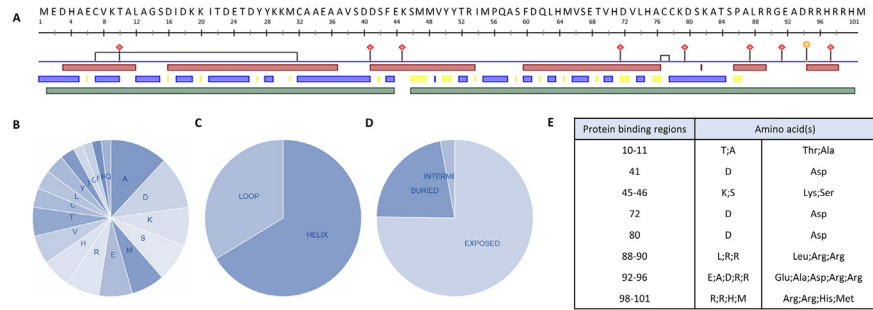
PAH-albumin adducts have been identified in several species (e.g. human, mice, rat and woodchuck), including two salmonid fish species [12, 13, 14]. To date, there are no reports of PAH-albumin adducts in Atlantic cod, in fact, there are no reports identifying the presence of albumin in the plasma of this species. The complete genome of Atlantic cod has been sequenced [15], and its full estimated proteome is available as the *Gadus morhua* EST database [16], however, the majority of these proteins remains unidentified. Therefore, in this study, we investigated the presence of albumin/albumin-like proteins in the plasma proteome of Atlantic cod, its theoretical potential for binding PAH metabolites and its use as a novel biomarker for environmental monitoring purposes. This was done by a desktop bioinformatic study followed by liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) sample analysis.

## 2. Results and discussion

### 2.1. Identification of a predicted albumin-like protein in Atlantic cod

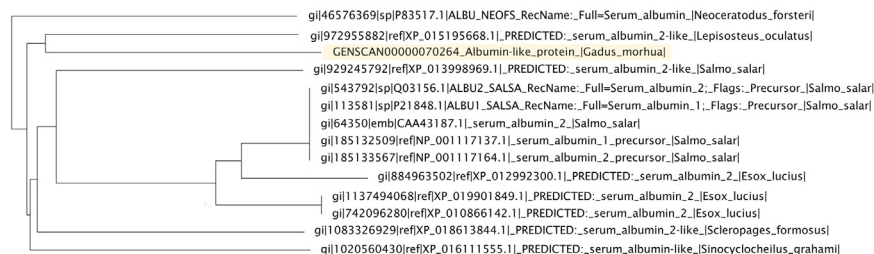
The general characteristics of albumin are that it is a globular protein, primarily consisting of alpha helices, and contains distinct binding domains for hydrophobic compounds [17]. Albumin proteins often function as carriers for a variety of endogenous components (e.g. hormones, fatty acids and steroid) and also play an

important role in maintaining the oncotic pressure of the body [17]. In humans, albumin is the dominating protein with levels up to 65% of the plasma proteome [17], while levels in different Teleost fish ranges from 0 to 60% [12]. This may be due to the fact that albumin is not essential for the maintenance of oncotic pressure in some fish species, and other proteins, such as the lipid-binding apolipoproteins, can function as the main transport proteins in its absence [18]. In general, lower levels of albumin/albumin-like proteins are observed in salt water fish compared to fresh water species, however, there are large knowledge gaps concerning the albumin/albumin-like proteins of lower vertebrates [19, 20]. Detection of albumins in Teleost fish have been reported as a difficult task due to the great variability of their characteristics and the low homology between different fish species and between mammalian and fish [19, 20]. To date, albumin/albumin-like proteins have been identified in 15 Teleost species (9 species of salmonids (i.e. *Salmo salar*, *Salmo trutta*, *Salmo gairdneri*, *Oncorhynchus mykiss*, *Oncorhynchus kisutch*, *Oncorhynchus gorbuscha*, *Oncorhynchus tshawytscha*, *Salvelinus fontinalis* and *Salvelinus alpinus*), American catfish (*Ameiurus nebulosus*), Australian lungfish (*Neoceratodus forsteri*), Spotted gar (*Lepisosteus oculatus*), Northern pike (*Esox lucius*), Asian arowana (*Scleropages formosus*) and Gold line fish (*Sinocyclichelus graham*)) [12, 19, 20, 21, 22], and have not been found in the plasma of 5 Teleost species (3 species of eel (*Anguilla dieffenbachia*, *Anguilla australis schmidti* and *Anguilla japonica*), Antarctic toothfish (*Dissostichus mawsoni*) and common carp (*Cyprinus carpio*)) [18, 19, 20]. By comparing the available NCBI information of Teleost albumin/albumin-like proteins to the Atlantic cod estimated proteome, 4 potential protein candidates were identified. Only 2 of these candidates contained predicted albumin domains and one was further identified as a phosphatidylinositol 4-phosphate 5-kinase type-1 protein. The last candidate, annotated as GENS SCAN00000070264, was a small protein of 101 Aa and contained only one domain assigned to the albumin superfamily. This albumin domain was estimated at Aa 20–83 by NCBI Blast, and at Aa 19–101 by PROSITE and InterPro (results not shown). Based on these findings, the GENS SCAN00000070264 protein was considered as the only albumin-like protein present in Atlantic cod that is homologous to albumins of other Teleost species. Its highest scoring homolog from the NCBI Blast search was the serum albumin 1 precursor of Atlantic salmon (*Salmo salar*) (results not shown). The matched sequence was 96% identical in a continuous segment of 83 Aa in the salmon albumin sequence area 383–465, and this segment included three binding sites of the salmon albumin. However, the Atlantic cod sequence only covers 17% of the full sequence of this salmon albumin. A NCBI search against the human serum albumin showed that the Atlantic cod albumin-like protein shared no sequence homology to this protein. The predicted protein structures of the albumin-like protein of Atlantic cod was investigated (Fig. 1), and found to contain mainly helices (Fig. 1C), eight possible binding regions (Fig. 1E) and display three



**Fig. 1.** Structure characteristics of the albumin-like protein of Atlantic cod (*Gadus morhua*). The structure features are shown in A: predicting the protein binding sites (red pins), polynucleotide binding region (orange pin), disulphide bonds (black lines), helices (red boxes), exposed areas (blue boxes), buried areas (yellow boxes) and disordered regions (green boxes). The amino acid composition (B), the secondary structure composition (C) and the solvent accessibility (D) are also presented, along with a list of the amino acid identification of the predicted protein binding regions (E).

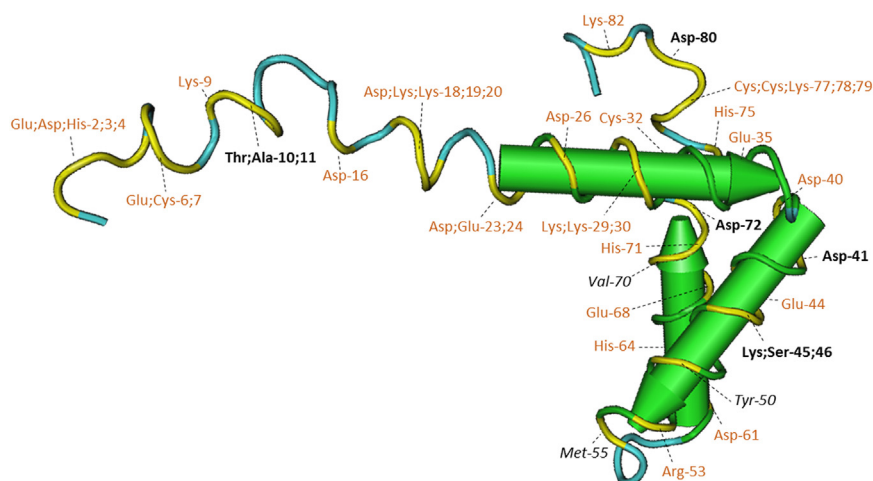
quarters of the protein as exposed regions (Fig. 1D). This information corresponds well to the general albumin characteristics. To gain further insight into the relationship of the Atlantic cod albumin-like protein to that of other Teleosts albumin/albumin-like proteins, all full Aa sequences available was compared in a phylogenic analysis (Fig. 2). The Teleost albumin/albumin-like proteins are primarily in the range of 608–613 Aa long, except for the albumin/albumin-like proteins of Gold line fish and Australian lungfish, notated with 209 and 101 Aa respectively. The albumin-like protein of Atlantic cod is therefore small in comparison to most reported albumin/albumin-like proteins. Studies on the lungfish albumin showed that this protein is evolutionary closer to the albumins of higher vertebrates compared to those of other fish [20]. In the present result, the albumin-like proteins of Atlantic cod and Spottet gar separated from the rest on the second branch, suggesting a different evolutionary development of these proteins compared to the others. However, it is important to notice that the full-length albumin sequences of Teleost species available for the phylogenic comparison is limited to only six species.



**Fig. 2.** The phylogenetic relationship between the albumin-like protein of Atlantic cod (*Gadus morhua*) and other full-length annotated albumin/albumin-like proteins of Teleosts.

## 2.2. PAH binding potential of the Atlantic cod albumin-like protein

Albumins high binding affinity for hydrophobic compounds allows it to bind exogenic hydrophobic contaminants such as PAHs. PAH-albumin adducts are generated via electrophilic substitution reactions at specific Aa sites of the albumin, forming a covalent bond between the two [7]. Some PAHs are found more likely to cause adductations as they present a more adduct friendly structural organizations (i.e. the bay-regions of benzo[*a*]pyrene) or they have higher frequency of specific reactive metabolic states, e.g. anti-diol epoxide metabolites [13]. The previously identified binding sites of PAH metabolites to albumin are primarily at Aa residues of Arg, Asp, Cys, Glu, His and Lys [9, 10, 13]. Throughout the sequence of the albumin-like protein of Atlantic cod, there were 1 Arg, 11 Asp, 4Cys, 7 Glu, 5His and 8 Lys residues, comprising 36% of the full sequence. The occurrence of PAH-Cys adduct can be considered less likely based upon the protein structure prediction, as all Cys appears to be involved in disulfide bonds, making them unavailable for potential adduct formation. The gathered information of all the potential binding sites of the Atlantic cod albumin-like protein are visualized in a 3D model (Fig. 3). In human serum albumin, PAH adducts bind almost exclusively within a binding pocket of subdomain 1B [13, 23], and based on the model prediction, the albumin-like protein of Atlantic cod may present similar binding pocket conformations. The occurrence of PAH adduct formation in the Atlantic cod albumin-like protein can therefore be considered as a possible event. The species differences regarding protein size (i.e. human albumin



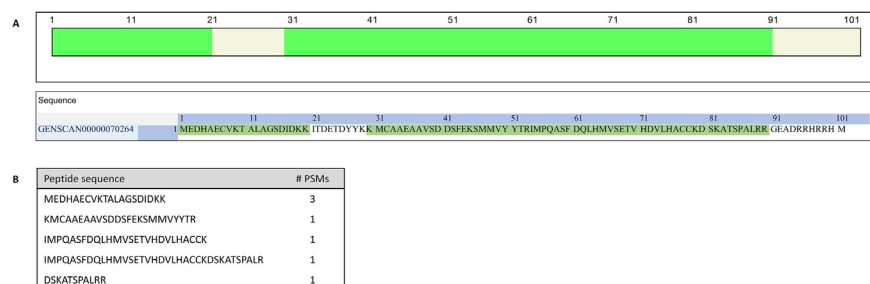
**Fig. 3.** The predicted 3D model of the albumin-like protein of Atlantic cod (*Gadus morhua*) based upon its amino acid sequence 1–85. The potential binding sites (yellow) were manually annotated based on the gathered knowledge from the known binding sites of its highest scoring Teleost homolog (NP\_001117137) (*italic black*), the protein structure investigation (**bold black**), and the location of the known PAH binding site residues (e.g. Arg, Asp, Cys, Glu, His and Lys) from other studies (*orange*).

consists of 585 Aa) and PAH metabolic pathways can potentially be accelerating and/or limiting factors of the adduct formation potential of the Atlantic cod albumin-like protein. Lower levels of PAH-albumin adducts (i.e. benzo[*a*]pyrene-His adducts) have previously been reported in fish compared to the levels found in humans [12].

### 2.3. Detection of PAH-albumin in Atlantic cod plasma

In a previous study, no albumin-like proteins of the Atlantic cod plasma proteome were identified among the characterized high abundant proteins (HAPs) of the Atlantic cod plasma proteome [24]. Further, it was found that a 14-kDa apolipoprotein and an apolipoprotein A-I were the two most dominating plasma proteins of Atlantic cod [24]. This led to the hypothesis that the Atlantic cod albumin-like protein is relatively low abundant and that the apolipoproteins may cover some of the features of the albumin/albumin like proteins.

In the current study, efforts were therefore made to identify the predicted albumin-like protein of Atlantic cod by analyzing fractionated plasma samples by LC-MS/MS analysis. These samples were collected from 16 individual fish, and for each sample, two protein fractions were obtained by immunodepletion chromatography prior to analysis. The resulting MS data was analyzed against the *Gadus morhua* EST database, and 360 proteins (1249 unique peptides) were identified. However, no identification of the albumin-like protein was obtained. This could be due to overshadowing effect of HAPs, despite the applied sample fractionation effort. Potential peptide spectrum matches (PSMs) for the albumin-like protein might therefore be lost or assigned to other proteins due to limitations of the MS technique and/or to software restrictions. A second analysis of the MS data was performed, with a database consisting of only the albumin-like protein (and seven common contaminant proteins that were identified in the first search). Here, the albumin-like protein was identified, based upon 7 individual PSMs from 6 of the 32 samples, covering 80% of the sequence (Fig. 4). A MS/MS spectra assigned to



**Fig. 4.** Identification of the albumin-like protein of Atlantic cod (*Gadus morhua*) in plasma. The sequence coverage at 80% as provided by Proteome Discoverer (A) and the list of detected peptide sequences and their peptide spectrum match (PSM) abundance (B) are reported.

the most identified peptide (i.e. MEDHAECVKTALAGSDIDKK) is shown in Fig. 5. These results support the proposed presence of an albumin-like protein in the plasma of Atlantic cod. Further work is required to improve the analytical detection rate of this protein. This is recognized as a challenging task due to the proteins predicted small size and low abundance, especially when considering the complex nature of blood plasma as sample matrix. Since frequencies of PAH-protein adduct events are generally low [7], the potential PAH-albumin biomarker in Atlantic cod is considered unlikely to be a biomarker tool of PAH contamination.

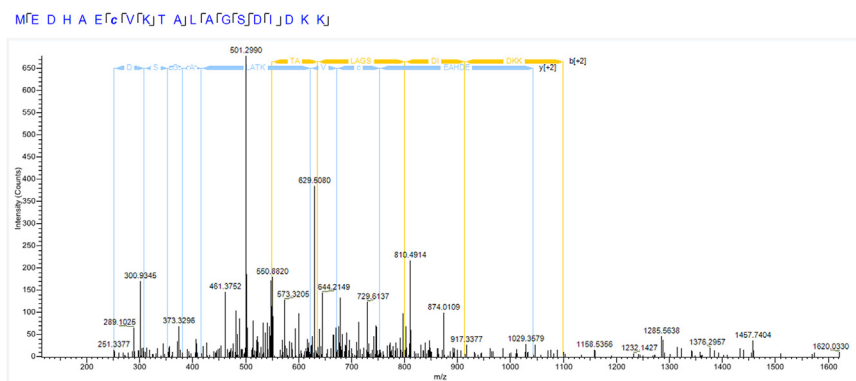
### 3. Conclusions

For the first time, an albumin-like protein is reported in the plasma proteome of Atlantic cod. The potential of this protein to be a biomarker of PAH exposure in Atlantic cod has been theoretically evaluated, due to its relevance in human studies. However, due to the low abundance of this protein and the related analytical challenges, it may not be easily applicable, especially when considering the low frequency of PAH-protein adduct events. This leads to the conclusion that detection of PAH-albumin adducts in Atlantic cod is unlikely to be suitable as biomarker tool for monitoring and risk assessments of PAH contamination.

## 4. Materials and methods

### 4.1. Desktop bioinformatics

The FASTA-files of the albumin/albumin-like protein homologs was collected from the NCBI (National Center for Biotechnology Information) protein database (available from <https://www.ncbi.nlm.nih.gov/>) generating a Teleost albumin database. This database was used to search for homologous proteins in the Atlantic



**Fig. 5.** Mass spectrometry/mass spectrometry spectra of one of the assigned MEDHAECVKTALAGSDIDKK peptide of the albumin-like protein of Atlantic cod (*Gadus morhua*), displaying the 2-charge fragment state.

cod (*Gadus morhua*) EST database (downloaded from Ensembl, available from <http://www.ensembl.org/index.html>, downloaded 30th Desember 2016, 77408 sequences) using Blast2GO (v. 4.0.7). The identified homologs (GENSCAN00000011784, GENSCAN00000023291, GENSCAN00000077583, GENSCAN00000070264) were searched in NCBI Blast (BLASTP 2.6.1+, non-redundant database) to find predicted protein identifications and domains. Additional domain investigation was performed by PROSITE (ExPASy Bioinformatics Resource Portal, Swiss Institute of Bioinformatics, available from <http://prosite.expasy.org/>) and InterPro (Protein sequence analysis & quantification, European Molecular Biology Laboratory, available from <http://prosite.expasy.org/>). An additional Blast search was performed to compare the Atlantic cod protein GENSCAN00000070264 against human serum albumin (AAA98797).

Phylogenetic analysis was performed on the Atlantic cod albumin-like protein (GENSCAN00000070264) and annotated full-length albumin/albumin-like proteins of other Teleost species by ClustalX (v. 2.0) and NJplot (v. 2.3).

The secondary structure analysis of the Atlantic cod albumin-like protein (GENSCAN00000070264) sequence was done by PredictProtein (Rostlab, available at <https://www.predictprotein.org/>).

The 3D model PDB-file of the Atlantic cod albumin-like protein (GENSCAN00000070264) was built by SWISS-MODEL (Biozentrum, Swiss Institute of Bioinformatics, available from <https://swissmodel.expasy.org/>), with template results: Template model 4po0.1.A; oligo-state monomer; sequence similarity 0.34; range 1–85; coverage 0.82; description Serum albumin; GMQE 0.61, QMEAN –1.67. VAST (Vector Alignment Search Tool, NCBI, available from <https://www.ncbi.nlm.nih.gov/Structure/VAST/vastsearch.html>), converted the PDB-file into Cn3D-file format, and the 3D model was visualized in Cn3D (v. 4.3, NCBI).

## 4.2. Sample preparation and LC-MS/MS analysis

Ethical approval of the experiment was obtained by the Norwegian Food Safety Authorities. Blood samples of wild Atlantic cod ( $n = 16$ ) was drawn (approximately 2 mL) using heparinized syringes (Na-heparin, 5000 IE/a.e./mL, LEO Pharma) and centrifuged at 5000 g for 5 min at 4 °C. The resulting plasma fraction was transferred into cryotubes, snap-frozen in liquid nitrogen and stored at –80 °C until further sample preparation.

The plasma sample was thawed and 40  $\mu$ L plasma were diluted with 360  $\mu$ L TBS, and desalted by the use of 3 kDa size exclusion filters according to the manufacturers specifications (Ultracel<sup>®</sup> 3 K Membrane, Amicon<sup>®</sup> Ultra Centrifugal Filters, Merck Millipore). Further, samples were fractionated by HDL immunocapture according to the manufacturers specifications (High Density



Lipoprotein (HDL) Depletion Column (IgY Kit), GenWay Biotech Inc.). Both resulting fractions were then buffer exchanged into 50 mM Ambic by use of the 3 kDa size exclusion filters according to the manufacturers recommendation. Protein concentration was measured in all sample fractions ( $n = 32$ ) by the Bradford method [25]. Volumes equivalent to 20  $\mu\text{g}$  proteins from the HDL depleted fraction and 10  $\mu\text{g}$  proteins from the HDL fraction, were transferred into new tubes. Further sample preparation included: sample reduction (1  $\mu\text{L}$  DTT (1 M), 45 min incubation at room temperature), alkylation (5  $\mu\text{L}$  IAA (1 M), 45 min incubation at room temperature), alkylation stop (5  $\mu\text{L}$  DTT (1 M), 45 min incubation at room temperature), and digestion with trypsin (4.29  $\mu\text{M}$  in 0.01% formic acid, Trypsin Gold, Promega, ratio 1:40 trypsin:protein, incubation overnight (18 h) at 37 °C). Plasma protein analysis was then performed using LC-MS/MS, as described in Pampanin et al. (2014) [26]. In brief, ultrahigh pressure liquid chromatography (UHPLC) coupled to LTQ Orbitrap XLTM Hybrid Ion Trap-Orbitrap Mass spectrometer (Thermo Scientific) was applied with total run-time of 250 min followed by a 90 min washing step and re-equilibration of the column. The mass spectrometer was calibrated (SUPELCO ProteoMass LTQ/FT-Hybrid ESI Pos. Mode Cal Mix MSCAL5-1EA, Thermo Scientific) prior to analysis.

The resulting raw data files were first analyzed by Proteome Discoverer 2.0 using the Sequest HT search engine against the *Gadus morhua* EST database (search 1). Trypsin was set as a digestion enzyme allowing for 2 missed cleavages. Precursor ion tolerance was set to 10 ppm and fragment mass tolerance to 0.6 ppm. Oxidation of methionine and carbamidomethylation of cysteine was set as fixed and static modifications, respectively. Peptide confidence filter was set to be at least medium, with the following combination of charge ( $z$ ) and X correlation: high significance 1.2 ( $z = 1$ ), 1.9 ( $z = 2$ ), 2.3 ( $z = 3$ ) and 2.6 ( $z \geq 4$ ); medium significance 0.7 ( $z = 1$ ), 0.8 ( $z = 2$ ), 1.0 ( $z = 3$ ) and 1.2 ( $z \geq 4$ ). Minimum peptide length was set to 6 amino acids. Furthermore, a decoy database search was performed with target false discovery rate, strict and relaxed parameters set to 0.01 and 0.05, respectively, and the cRAP database (downloaded from <http://www.thegpm.org/crap/>), 30th December 2016) was used to identify potential common contaminants in the dataset.

A second search (search 2) was performed on the same raw MS data in Proteome Discoverer 2.0 using the Sequest HT search engine against a database of 8 proteins: the albumin-like protein of Atlantic cod (GENSCAN00000070264) and the seven common contaminants detected in search 1 (CAS1\_BOVIN; ALBU\_BOVIN; ALBU\_HUMAN; TRYP\_PIG; K22E\_HUMAN; K2C1\_HUMAN; CASB\_BOVIN). The other parameters were the same as for search 1, with the exception of peptide confidence that was set to be at least high, with the following combination of charge ( $z$ ) and X correlation: high significance 1.2 ( $z = 1$ ), 1.9 ( $z = 2$ ), 2.3 ( $z = 3$ ) and 2.6 ( $z \geq 4$ ).

## Declarations

### Author contribution statement

Karianne Skogland Enerstvedt: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Magne O. Sydnes, Daniela M. Pampanin: Conceived and designed the experiments; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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