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Rapid Determination of Fish Species of Raw and Heat-Treated Fish Meat Using Proteomic Species-Specific Markers

Running title: Rapid Determination of Fish Using Species-Specific Markers

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SUMMARY

Research background. The main issue regarding authenticity of fish meat lies mainly in misleading labelling or species substitution like replacing valuable fish meat with species of lower value or species originating from illegal fishing. For these reasons, the need for adequate analytical methods for the detection of food fraud has arisen.

Experimental approach. This study aimed to differentiate six fish species—carp, mackerel, pike, pollock, salmon, and trout—based on differences in their protein composition using two mass spectrometry methods. Matrix-Assisted Laser Desorption/Ionization – Time Of Flight Mass Spectrometry (MALDI-TOF MS) was employed to identify characteristic species-specific *m/z* values for raw and cooked fish meat discrimination. Additionally, Liquid Chromatography – Electrospray

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Ionization – Quadrupole – Time Of Flight (LC-ESI-Q-TOF) was used to determine specific amino acid sequences in carp and salmon, selected as model species.

Results and conclusions. Distinct species-specific *m/z* markers were identified for all six fish species, enabling their differentiation in both raw and processed forms. In carp and salmon, hundreds of peptide sequences were detected, leading to the identification of a panel of peptide markers that determine both fish species and meat processing type. The findings confirm that mass spectrometry-based proteomic approaches can serve as effective tools for fish meat authentication.

Novelty and scientific contribution. This study demonstrates the feasibility of using two complementary mass spectrometry techniques for reliable and rapid fish species authentication. The identification of specific peptide markers and species-specific *m/z* values contributes to improving food authenticity control, offering a powerful approach for detecting fish meat adulteration.

Keywords: fish meat; fish species; species-specific *m/z* values; peptide markers; mass spectrometry; food authentication

INTRODUCTION

In the case of fish and seafood, the main issue regarding authenticity seems to be misleading labelling or species substitution (replacing a more expensive fish with a cheaper one). Specifying the species is a mandatory requirement in the vast majority of legislative regulations. Especially in processed products, where visual identification may not be possible in some cases, the identity of the animal can be falsified. There is typically an economic incentive to replace valuable materials with species of lower value or species originating from illegal fishing. Another problem is the fact that many species of seafood are sold under a collective name (*1–4*).

The methods used for meat authentication are generally based on DNA or protein analysis. Molecular techniques based on DNA analysis have experienced tremendous development in recent decades. They overcome some limitations of methods based on protein analysis, such as protein denaturation during heat treatment of meat, which can lead to changes in the antigenicity of molecules and their electrophoretic mobility (*5,6*). However, similar challenges may arise with DNA barcoding when distinguishing closely related species. For example, although mitochondrial cytochrome c oxidase (COI) subunit 1 DNA barcoding successfully identified 14 of 16 freshwater fish species from Lake Wivenhoe (Queensland, Australia), two undifferentiated species from the family Terapontidae, which have identical COI gene sequences, could not be distinguished using this method (*7*). This highlights a limitation of DNA barcoding in differentiating closely related species, a problem that may

also exist in protein-based methods. The digital polymerase chain reaction (dPCR) and its modified form ddPCR (droplet digital PCR) are the oldest used DNA amplification technologies that use a water-oil emulsion drop system (*5,8*). Doi *et al.* (*9*) used ddPCR for the detection of environmental DNA (eDNA) originating from an invasive fish species – the bluegill sunfish (*Lepomis macrochirus*). Furthermore, ddPCR was applied on the identification and quantification of the highly valued silver pomfret (*Pampus argenteus*), whose falsification is a serious worldwide problem (*10*) and also for analysing of marine products from cod (*Gadus chalcogrammus*), which is of great commercial importance (*11*). PCR analysis with restriction fragment length polymorphism (PCR-RFLP) is the most widely used method for identification of meat species including fish (*12–14*). This assay has been optimized to differentiate three closely related gadoid fish species: Alaska pollack, Pacific cod and Atlantic cod in commercial seafood products (*15*). Lin and Hwang (*16*) successfully identified eight tuna species in canned products using this technique. Species-specific PCR can be used to identify the taxonomical origin of fish meat and seafood products. For example, Kim *et al.* (*17*) differentiated three related grouper fish species: *Epinephelus septemfasciatus*, *E. bruneus* and *E. akaara*. Multiplex PCR is a method that enables simultaneous identification of several species at the same time. This technique was applied to distinguish seven Clupeiform species including several economically important fishes *e.g.* herring and sardines (*18*). Real-time PCR has also found application in the case of fish meat authentication. The method was developed for the differentiation and quantification of two closely related tuna species (bigeye tuna – *Thunnus obesus*, yellowfin tuna – *Thunnus albacares*) in canned products (*19*). The combination of real-time PCR and multiplex PCR was used for the identification of eight ecologically and economically important freshwater fish species Hulley *et al.* (*20*).

Traditional techniques based on protein analysis include immunological, chromatographic, spectroscopic, and electrophoretic methods. The U.S. Food and drug administration (FDA) conducts the Regulatory fish encyclopaedia (*21*) that is serving as a repository of information on protein analyses for fish identification. Traditional protein methods face challenges due to the denaturation or degradation of proteins that often occurs during the sample preparation process. This makes these methods generally unsuitable for identifying proteins in processed meat. However, in some studies, fish species were identified by Enzyme-Linked Immune Sorbent Assay (ELISA) using antibodies against muscle proteins. It involved distinguishing canned sardines from other fishes such as herring, mackerel, anchovy (*22*) and identifying individual species of flatfish (*23*). Red snapper was also identified using this technique (*24*), as well raw and processed grouper meat was distinguished from cheaper fish species (*25*).

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used by a group of Pineiro *et al.* (*26*) for distinguishing of 15 species in raw and cooked conditions. Also, the combination of SDS-PAGE with isoelectric focusing was successful in species identification of unknown samples (*27,28*). Martinez and Friis (*29*) used two-dimensional electrophoresis (2-DE) to investigate the authenticity of fishes and shrimp and also to assess their freshness using separated myofibrillar proteins. Berrini *et al.* (*30*) distinguished four species of fishes, which sold under the same trade name "perch", using a method focusing on sarcoplasmic proteins.

High-Performance Liquid Chromatography (HPLC) methods for determining the type of meat typically rely on analysing protein, peptide, or amino acid profiles unique to different meat types. For instance, 31 fish species were distinguished through HPLC analysis of water-soluble sarcoplasmic muscle proteins (*31*). However, it was found that heat treatment (cooking) had a relatively significant effect on the quality of the chromatograms (*31*). Chou *et al.* (*32*) developed a method applicable to fresh and cooked meat for the routine discrimination between meat products from 15 common animal species (mammals, birds, and fishes) based on HPLC with electrochemical detection using copper nanoparticles.

Currently, mass spectrometry (MS) techniques play a key role in the analysis of proteins and peptides in food products, including the investigation of the authenticity of meat and meat products. For example, Volta *et al.* (*33*) distinguished three freshwater fish meat species (*Alosa agone*, *Coregonus macrophthalmus*, and *Rutilus rutilus*) according to the differences in the spectra of muscle tissue using MALDI-TOF MS.

In this work, the proteomic approach using MALDI-TOF and LC-ESI-Q-TOF mass spectrometry was tested to distinguish six selected (three marine and three freshwater) fish species. The samples were digested with trypsin without additional protein extraction before proteomic analyse using both mass spectrometry methods. This approach was successfully used for preparation of different samples of taxonomical origin (*34,35*). The obtained data were evaluated using the PostgreSQL database system created in our laboratory, which was accessed using the pgAdmin interface (*36*). Species-specific markers (*m/z* values and amino acid sequences) enabling reliable identification of fishes were found. Also, the possibility of distinguishing between raw and heat-treated meat was investigated. The goal was to distinguish between individual species even in the case of heat treatment, and to try to distinguish between raw and heat-treated meat of the same species.

MATERIALS AND METHODS

Reagents and materials

Acetonitrile (ACN) (LC-MS grade), 2,5-dihydroxybenzoic acid (DHB), formic acid (FA), and trifluoroacetic acid (TFA, suitable for HPLC-MS) were purchased from Sigma-Aldrich (Burlington, Massachusetts, USA). Ammonium hydrogen carbonate (AHC, suitable for HPLC-MS) was obtained from Lachema (Brno, Czech Republic). Peptide Calibration Standard II was purchased from Bruker Daltonics (Bremen, Germany). Pierce Trypsin Protease MS Grade was obtained from ThermoFisher Scientific (Waltham, Massachusetts, USA). The commercially available reverse phase C_{18} ZipTip pipette tips were purchased from Millipore Corporation (Burlington, Massachusetts, USA). The water was purified with a Milli-Q water purification system from Millipore Corporation (Burlington, Massachusetts, USA).

Reference samples of fish meat

The meat from six selected fish species were analysed. Among them were three freshwater species: common carp (*Cyprinus carpio*), northern pike (*Esox lucius*), rainbow trout (*Oncorhynchus mykiss*), and three marine species: Atlantic mackerel (*Scomber scombrus*), Alaska pollock (*Theragra chalcogramma*) and Atlantic salmon (*Salmo salar*). Freshwater fish species were purchased from the local fish store named Štičí líheň ESOX spol. s. r. o. in Tábor (Czech Republic), marine species were from wholesale chains Albert and Lidl. Three individuals of each species (biological replicates) were analysed in this work.

Two types of samples were prepared for each fish: raw and cooked meat. The heat-treated samples were prepared by boiling 1–2 g of cut fish meat in boiling water for 10 min. Both types of samples were subsequently stored in a freezer (-80 °C). These primary samples were later sampled for measurement on the day of analysis. Three samples (technical replicates) were weighed (approx. 1 mg) for each individual. In total there were nine samples of raw meat and nine samples of cooked meat from each fish species, which were subsequently analysed.

Sample preparation

The 1 mg of each sample was digested in 20 µL of 50 mM AHC containing 0.02 mg/mL of trypsin at 37 °C with constant shaking for two hours. After two hours, the cleavage was terminated by adding of 1 µL of 10 % TFA solution to a final concentration of 0.5 % TFA. After the trypsin digestion, the samples were purified and concentrated on reverse phase ZipTip C18. After purification, 10 µL of each purified sample were obtained.

MALDI-TOF MS measurements and data acquisition

2 µL of purified peptide sample were mixed with 7.5 µL of DHB matrix solution (8.5 mg of DHB in 0.5 mL of mixture of acetonitrile/0.1 % TFA in water). 1.3 µL of the resulting mixture was spotted thrice on the stainless steel MALDI target and air-dried. Mass spectra were acquired using MALDI-TOF Autoflex Speed mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a Nd:YAG laser (355 nm) in positive reflector mode. The obtained spectra contained peaks in the 900–4500 *m/z* interval. The corresponding spectrum was obtained from a total of 7000 shots for each spot.

LC-ESI-Q-TOF MS conditions and data acquisition

Measurements were carried out using UHPLC Dionex Ultimate3000 RSLC nano (Dionex, Bremen, Germany) connected to ESI-Q-TOF Maxis Impact mass spectrometer (Bruker Daltonics, Germany). Purified and air-dried samples (after trypsin digestion, see Sample preparation) were dissolved in 10 µL of mixture of 3 % acetonitrile and 0.1 % formic acid. 3 µL of the solution were loaded into an Acclaim PepMap 100 C18 trap column (100 µm x 2 cm, size of reverse phase particles 5 µm; Dionex, Bremen, Germany) with a flow rate of 5 µL/min of 3 % the mobile phase B for 7 minutes. The peptides were then eluted from the trap column into Acclaim PepMap RSLC C18 analytical column (75 µm x 150 mm, size of reverse phase particles 2 µm; Dionex, Bremen, Germany) using the following gradient: 0–5 min 3 % B, 5–35 min 3–35 % B, 37 min 90 % B, 37–50 min 90 % B, 51 min 3 % B, 51–60 min 3 % B. The mobile phase A consisted of 0.1 % formic acid in water and mobile phase B of 0.1 % formic acid in acetonitrile. The flow rate during gradient separation was set at 0.3 µL/min. The peptides were eluted directly to an ESI source – Captive spray (Bruker Daltonics, Bremen, Germany). Measurements were carried out in positive ion mode with a precursor selection in the range of 400–1400 Da; up to 10 precursors were selected for fragmentation from each MS spectrum.

MS spectrum was recorded every 3 s, MS/MS spectra were collected at 4–16 Hz depending on precursor intensity. Dynamic precursor exclusion was set to 1 min, preferred number of precursor charges was 2–5. Singly charged precursors were excluded from fragmentation. Collision-induced MS/MS spectra were recorded in the range 50–2200 *m/z*. Mass spectra were extracted by DataAnalysis 4.1 (Bruker Daltonics, Bremen, Germany) and loaded into Proteinscape 4.2 (Bruker Daltonics, Bremen, Germany) and later into Mascot 2.4.1 (Matrix Science, Boston, USA), which was used for protein identification. The identification was carried out against a single-species database containing the proteome of the investigated species (*Cyprinus carpio, Salmo salar*; was downloaded from the Uniprot website, 4/4/2022) (*37*), which was supplemented with common laboratory contaminants. There were following identification parameters: enzyme trypsin (one missed cleavage

site was allowed), oxidation of methionines as a variable modification, accuracy of assigning precursors 10 ppm and fragments 0.05 Da. Identified peptides and proteins were filtered to maintain a false positive identification rate of 1 %. All samples were analysed by LC-ESI-Q-TOF MS in three repetitions to obtain characteristic peptide profiles.

Searching for species-specific markers

To distinguish individual types of fish meat, species-specific markers were identified as *m/z* or peptides that occurred with certain frequency in spectra obtained from one specie but were absent in the spectra of other fish species. The frequency represents a number of spectra in which the particular *m/z* values or amino acid sequences occurred. Then these peptides or *m/z* values can be considered species-specific markers within the selected group of fishes. Mass spectra were processed using two complementary methods: MALDI-TOF and LC-ESI-Q-TOF.

For MALDI-TOF data processing, the mMass software (version 5.5.0) was utilized (*38*). The involved spectrum smoothing, baseline correction, and manual peak selection, where 80–110 peaks were selected for each MALDI-TOF spectrum. The *m/z* values from spectra obtained for a single species were then recorded in Microsoft® Excel® for further analysis.

For managing and analysing the extensive data, we used the PostgreSQL object-relational database system (version 2022.4.4) with pgAdmin 4 (version 6.21), an open-source graphical administration tool for PostgreSQL (*36*). The analysis focused on identifying *m/z* values that appeared consistently across spectra for each species. This process was adjusted to include only peaks present in a specific frequency, set in this study to a minimum of 23 out of 27 MALDI spectra (three individuals, three technical repetitions, and three spots per technical repetition).

Similarly, Excel data files containing results (identified peptides and their corresponding proteins) from LC-ESI-Q-TOF MS were processed using comparable steps. Species-specific peptides were identified as those consistently present in all nine Excel data files corresponding to a single species (three individuals, three technical repetitions, where each technical replicate was injected once).

RESULTS AND DISCUSSION

Results from MALDI-TOF mass spectrometry

MALDI-TOF MS measurements were conducted on all six fish species, analysing both raw and cooked samples. This analysis identified *m/z* values that serve as species-specific markers, characterizing each fish species.

Searching for species-specific markers by MALDI-TOF MS

The list of peptides (*m/z* values) was obtained for each of the fish species using the data evaluation described in Searching for species-specific markers. In the next step, values were compared between individual species in order to find characteristic values for one specific species, i.e. a given *m/z* value occurred in one species, but not in any of the others. The list of characteristic values for raw and cooked meat samples is shown in Table 1. Fig. 1 shows the MALDI-TOF MS spectra for carp, pollock, and salmon, highlighting the differences in their characteristic *m/z* values. These spectra serve as a visual representation of some species-specific markers identified in our analysis.

The distinction of fish species using species-specific protein patterns by MALDI-TOF MS has been previously demonstrated in different studies (*39–42*). These studies employed a simpler approach without trypsin digestion, analysing extracted proteins directly. Enzymatic cleavage was only used in a subsequent step for protein biomarker identification. This method has proven effective for distinguishing species, even without the need for tryptic digestion, particularly for species that are not closely related, such as the six species in our study.

Our approach differs fundamentally in using trypsin digestion prior to MALDI-TOF MS measurements. This technique, known as in-sample digestion, fragments proteins into peptides, resulting in spectra with a broader range of information and a higher resolution. While the approach of Mazzeo *et al.* (*39*) identified markers in the >11000 *m/z* range, corresponding to small proteins like parvalbumins, or Stahl and Schroeder (*40*), who collected spectra in the mass range 2–20 kDa, our analysis focuses on the 900–4500 Da range, capturing differences across the entire proteome rather than targeting specific proteins.

Spielmann *et al.* (*42*) explored the use of MALDI-TOF MS for processed meat and developed a database of species-specific fish proteins using the Biotyper tool. While effective, the Biotyper tool is proprietary and requires a paid license, making it less accessible. In contrast, the approach described in this paper utilizes PostgreSQL, an open-source and entirely free database system, which not only reduces costs but also provides broader accessibility to researchers and laboratories.

Although the distinction of the six tested species could likely also be achieved using simpler methods, this method demonstrates strong potential in the context of heat-treated fish meat, where the higher resolution and broader marker identification provide added value. By focusing on peptide-level markers and leveraging accessible tools, the shown approach offers a cost-effective and innovative alternative for fish meat authentication.

Markers were then searched for different types of cooked fish meat in the same way. In this analysis, at least two specific markers were found for each species. The list of characteristic values of the cooked meat samples is shown in Table 1. A slightly bigger number of characteristic values was found for cooked meat. This can be explained by the fact that proteins in their native structure are not as easily accessible to trypsin cleavage in some positions as the loosened, thermally denatured proteins. Some *m/z* values are identical for raw and cooked fish meat. These values can be considered as characteristic markers for the given species regardless of the type of meat processing (underlined bold values in the Table 1). The other half of the *m/z* values, which do not match between raw and cooked meat, proves that some markers differ depending on the type of processing to distinguish the given species. The obtained results prove the feasibility of the method also for cooked meat and at the same time confirm the stability of some MALDI-TOF MS markers during heat treatment (markers found for raw and cooked meat at the same time), mentioned in the literature (*39,40*).

In the next step, the markers that could distinguish between raw and heat-treated meat of the same species were searched. The search for markers was carried out sequentially for all investigated species and its result is shown in Table 2. According to the results, the raw fish meat samples contained more specific markers compared to their cooked counterparts. Underlined bold values represent peaks already included in the previous two tables, where markers for interspecies discrimination of raw or cooked meat. These markers are with a high discriminatory value, which, if present in a sample, can determine not only the type of fish meat, but also its type (raw or heat treated). Such markers were found for raw meat of carp, mackerel, and pike, in the case of cooked meat of carp and mackerel.

Results from LC-ESI-Q-TOF mass spectrometry

The LC-ESI-Q-TOF analyses were performed only on carp and salmon samples, both raw and cooked (a total of 36 samples were analysed – nine for each type of processing of these species). The reason was that the used protein database Uniprot (which peptides and proteins are searched against) are not complete in the category of fish species. Therefore, the only two species whose protein sequences are available in the database were selected. This method can detect the amino acid sequences of peptide fragments and identify the protein from which the fragments originated, allowing more accurate data than *m/z* values commonly obtained by MALDI-TOF MS.

Identified proteins

Over 100 proteins (100–150) detecting by at least two peptides (with the length from 7 to 30 amino acids) were identified in each of the samples. The same proteins were mostly identified for cooked and raw meat of the same species, but the number of found peptides differed between them, which again indicates slightly different cleavage by trypsin of the heat-treated proteins. Table 3 shows selected identified proteins in raw and cooked carp meat. Similarly, Table 4 shows proteins for salmon.

The proteins with the biggest number of identified peptides include myofibrillar proteins, which are the most abundant proteins in fish muscles: myosin, actin, nebulin, titin, and tropomyosin. All proteins from this group participate in a process of muscle contraction, which they either directly ensure or fulfil a regulatory function.

A big number of proteins from the second most represented group of proteins in fish meat was detected: sarcoplasmic proteins. Mainly enzymes belonging to the group of these water-soluble proteins were identified, *i.e.* creatine kinase (EC 2.7.3.2), glycogen phosphorylase (EC 2.4.1.1), SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase; EC 7.2.2.10), fructose-1,6-bisphosphate-aldolase (EC 4.1.2.13), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), and phosphofructokinase (EC 2.7.1.11). Other sarcoplasmic proteins as myoglobin, α and β subunits of haemoglobin, and parvalbumin were found. Parvalbumin is an important sarcoplasmic protein used for fish meat authentication, and it is the main allergen of fish meat.

The third group of very little represented stromal proteins was represented by two types of collagens, type I and VI. In both fishes, carp and salmon, significantly more peptides belonging to collagens were found in cooked meat.

Searching for characteristic peptide sequences

Evaluation of the LC-ESI-Q-TOF data against the Uniprot database also enables a closer look at the peptide fragments. The found specific sequences of peptide fragments were analysed (similarly to the search for MALDI-TOF *m/z* markers) by the program pgAdmin 4, which works with the PostgreSQL database system (see Searching for species-specific markers).

Distinguishing between carp and salmon

The evaluation was carried out separately for raw carp and salmon samples and separately for cooked ones, using the identical procedure. Firstly, it was determined which peptides were found in all samples of one species (*i.e.* nine samples: three biological individuals for one species, three

technical repetitions from each individual) using the software tool pgAdmin 4. Secondly, this was followed by the determination of characteristic sequences for the given species, which were found in all samples of one species and at the same time in not a single sample of the other. These characteristic sequences were approximately 400 for carp and 550 for salmon in the raw meat, and approximately 400 sequences for carp and 600 for salmon in the cooked meat. At this point it is important to emphasize that "characteristic" means specific only to the other compared species in this case, i.e. sequences of carp are characteristic to salmon and vice versa. This is a proposal of a new method by which the fish meat could be identified, after obtaining the measured data from a sufficient number of fish species. The results obtained for carp and salmon indicate that this method of identification can work. The advantage over MALDI-TOF MS is that it is possible to determine the specific peptide sequences responsible for distinguishing fish species and to identify the protein of origin from which these peptides are derived.

The most significant proteins, in terms of species discrimination, can be determined based on the number of the found characteristic sequences. Table 5 shows the five proteins with the largest number of characteristic sequences for raw and cooked salmon and carp meat.

In raw carp samples, significantly more characteristic sequences came from glycogen phosphorylase (10) than in cooked ones (4). Some proteins did not provide any characteristic sequences in the cooked carp meat samples – for example, the ryanodine receptor (a receptor associated with calcium channels) or the enzyme malate dehydrogenase. Both proteins had two characteristic sequences in the raw carp samples. The raw salmon meat contained more characteristic sequences for the enzymes phosphoglucomutase (8 versus 3) and glycogen debranching enzyme (8 versus 2) in comparison to cooked meat.

On the contrary, cooked carp and salmon samples included significantly more characteristic sequences originating from two proteins: parvalbumin and collagen. This mentioned two proteins were of great importance in distinguishing the cooked species (altogether around 20 characteristic sequences), while in the raw carp no characteristic sequence from these two proteins and in salmon only two from parvalbumin and one from collagen were found. In carp, 11 characteristic sequences of glyceraldehyde-3-phosphate dehydrogenase can be used for distinguishing cooked meat, on the contrary, not a single one for distinguishing raw meat. Similarly, lactate dehydrogenase contains four characteristic sequences for distinguishing cooked meat, but none for raw. However, unlike parvalbumin and collagen, the same situation for these two enzymes was not observed for salmon samples.

Distinguishing between carp and salmon including the type of meat processing

Finally, it was attempted to perform an analysis of characteristic sequences including the type of processing (raw carp and salmon were compared at the same time to their cooked meat). Only a few characteristic sequences specific to each sample type (*e.g.* raw carp) were identified: 17 characteristic sequences for raw carp, 40 for cooked carp, 10 for raw salmon, and only 5 for cooked salmon. These markers, in the form of characteristic sequences, provide discriminatory value, as they meet strict criteria: a given characteristic sequence is presented in all nine samples of one material (*e.g.* raw carp), but it is not found in a single sample of the other materials (cooked carp, raw and cooked salmon). The characteristic sequences for each fish can be found in Supplementary material (Tables S1–S4).

Seven of the 40 characteristic sequences for boiled carp came from parvalbumin, which demonstrates both properties of parvalbumins reported: its interspecies variability and thermostability. Owing to interspecies variability, it is possible to distinguish between carp and salmon, thanks to thermostability it is possible to find parvalbumin fragments in cooked meat. Another seven of the 40 characteristic sequences for boiled carp came from glyceraldehyde-3-phosphate dehydrogenase. Characteristic sequences originally belonging to collagen were found in both cooked carp and salmon samples.

Comparison of markers found by both mass spectrometric methods

A possible similarity of the found markers between MALDI-TOF and LC-ESI-Q-TOF mass spectrometry was studied. The *m/z* markers obtained from MALDI-TOF MS (mentioned in Table 1 and Table 2) were compared to the list of the characteristic peptide sequences (their *m/z*) obtained by LC-ESI-Q-TOF.

Since the ions created by MALDI ionization acquire a uniform charge of +1 and the ions created by electrospray ionization can be multiply charged, it was not possible to directly compare the *m/z* values obtained by these two methods. The molecular weights of the peptide fragments were compared after charge subtraction. For MALDI-TOF, these values were easily obtained by subtracting the mass of one proton (MH^+=1 Da), for LC-ESI-Q-TOF the mass of the peptide fragment was obtained directly from the results exported by Mascot 2.4.1. The tolerance was set to ±0.3 Da. Seven and three characteristic MALDI-TOF MS markers were obtained for raw carp and salmon samples respectively (see Table 1). Of these, three values for carp and none for salmon were found in the LC-ESI-Q-TOF markers in the form of characteristic sequences within the required tolerance. Their overview is shown in Table 6.

In cooked samples of the same fish species, 10 markers for carp and two for salmon were obtained by the MALDI-TOF method (see Table 2). Within the same tolerance, four out of ten carp markers were found in LC-ESI-Q-TOF data as well. However, not a single match was found for salmon.

CONCLUSIONS

Using MALDI-TOF MS, species-specific markers in the form of characteristic *m/z* values were identified for each species. Additionally, markers distinguishing between raw and cooked meat of the same species were determined. The six markers with a high discriminatory power for carp, mackerel and pike that, in addition to the species identification, would also determine the type of processing (raw or cooked) were found.

By LC-ESI-Q-TOF, only carp and salmon samples (raw and cooked meat) were analysed. A large number of species-characteristic amino acid sequences: in raw samples, about 400 for carp and 550 for salmon, in cooked samples about 400 sequences for carp and 600 for salmon were found. The most characteristic sequences came from myosin, actin, nebulin, titin, and sarcoplasmic enzymes. The search of characteristic sequences was performed by including both raw and cooked carp and salmon samples. In this case, only tens of characteristic sequences were obtained for the given fish species and method of meat preparation (e.g. for raw carp meat).

Since one of the most common types of falsification of fish meat in general is species substitution, when meat of a more expensive species is replaced by meat of a cheaper species, the results of this work can find application in a food analysis. The identified species-specific markers have potential use in assessing the authenticity and taxonomical origin of fish products to ensure adequate quality and safety, especially in cases where morphological features are lost during fish meat processing, or DNA and protein degradation has occurred due to high temperatures during cooking process.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHORS' CONTRIBUTION

Alena Meledina conducted the main experiments, prepared tables and figures, and wrote the initial draft of the manuscript. David Straka contributed to the experimental design and provided critical revisions to the manuscript. Filip Soucek assisted in sample preparation and contributed to data interpretation. Tatiana Anatolievna Smirnova performed data analysis, conducted additional laboratory analyses, and carried out the statistical analysis. Stepanka Kuckova supervised the entire study, conceived and designed the research, provided funding and resources, and finalized the manuscript for submission. All authors have read and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at: www.ftb.com.hr.

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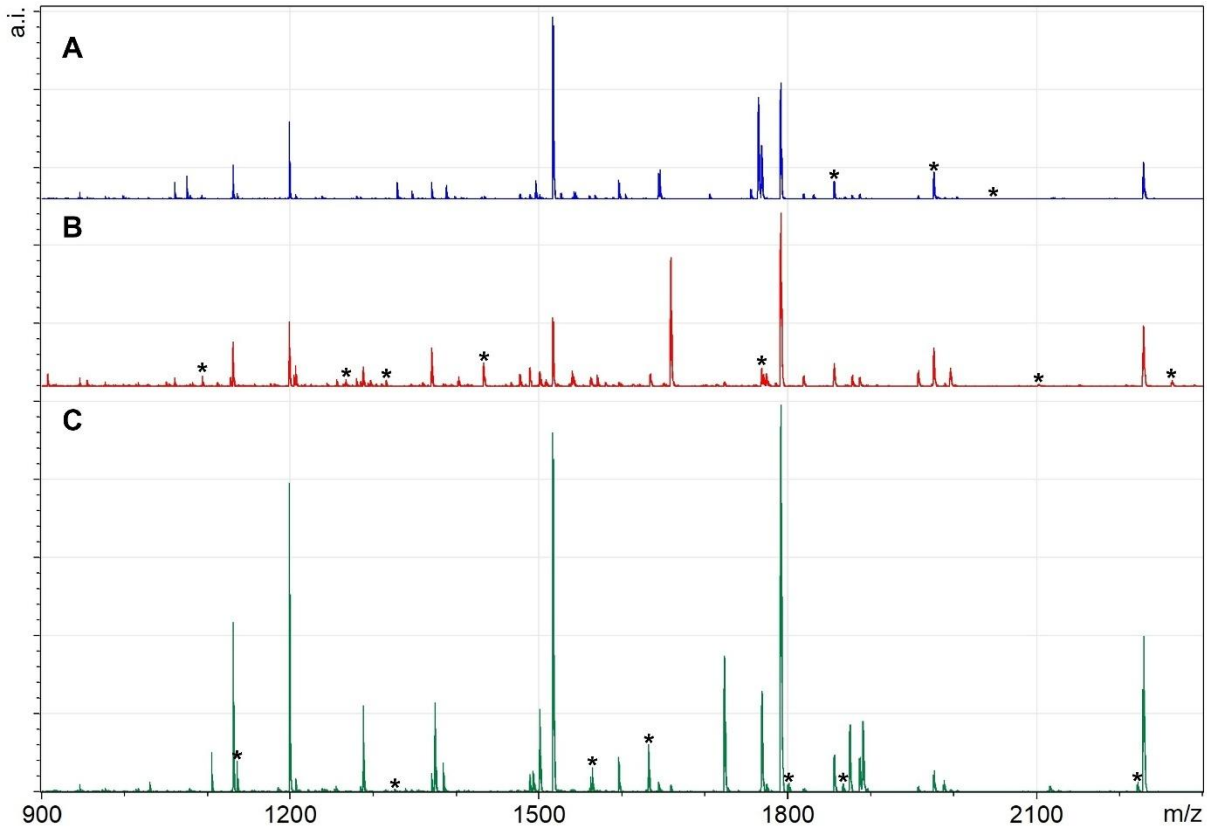

Fig. 1. MALDI-TOF MS spectra of carp (red), pollock (green), and salmon (blue). Characteristic *m/z* values for raw meet are indicated with asterisks (*)

Table 1. Characteristic *m/z* values for raw and cooked meat of individual types of fish (underlined bold values are the same for raw and cooked fish meat)

	Fish species	Characteristic *m/z* values								
Raw meat	Carp	**1093.7**	1263.6	1309.7	1432.7	**1770.8**	**2101.1**	2263.3		
	Mackerel	**934.6**	**1269.7**	**1380.8**	**1397.9**	**1411.8**	1463.7	**1777.0**	1836.0	**1932.2**
		2023.2	2095.2	**2291.4**	2638.6	4048.3				
	Pike	1226.6	**2587.5**	**4165.5**						
	Pollock	**1142.8**	**1325.8**	1564.3	**1632.2**	**1800.5**	**1866.6**	**2221.6**	2553.6	2635.2
	Salmon	1868.2	1980.4	**2059.1**						
	Trout	1109.9	**3175.6**							
Cooked meat	Carp	**1093.7**	1137.5	1180.5	1633.6	**1770.8**	**2101.1**	2185.0	2317.1	2484.2
		2732.1								
	Mackerel	**934.6**	1050.6	1115.7	1239.8	**1269.8**	1296.8	1308.7	**1380.9**	**1397.9**
		1411.8	1506.8	1725.2	**1777.1**	1838.2	**1932.1**	**2291.5**	2392.4	
	Pike	1355.9	**2587.5**	2723.7	3458.3	**4165.5**				
	Pollock	**1142.9**	**1325.9**	1339.0	1384.0	1409.1	1561.2	**1632.2**	1774.6	1780.7
		1800.5	**1866.7**	1890.6	1895.7	**2221.7**	3183.5			

	Salmon	**2059.1**	3291.4							
	Trout	1344.6	1565.7	**3175.6**						

Table 2. Characteristic *m/z* values for differentiating raw and cooked fish meat of individual fish species (underlined bold values are usable to characterize the type of fish as well as the type of processing)

Fish species	Type of meat	Characteristic *m/z* values						
Carp	raw	1050.6	1127.7	**1309.8**	1383.7	1500.8	1561.9	1569.9
		1877.3	2480.2					
	cooked	1661.6	**2184.9**	2446.1	**2732.1**			
Mackerel	raw	914.5	1028.6	1127.7	1643.9	1650.9	**2023.2**	2115.1
	cooked	909.6	**1239.8**	2216.4				
Pike	raw	1127.7	**1226.8**	1358.9	1402.8			
	cooked	1279.9	1908.4					
Pollock	raw	1254.9	1740.0					
	cooked	1470.0						
Salmon	raw	1002.5	1050.6	1067.6	1127.7	1269.7	1296.8	1339.7
		1397.8	1411.8	1506.9	1682.0	1777.0	1932.0	4048.3
	cooked	1135.5	1237.6	1476.7	1488.7	1754.8	1854.7	2003.0
		2384.2	2512.4					
Trout	raw	1240.8	1358.8	1536.1	1560.0	1705.0		
	cooked	1400.6	2118.9	2406.1	2438.0	3414.6	3471.7	

Table 3. Selected proteins identified in raw and cooked carp meat

Access code	Protein	No. of peptides	
		raw	cooked
A0A8C1U661	Myosin heavy chain, fast skeletal muscle-like	124±9	122±9
A0A8C2BNT8	Myosin, heavy chain b	65±4	60±6
A0A8C1U0K1	Nebulin	59±9	86±17
A0A8C1WNY8	ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 1, like	35±2	38±5
A0A8C1N3F8	Creatine kinase M-type	35±3	37±3
A0A8C1T5E4	Actin alpha 1, skeletal muscle	33±4	36±4
A0A8C1X499	Actinin alpha 3b	31±3	32±3
A0A8C1USJ2	Phosphorylase, glycogen, muscle A	29±4	29±5
A0A8C1V0Y0	Myosin regulatory light chain 2, skeletal muscle isoform-like	25±2	-
A0A2U9IYA4	Fructose-bisphosphate aldolase	23±3	23±4
A0A8C1J152	Myosin light chain 3, skeletal muscle isoform-like	22±3	16±2
A0A8C1FTE8	Alpha-tropomyosin	22±1	19±3
A0A8C1W232	Myosin light chain 1, skeletal muscle isoform-like	17±2	-
A0A8C1TSP1	EF-hand calcium binding domain 7	16±3	-
A0A8C1S5P5	Calsequestrin 1a	8±1	-
A0A8C1RRE4	Glyceraldehyde-3-phosphate dehydrogenase	-	24±2
A0A8C1S397	Enolase 3 (beta, muscle)	-	19±5
A0A8C1ZWS8	Myosin binding protein C, fast type b	-	19±2
Q8UUS2	Parvalbumin	-	10±1

Table 4. Selected proteins identified in raw and cooked salmon meat

Access code	Protein	No. of peptides	
		raw	cooked
A0A1S3QIW0	Myosin heavy chain, fast skeletal muscle-like	156±16	156±13
A0A1S3NZ45	Titin-like	137±15	83±18
A0A1S3NZK3	Nebulin isoform X11	67±8	90±17
B5DG55	Alpha-1,4 glucan phosphorylase	60±5	35±4
A0A1S3NEY1	Calcium-transporting ATPase	38±4	36±5
A0A1S3SB73	Actin, alpha cardiac	36±6	34±7
B5DGP2	Creatine kinase	28±3	26±3
A0A1S2WZE0	Fructose-bisphosphate aldolase	28±5	32±5
B5DGR3	Glyceraldehyde-3-phosphate dehydrogenase	27±4	29±3
Q91472	Fast myotomal muscle tropomyosin	25±4	-
B5DGU1	Pyruvate kinase	25±2	26±4
A0A1S3LCK1	ATP-dependent 6-phosphofructokinase	24±3	19±3
Q7ZZN0	Myosin regulatory light chain 2	21±3	19±2
A0A1S3P5Q0	Triosephosphate isomerase	20±1	-
A0A1S3QZX8	Glycogen debrancher	18±5	-
A0A1S2WZE3	2-phospho-D-glycerate hydro-lyase	-	33±4
A0A1S3NGD5	Myosin-binding protein C, fast-type-like	-	29±5
B5DG39	L-lactate dehydrogenase	-	13±2

Table 5. Proteins containing the most species-characteristic sequences in raw and cooked carp and salmon samples

Fish	Access code	Protein	No. of characteristic sequences	
			raw	cooked
Carp	A0A8C1U661	Myosin heavy chain, fast skeletal muscle-like	62	50
Carp	A0A8C2BNT8	Myosin, heavy chain b	32	24
Carp	A0A8C1U0K1	Nebulin	26	33
Carp	A0A2U9IYA4	Fructose-bisphosphate aldolase	19	-
Carp	A0A8C1WNY8	Calcium-transporting ATPase	19	16
Carp	A0A8C1N3F8	Creatine kinase M-type	-	20
Salmon	A0A1S3QIW0	Myosin heavy chain, fast skeletal muscle-like	90	86
Salmon	A0A1S3NZ45	Titin-like	86	59
Salmon	B5DG55	Alpha-1,4 glucan phosphorylase	37	-
Salmon	B5DGP2	Creatine kinase	31	30
Salmon	A0A1S3NZK3	Nebulin isoform X11	30	44
Salmon	A0A1S2WZE3	2-phospho-D-glycerate hydro-lyase	-	20

Table 6. Comparison of similarity of found markers between both mass spectrometric methods for raw and cooked meat samples of carp

	Species	Fragment mass [Da]		Peptide	Protein
		MALDI-TOF	LC-ESI-Q-TOF		
Raw	Carp	1092.7	1092.56	K.GFTLPTTNSR.G	Creatine kinase, muscle b
	Carp	1262.6	1262.64	K.VAFNQVADIMR.A	LanC synthetase component C-like
	Carp	1308.7	1308.68	R.IDFDAFLPMLK.S	Myosin light chain 3, skeletal muscle isoform
Cooked	Carp	1092.7	1092.56	K.GFTLPTTNSR.G	Creatine kinase, muscle b
	Carp	1136.5	1136.61	K.NALAHAVQSAR.H	Myosin heavy chain, fast skeletal muscle
	Carp	1179.5	1179.55	R.LQTENGEFSR.Q	Myosin heavy chain, fast skeletal muscle
	Carp	2184.0	2184.03	K.GILGYTEDQVVSTDFNGDVR.S	Glyceraldehyde-3-phosphate dehydrogenase

SUPPLEMENTARY MATERIAL

Table S1. Characteristic peptides for raw carp samples

Protein origin	Peptide	*m/z*
Actinin alpha 3a; Actinin alpha 3b; Alpha-actinin-3	R.FAIQDISVEETSAK.E	769.39
A kinase (PRKA) anchor protein 12b	K.EESQAESKAEPK.V	666.82
Alpha-tropomyosin; Tropomyosin alpha-1 chain-like; Tropomyosin alpha-1 chain-like	K.DAQEKLELAEK.K	637.33
ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting	K.FTLEFSR.D	450.23
ATP citrate lyase b	K.LSTIEFK.S	419.24
Phosphorylase, glycogen, muscle A	R.HLEIIYEINRR.H	485.94
EF-hand calcium binding domain 7	R.DIFDFAALK.E	520.27
	K.AIGGIILTASHNPGGPSGDFGIK.F	727.05
Glycogen debranching enzyme-like	R.VLDWINPTGR.E	585.81
Malate dehydrogenase 2, NAD (mitochondrial)	R.FTFSLLDAMNGK.E	672.33
Myosin heavy chain, fast skeletal muscle-like; Myosin, heavy chain b	K.SRVTFQLSAER.S	647.34
SH3 domain binding glutamate-rich protein	K.QQDVVGFLEALK.I	673.87
Uncharacterized protein	K.TLITDTVFK.I	519.30
Uncharacterized protein	-.MLMSHLEEPK.L	623.79
Uncharacterized protein	R.LNVSSTVTSTVLK.I	674.89
Uncharacterized protein	R.YFLTLENVTGSK.T	686.36
Uncharacterized protein	R.YSVTGLETGAEYK.F	709.34

Table S2. Characteristic peptides for cooked carp samples

Protein origin	Peptide	*m/z*
Adenosine monophosphate deaminase 1 (isoform M)	K.LAGWFNK.H	418.23
Adenylate kinase isoenzyme 1	K.IGAPALLLYIDAKAETMVQR.L	725.07
Calsequestrin 1a	K.SQKSEHYQEYEDAAEEFHPHIK.F	676.31
Glucose-6-phosphate isomerase b	K.ILVANFLAQTEALMK.G	831.46
	K.SITDVVNVGIGGSDLGPLMVTEALKPYSK.G	987.52
Glyceraldehyde-3-phosphate dehydrogenase	K.VIHDNFVIIEGLMSTVHAITATQK.T	660.10
	R.VCDLMAHMASKE.-	667.80
	R.VIISAPSADAPMFVMGVNHEKYDNSLK.V	734.12
	R.VIISAPSADAPMFVMGVNHEK.Y	738.37
	K.WGDAGANYVVESTGVFTTIEK.A	748.70
	R.SSIFDAGAGIALNDHFVK.L	931.47
	K.AAADGPMKGILGYTEDQVVSTDFNGDVR.S	976.13
Collagen, type I, alpha 2	R.GNPGPAGALGAQGPIGNR.G	802.42
	R.GPIGNIGMPGMTGPQGEAGR.E	956.95
	R.GPLGNIGMPGMTGPQGEAGR.E	956.95
Creatine kinase, mitochondrial 2a (sarcomeric)	K.TVGMVAGDEESYEVFAEIFDPVIKDR.H	972.80
Creatine kinase, muscle b	K.DIYNKLR.S	461.26
	K.GFTLPTTNSRGER.R	479.25
	K.VLTKDIYNK.L	547.32
Enolase 3, (beta, muscle)	K.FTGSVDIQVVGDDLTVTNPK.R	1053.04
Myomesin 1a (skelemin)	K.SDDVLIFDIGK.I	611.32
Myomesin 1b	K.ATNQSSLVLIGDVFK.Q	796.43
Myomesin 2a	R.FVVHGLVPGDTYVFR.V	569.30
Myosin binding protein Hb	K.VNLVVPFSGKPQPVVSWTK.D	694.73
Myosin heavy chain, fast skeletal muscle-like	R.TLEDQLSEIKTK.S	702.88
	R.ARLQTENGEFSR.Q	704.35

Nebulin	R.SDAVYKADLEWIR.G	522.60
	R.DIASDYKYK.L	551.77
Parvalbumin-2-like	K.SGFIEEDELKLFLQNFAAGAR.A	785.73
Parvalbumin-7-like; Parvalbumin 7	K.FFDVVGLK.A	462.76
	K.IGIDEFEALVHE.-	686.34
Parvalbumin beta-like; Parvalbumin	K.AFAIIDQDK.S	510.77
	K.IGVDEFTALVK.A	596.33
	K.AFAIIDQDKSGFIEEDELK.L	723.36
	K.AFAIIDQDNSGFIEEEELKLFLQNFK.A	765.39
Pyruvate kinase M1/2b	K.TTGSAFIQTQQMHAAMAETLLEHLCLLDIDSEPTVSR.N	1015.24
Rhotekin 2a	R.NIATRSTVSSCSSLAMEIK.R	500.26
Triosephosphate isomerase 1b	K.FFVGGNWK.L	477.74
Troponin C type 2 (fast)	K.AAFDMFDTDGGGDISTKELGTVMR.M	845.38
Uncharacterized protein	K.AGTKIELPADITGKPEPK.V	467.01

Table S3. Characteristic peptides for raw salmon samples

Protein origin	Peptide	*m/z*
Alpha-1,4 glucan phosphorylase	R.HLEIIYEINRR.F	485.94
ATP-dependent 6-phosphofructokinase	R.TFILEVMGR.H	533.29
Glycerol-3-phosphate dehydrogenase	K.NIVAVGAGFCDGLGFGDNTK.A	977.97
Malate dehydrogenase	R.FTFSVLDAMNGK.E	665.33
Myosin-7-like	M.EGDLNEMEIQLSHSNR.Q	624.62
	-.MEGDLNEMEIQLSHSNR.Q	673.63
Myosin-binding protein C, fast-type-like	K.LLDDYHVVVGER.V	472.25
Titin-like	R.FSLTIFR.A	442.25
	R.VLDSPSMPANFAIK.E	745.38
TSC22 domain family protein 3	-.MSTEIFK.T	428.22

Table S4. Characteristic peptides for cooked salmon samples

Protein origin	Peptide	*m/z*
Annexin	R.SLLLALVQAK.R	528.34
Collagen alpha-3(VI) chain-like isoform X1; Collagen alpha-3(VI) chain-like isoform X2; Collagen alpha-3(VI) chain-like; Collagen alpha-3(VI) chain-like	R.SQEGVPQMLILLSGGR.S	562.30
Protein S100	K.DLLNAELGEIMGK.N	701.86
Troponin C, skeletal muscle	K.NADGMLDFDEFLK.M	757.84
Voltage-dependent anion-selective channel protein 3; Voltage-dependent anion-selective channel protein 3	K.LTLSALIDGK.N	515.81