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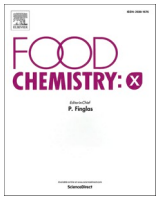

Discovery of mass spectral peak markers and protein biomarkers in fish muscle exudates for rapid and precise recognition of fish species via magnetic beads (MBs) and mass spectrometry

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ABSTRACT

In this study, muscle exudates from five fishes belonging to the family Sciaenidae, in the order Perciformes, were analyzed as models for the discovery of biomarkers by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). MagSi-weak cation exchange magnetic beads (WCX-MBs) were utilized for the enrichment of proteins from fish exudate samples, allowing protein biomarkers to be identified and subsequently used for fish species differentiation. Buffers with pH ranging from 4.0 to 9.0 can provide an environment for proteins in fish muscle exudate to bind to the WCX-MBs. The optimal enrichment based on WCX-MBs can be achieved when the exudate samples are diluted 100folds. More species-specific biomarkers in mass spectra can be identified when using WCX-MBs. The number of ions that can be considered as peak markers and can differentiate the analyzed fishes increases from 38 to 121 when using WCX-MBs to isolate peptides/protein in fish muscle exudate. Particularly, eight peak markers in mass spectra were assigned to be specific to *Nibea albiflora* (NA), three peak markers specific to *Larimichthys crocea* (LC), two peak markers specific to *Miichthys miiuy* (MM), seven peak markers specific to *Collichthys lucidus* (CL), and six peak markers specific to *Larimichthys polyactis* (LP). Furthermore, five proteins were identified based on the characterization of tryptic peptides and their potential to be biomarkers, of which four proteins specific to CL and one specific to LC were identified. The single-blind samples analysis demonstrated that these species-specific peak markers and protein biomarkers can be successfully utilized for corresponding fish recognition. The utilization of WCX-MBs can improve the discovery of fish species-specific biomarkers in fish muscle exudate samples. The present protocol holds potential of being a rapid and accurate identification tool for recognition of fish species.

1. Introduction

Seafood intake is vital for human health. Critselis et al. elucidated the long-term effects of high fish intake rich in n-3 fatty acids for deterring cardiovascular disease (CVD) and related adverse outcomes in healthy individuals (Critselis et al., 2023). It was found that high intake of seafood and particularly small fish rich in n-3 fatty acids is associated with a lower risk of 10-year fatal and non-fatal CVD (Bae, Lim, & Lim, 2023). Consumers are increasingly becoming aware of the beneficial health effects of fish, which has significantly contributed to the growth

Abbreviations: AmAc, ammonium acetate; CL, *Collichthys lucidus*; COX, cytochrome *c* oxidase; CVD, cardiovascular disease; EDC, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydro-chloride; ETC, electron transfer chain; IgEs, immunoglobulin E antibodies; IMS, immunomagnetic separation; LAMP, loop-mediated isothermal amplification; LC, *Larimichthys crocea*; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LP, *Larimichthys polyactis*; Mag Si-WCX, magnetic silica beads coated with a weak cation exchange surface; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MBs, magnetic beads; MES, sodium citrate and 4-morpholineethanesulfonic acid; MM, *Miichthys miiuy*; MS, mass spectrometry; Mw, molecular weight; NA, *Nibea albiflora*; NaCl, sodium chloride; NaOH, sodium hydroxide; PBS, phosphate buffered saline; PCA, principal component analysis; PLS-DA, partial least squares discrimination analysis; RCCC, renal clear cell carcinoma; RT, room temperature; SA, sinapic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SIB, swiss institute of bioinformatics; SNR, signal-to-noise ratio; SWATH, sequential window acquisition of all theoretical fragment ion spectra; TFA, trifluoroacetic acid; UPLC-Q-TOF, ultrahigh-performance liquid chromatography quadrupole time-of-flight; VIP, variable importance in projection; WCX-MBs, magSi-weak cation exchange magnetic beads..

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in consumption of fish (De Battisti et al., 2013). With the globalization of markets, economically motivated adulteration and/or fraudulent labeling are becoming important issues (Kroetz et al., 2020).

In today's market for convenience purposes, many fishes have been processed as fillets, making the recognition of fish species hard especially for closely related fishes, due to similar textural appearance. Some fishes like cod fish have been widely recognized as high-quality fish with high nutritional value and high economic benefits, making them susceptible to seafood fraud. The growth in fisheries consumption has brought increased cases in food fraud and adulteration (Moyer, DeVries, & Spink, 2017), which threatens consumer health and legitimate interests, making fish identification and origin assessment an important prerequisite to ensure adequate quality and safety control measures. Control of fish fraud is still a challenge especially for fishes with high market value (Donlan & Luque, 2019).

The establishment of rapid and accurate detection methods for identifying fish species is an important and significant aspect in seafood analysis that still needs attention and has continuously developed over the past years. Visible loop-mediated isothermal amplification (LAMP) has been utilized to identify common cod and oilfish in the Chinese market (Li, Xie, Yu, Wang, & Chen, 2021). The ^{1}H NMR metabolic profiling of fish muscle tissue was performed for the distinguishment of fish species susceptible to adulteration (Saglam, Paasch, Horns, Weidner, & Bachmann, 2024). Deep learning has been utilized to develop a class-aware fish species recognition method (Alaba et al., 2022).

The simplification of pretreatment is an important issue for fish analysis. Biomaterials extracted from fish skin have been analyzed via MALDI-TOF MS for differentiation and authentication of fishes at the species level (Bi et al., 2019). A one-step pretreatment method, that is, boiling tissue sample in 0.1 M trifluoroacetic acid (TFA) for 5 min, combined with comparison of mass spectral patterns, can be used for super-fast seafood authenticity analysis (Wang & Bi, 2021). Directly analyzing exudates can greatly simplify the pretreatment process for enhanced analysis efficiency. Fish species identification has been achieved by molecularly transferring the substances on fish skin or muscle tissues on to the surface of a MALDI-target plate for detection (Shao & Bi, 2020). Muscle tissue exudate has been found capable of providing valuable information about the potential pathways and processes involved in the post-slaughter ageing period of meat (C. Wang, Li, Zhao, Bi, & Xie, 2022). Pretreatment of fish samples using procedures that are less complicated holds potential of enhancing analysis efficiency and reducing analysis costs.

When studying complex biological samples, biomarkers can be identified to assist in analyses. A high-intensity-focused-ultrasound-assisted tryptic digestion method was employed in conjunction with selected MS/MS ion monitoring techniques to monitor species-specific peptide biomarkers in Merlucciidae fishes (Carrera et al., 2011). Ambient liquid extraction surface analysis mass spectrometry (LESA-MS) was employed to analyse five heat-treated meats, including beef, pork, horse, poultry and turkey, in order to identify heat-stable species-specific peptide markers for the purpose of distinguishing raw and cooked meat (Montowska, Alexander, Tucker, & Barrett, 2014). A combination of ambient desorption electrospray ionization mass spectrometry (DESI-MS) and a liquid extraction surface analysis-mass spectrometry (LESA-MS) analytical method was employed to analyse skeletal muscle proteins derived from standard proteins and raw meat mixtures, for species-specific analysis via the differentiation of peptide peaks specific to individual proteins (Montowska, Rao, Alexander, Tucker, & Barrett, 2014). Biomarkers have been identified to assess the health of fish in coastal and marine ecosystems (Kroon, Streten, & Harries, 2017).

Multivariate analysis methods such as principal component analysis (PCA) have been utilized for assisting the discovery of biomarkers. Peptide biomarkers were discovered via the combination of sequential window acquisition of all theoretical fragment ion spectra (SWATH)-ultrahigh-performance liquid chromatography quadrupole time-of-flight (UPLC-Q-TOF) mass spectrometry (MS)-based proteomics and chemometrics for the discrimination of shrimp species of the order Decapoda: *Marsupenaeus japonicus*, *Fenneropenaeus chinensis*, and *Litopenaeus vannamei* (Hu et al., 2018). Protein biomarkers for three tuna species, skipjack, bigeye and yellowfin tuna, were identified by SWATH-MS-based proteomics analysis and hierarchical clustering, with 14 proteins screened as potential biomarkers for tuna species identification (Hu et al., 2022a). However, capturing a specific protein biomarker from biofluids is not an easy task.

The removal of possible interfering factors before mass spectrometric analysis of a complex biological sample is crucial for the analysis of biosamples. Magnetic beads (MBs) assisted methods, compared with conventional separation methods, have shown excellent performance in the separation of complex components of biological materials, enabling both separation and enrichment. Biofunctional MBs can be used for the enrichment of target components in biological materials. We utilized MBs functionalized with anti-human Immunoglobulin E antibodies (IgEs) to perform IgEs immunomagnetic separation (IMS) in blood samples for capturing allergens from seafood protein extracts (Zhao et al., 2021).

It has been discovered that it is possible to detect proteins from fish muscle exudates, and the number of identified proteins is variable. One hundred and nine proteins have been identified in *Larimichthys crocea* muscle exudate by MALDI-TOF MS and LC-MS/MS (C. Wang et al., 2022). Although it's promising to discover biomarkers from fish muscle exudate samples, studies on the utilization of MBs for biomarker discovery in food matrices are limited to the best knowledge of the authors.

The present study aimed to demonstrate a protocol for discovering biomarkers from fish muscle exudate samples by utilizing MBs. It is well known that more closely related seafood species are difficult to identify by morphological characteristics, and are susceptible to food fraud. Herein, muscle exudates of five fishes belonging to the family Sciaenidae in the order Perciformes were studied. MagSi-weak cation exchange magnetic beads (WCX-MBs) were utilized for extracting proteins in the exudates of fish. The direct analysis of fish exudate sample without WCX-MBs enrichment was carried out as a control. The isolated proteins were analyzed by MALDI-TOF MS to verify the feasibility of identifying fish species by analyzing muscle exudates. Species-specific peak markers and protein biomarkers in mass spectra were screened. Furthermore, the developed approach was applied for examining the validity of the discovered species-specific peak markers for fish species recognition, to show their potential as species-specific markers. Potential biomarkers were characterized in the studied exudate samples of fishes by analyzing tryptic digests by MALDI-TOF MS. This is the first study to discover biomarkers in fish muscle exudates for rapid and precise recognition of fish species via magnetic beads (MBs) and MALDI-TOF MS. It is promising to apply the present protocol for tackling challenges involving food fraud.

2. Material and methods

2.1. Chemicals and fish samples

Ammonium acetate (AmAc) was obtained from Shanghai Titan Scientific Co., Ltd. (Shanghai, China). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydro-chloride (EDC, 99.0%), n-hexane (C_6H_{14}, ≥98.0%), phosphate buffered saline (PBS), sodium hydroxide (NaOH, 98%) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Sodium chloride (NaCl) was obtained from Meryer Technologies Co., Ltd. (Shanghai, China). Sodium citrate and 4-morpholineethanesulfonic acid (MES, ≥99%) were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Trifluoroacetic acid (TFA, ≥99.9%) was purchased from Fisher Scientific Inc. (Loughborough, UK). Sinapinic acid (SA, ≥99.0%), myoglobin from equine skeletal (≥98%) and cytochrome from equine heart (≥95%), were purchased from Sigma-Aldrich (St Louis, USA). Acetonitrile (99.90%) was obtained from Shanghai Titan Scientific Co., Ltd.

(Shanghai, China). Trypsin from bovine pancreas (≥10,000 BAEE units/mg protein) was purchased from Sigma-Aldrich (Saint Louis, USA). Tween 20 was obtained from Beijing Coolaber Technology Co., Ltd. (Beijing, China). Deionized (DI) water was produced by a Milli-Q deionized water system (0.22 μm, Millipak Express 40, Damstad, Germany), and used in all aqueous solutions. Magnetic silica beads coated with a weak cation exchange surface (Mag Si-WCX, 20 mg/mL, 400 nm in diameter) were obtained from Xi'an Ruixi Biological Technology Co., Ltd. (Xi'an, Shaanxi, China).

Fish samples of yellow drum (*Nibea albiflora*, NA), large yellow croaker (*Larimichthys crocea*, LC), brown croaker (*Miichthys miiuy*, MM), light maigre (*Collichthys lucidus*, CL), and small yellow croaker (*Larimichthys polyactis*, LP) were obtained from local fishery stores in Zhoushan fishing grounds (Zhoushan, Zhejiang Province, China), in November 2021 and delivered to the laboratory in 48 h prior to filleting. NA and MM were from Zhoushan Gouqi Umami seafood shop (Zhoushan, Zhejiang Province, China). LC, LP and CL were from Zhoushan old fisherman seafood shop (Zhoushan, Zhejiang Province, China). Six samples of each fish species were used as biological replicates. After filleting, the non-posterior fish muscle was cut into pieces with weight of 12.0 ± 4.0 g/each for fish CL and LP, and 28.0 ± 5.0 g/each for fish MM, NA and LC, and separately stored in sealed bags at −20 °C for further use.

2.2. Collection and lipid-removal of exudate from thawed muscle tissue

Fish muscle exudate was collected after thawing the fish muscle sample at room temperature (RT) for 2 h. The thawed muscle tissue secretions of each fish sample were mixed, and were divided into three portions for replication analysis in one run of experiment. The collected fish muscle exudate sample was mixed with hexane (1:1, *v*/v), and centrifuged at 20,000*g* for 5 min at RT to remove the lipids. The upper layer was discarded, and the extraction was repeated three times. The bottom protein layer was then collected for further analysis. The lipid-removed fish muscle exudate samples were divided into at least two portions for direct MALDI-TOF MS analysis and for the WCX-MBs coupling, respectively.

2.3. Extraction of proteins from exudates of fish muscle by WCX-MBs

WCX-MBs suspension was activated by refering to the manufacturer's instructions with necessary modification. Briefly, WCX-MBs suspension was resuspended on a vortex mixer (VORTEX-5, Kylin-Bell, China) for 3–5 s. 20 μL of the WCX-MBs suspension was transferred to an EP centrifuge tube and the supernatant was discarded by placing the sample on a magnetic separation rack (BMB-01, 16 holes, for 1.5–2 mL Eppendorf microcentrifuge tubes, BioMag Scientific Inc., Wuxi, Jiangsu Province, China). 200 μL of AmAc buffer (50 mM, pH 4.0, with 1.0 M NaCl) was added to the above-treated MBs pellets and thoroughly resuspended, magnetically separated and the supernatant discarded. The MBs were then washed twice by using AmAc buffer (50 mM, pH 4.0, with 1.0 M NaCl) to activate MBs pellets.

Exudate samples were diluted with 50 mM sodium citrate buffer (pH 5.0), at folds of 40, 60, 80, 100, and 200, respectively. 200 μL of diluted exudate sample was added to the activated WCX-MBs pellets. The mixture was shaken at 800 rpm on an incubator (Eppendorf ThermoMixer C, Eppendorf, Hamburg, Germany) for 30 min at RT to extract proteins in exudate samples, then magnetically separated and the pellets were collected. 10 μL of TFA solution (1%, *v*/v) was added to the MBs pellets for the elution of proteins, then resuspended and magnetically separated, and the supernatant was collected for MALDI-TOF MS analysis.

To identify the eluted proteins, 8 μL of the eluted solution was digested with 0.2 μL of trypsin solution (2 mg/mL in 10 mM PBS buffer (pH 7.4), protein: trypsin = 30:1, *w*/w) at 37 °C for 14 h. The digestion was terminated by adding TFA with a concentration of 0.4% (*v*/v). The obtained digests were analyzed by MALDI-TOF MS.

2.4. Buffers at different pH for the binding of proteins in exudate samples

AmAc buffer (50 mM, pH 4.0), sodium citrate buffer (50 mM, pH 5.0), MES buffer (50 mM, pH 6.0), PBS buffer (50 mM, pH 7.2–7.4), sodium citrate buffer (50 mM, pH 8.0), and sodium phosphate buffer (50 mM, pH 9.0) were separately utilized to study the effect of buffer's pH on the attachment of proteins in fish exudate samples to WCX-MBs. Exudate samples were diluted 100 folds with buffers at different pH. The activated WCX-MBs pellets (obtained from 20 μL of WCX-MBs suspension) were utilized for coupling proteins from fish muscle exudate samples.

2.5. MALDI-TOF MS analysis of proteins extracted from fish exudate by MBs

1 μL of the above obtained protein eluent and enzymatic products were deposited on a Bruker target plate (MSP 96 target grinding steel), respectively. After drying at RT, 1 μL of SA (15 mg/mL) in a mixture of acetonitrile/water/TFA (500:499:1, *v*/v) as matrix was dropped on top of the sample's surface, and left to dry at RT for MALDI-TOF MS analysis. The laser frequency was set to 20 Hz and the laser intensity was set to 55%. The detector gain was set to 10×. The laser attenuator was set to 35% bias and 40% range. Aqueous protein solutions containing cytochrome *c* (2 mg/mL) and myoglobin (4 mg/mL) were used as external standards and the instrument was calibrated with ions of cytochrome *c* (m/z = 6181 and 12,361) and myoglobin (m/z 8476 and 16,952). Mass spectral peaks with a signal-to-noise ratio (S/N) ≥ 3 were acquired over a mass range of 2000–20,000 Da for eluted protein samples or 0–6000 Da for digested products, respectively. All raw mass spectral data were acquired on a Bruker MicroFlex LRF mass spectrometer (Bremen, Germany) and analyzed in both linear and positive ion modes. At least six mass spectra were collected for each fish species. All the experiments were conducted in at least triplicates.

2.6. Discovery of peak markers in mass spectra

Peak markers in mass spectra were screened by referring to a previous report with minor modification (Hu et al., 2022b). In detail, peaks from MALDI-TOF mass spectra that held variable importance in projection (VIP) value >1.0 and specifically detected from one particular fish species, were chosen as peak markers and identified by using UniProt database. For the peptide markers' discovery, the following conditions were taken into account. 1) Variables with VIP value >1.0 were considered statistically significant in the model; 2) Variables with a small range of Jack-knife-based confidence intervals are more plausible for the model; 3) The variables with final fold change >1.5 or < 0.67, and $p < 0.05$ were selected.

2.7. Identification of potential fish species-specific biomarkers

Identification of protein biomarkers was achieved by using the search tool on *https://www.uniprot.org* for searching against the UniProt database and assigning the measured ion peaks. Mass spectrometric data analysis of tryptic digests was performed with flexAnalysis from Bruker and the FindMod tool available on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (*http:/lexpasy.org/*). The masses and intensities of special peaks were sent to the FindMod tool for database searching. Experimentally measured peptide masses were compared with the masses of the theoretical peptides calculated from a specified Swiss-Prot/TrEMBL entry to finish the peak identification.

2.8. Analysis of exudates of single-blind fish samples

Two fish samples were randomly selected from the studied fishes and analyzed as single-blind samples. Fish muscle exudates of two fish samples were collected, the lipid-removed, WCX-MBs enriched and analyzed by MALDI-TOF MS at optimized conditions.

2.9. Statistical analysis of the mass spectral data

FlexAnalysis 3.4 user manual revision 1 software (Bremen, Germany) and MALDIquant R package were utilized to extract and process raw mass spectral data. Peaks with S/N $\geq$ 3 were extracted and subjected to *www.metaboanalyst.ca* for PCA, partial least squares discrimination analysis (PLS-DA), and VIP analysis. Mass spectral peaks were processed with mMass open source MS tool (*http://www.mmass.org/*), to obtain the averaged mass spectrum. Multivariate statistical analysis of the matrices was performed by using SIMCA-P 14.1 (MKS Umetrics, Umeå, Sweden) and MetaboAnalyst 5.0 (McGill University, Montreal, Canada).

3. Results and discussion

3.1. Extraction of proteins in exudates by WCX-MBs

The goal of this study was to discover biomarkers in fish muscle exudates for facile and accurate recognition of fish species. The fishes, *Nibea albiflora* (NA), *Larimichthys crocea* (LC), *Miichthys miiuy* (MM), *Collichthys lucidus* (CL), and *Larimichthys polyactis* (LP) belonging to the family Sciaenidae, in the order Perciformes, were studied as models of which, LC and LP belong to the genus Larimichthys. LC and LP are important aquaculture species in China that have a wide difference in price due to consumer preferences for LP. The recognition of fish species will assist in reducing possible adulteration issues, for instance those associated with substitution of LP with LC.

Herein, WCX-MBs combined to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) was utilized for the recognition of fish species by the analysis of exudates. Specifically, WCX-MBs with a size of ca. 400 nm, modified with silica and cationic groups were utilized to isolate proteins in fish exudate samples in order to enhance the recognition of fish species. The WCX-MBs are supposed to be able to physically adsorb positively charged proteins and peptides in a controlled pH environment, that is, peptides and proteins can bind to the cationic groups on the surface of the WCX-MBs while other components can be discarded under the assistance of magnetic separation, to achieve an improved detection of exudate samples.

3.2. Optimization of coupling of proteins in exudate by WCX-MBs

The experimental conditions for the coupling of proteins in exudate by WCX-MBs were optimized. The pH environment for promoting protein and peptide binding to MBs was firstly taken into account.

During the experiment, buffers with different pH were utilized. NA fish muscle exudate was randomly selected as a model. Fig. 1A plots the MALDI-TOF mass spectrometric profiles of the absorbed proteins in NA fish muscle exudate by using WCX-MBs in buffers with different pH environments. It can be found that the MALDI-TOF MS profiles for proteins are optimal when sodium citrate buffer solution (50 mM, pH 5.0) was utilized, indicated by a relatively high number and high intensity of mass peaks in the obtained mass spectrum (Table A.1 in Appendices). The optimal adsorption at pH = 5 may be attributed to the fact that most of the proteins in the analyzed fish muscle exudates exhibit relatively high theoretical isoelectric point (PI) values. For instance, only 10 of the 109 proteins identified in exudate samples of LC exhibit PI values below pH 5 (C. Wang et al., 2022). When the value of the pH of the adsorption environment is below the proteins' PI values, the protein molecules carry net positive charges. The positively charged proteins tend to bind to the functional groups on the surface of the WCX-MBs which is negatively charged under the utilized conditions in the present study. Alternately, it can be seen that all the utilized buffers in the present study, with pH ranging from 4.0 to 9.0 can provide an environment for protein and peptides in fish muscle exudate to bind to the utilized WCX-MBs.

3.3. Effect of dilution of exudate sample on the MS detection

In order to investigate the impact of dilution on MS analysis, the exudate samples of fish muscle were diluted with different folds. Fig. 1B shows the representative MALDI-TOF mass spectra for fish exudate samples of NA after diluting with sodium citrate buffer (50 mM, pH 5.0). Table 1 lists the number of peaks with signal-to-noise ratio (SNR) $\geq$ 3 in MS spectra of fish muscle exudates with and without using WCX-MBs. It can be found that optimal enrichment conditions based on WCX-MBs can be achieved when the exudate samples are diluted 100-folds, indicated by the improved mass signals in the obtained mass spectra as listed in Table B.1 in Appendices.

3.4. Enhanced differentiation of fishes by coupling the proteins in the fish muscle exudate via WCX-MBs

The differentiation of the analyzed fishes were investigated with and without using WCX-MBs. Fig. 1C&E show representative MALDI-TOF mass spectra of muscle tissue exudates of five analyzed fish species with and without using WCX-MBs. It can be observed that the intensity and number of ion peaks in the mass spectra for fish muscle exudate without using WCX-MBs were relatively low and less. After isolating proteins in exudate with WCX-MBs, the No. of peaks for the averaged MALDI-TOF mass spetrum (n = 6) with signal-to-noise ratio (SNR) $\geq$ 3 increases from 83 to 163 in the muscle exudate of LP, from 106 to 151 for MM, from 100 to 153 for CL and from 102 to 139 in NA as listed in Table 1. It can be concluded that the fish muscle exudate samples enriched with WCX-MBs show more MS peaks in the obtained mass spectra, illustrating the effective capture of proteins and/or peptides from fish muscle exudates by WCX-MBs.

The mass spectral data processed by SIMCA software were utilized for screening the markers for distinguishing fish species. Fig. 2A&C show the PCA score plots for the MALDI-TOF mass spectra of muscle exudates, with and without WCX-MBs enrichment, collected from five fish species, respectively. It can be seen that most of the analyzed five fish species in the PCA score plot in Fig. 2A have varying degrees of overlap. Fig. 2C plots the PCA scores for the mass spectra after coupling exudate proteins with WCX-MBs. It can be seen that the points representing the MALDI-TOF mass spectra of the five fish species can be successfully differentiated. The results indicate that the coupling of fish exudate protein with WCX-MBs shows a better discrimination of the analyzed fish species.

A VIP score is a measure of a variable's importance in the PLS-DA model. It summarizes the contribution a variable marker has to the model. Only those ion peaks with a VIP score over a particular threshold value (typically 1) are considered important for the discrimination among the analyzed samples (Wold, Sjöström, & Eriksson, 2001). Fig. 2B&D illustrate the ion peaks of muscle exudate with the VIP values. As shown in Fig. 2B, 38 ions with VIP values>1.0 can be considered as peak markers that cause differences among the analyzed fishes. However, as shown in Fig. 2D, there are 121 ions with VIP values>1.0 in fish muscle exudate using WCX-MBs, indicating that much more potential peak markers can be promisingly found after enrichment and/or purification of proteins from fish muscle exudate using WCX-MBs.

3.5. Discovery of fish-species peptide markers in mass spectra

In the present study, WCX-MBs were utilized to discover mass spectral peak markers in exudate of fish muscle. The isolation and/or

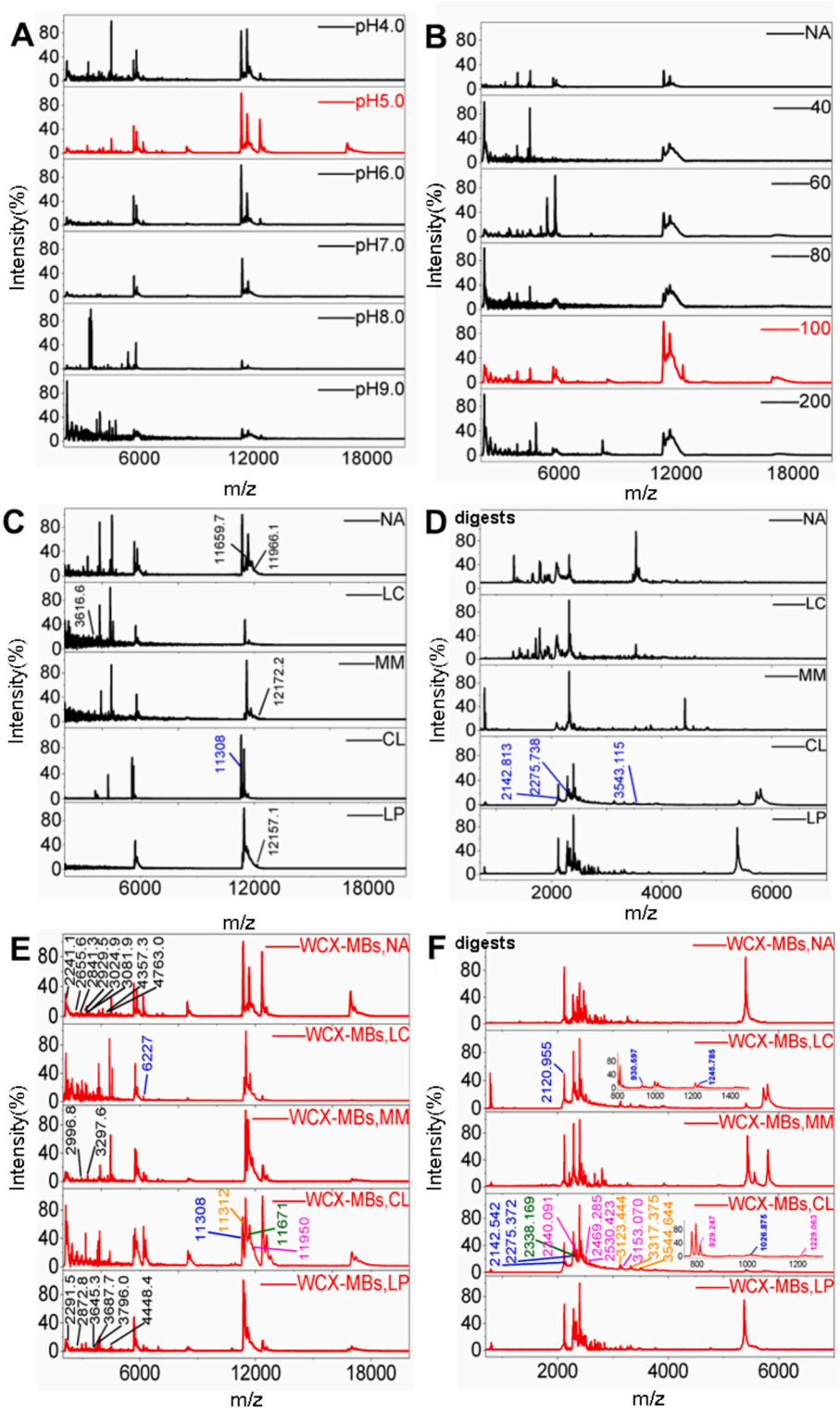

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Fig. 1. (A) Representative MALDI-TOF mass spectra for fish exudate samples of NA after protein extraction by using WCX-MBs in different pH environments. The pH of the utilized buffer was labeled as inlets. (B) Representative MALDI-TOF mass spectra for fish exudate samples of NA after protein extraction by using WCX magnetic beads after diluting the exudates with sodium citrate buffer (50 mM, pH 5.0). The fold of dilution is labeled as inlets. The MBs pellets were added into diluted exudate sample. The mixture was shaken to extract proteins in exudate samples, then magnetically separated and the pellets were collected. 10 μL of TFA solution (1%, *v*/v) was added to elute the proteins on MBs pellets for further analysis by MALDI-TOF MS. (C) Representative MALDI-TOF mass spectra of muscle tissue exudates from five fish species, NA, LC, MM, CL, and LP under optimized conditions. (D) Representative MALDI-TOF mass spectra for tryptic digests of muscle tissue exudates from five fish species, NA, LC, MM, CL, and LP under optimized conditions. (E) Representative MALDI-TOF mass spectra of muscle tissue exudates from five fish species, NA, LC, MM, CL, and LP with enrichment by WCX-MBs (red) under optimized conditions. (F) Representative MALDI-TOF mass spectra for tryptic digests of muscle tissue exudates from five fish species, NA, LC, MM, CL, and LP with enrichment by WCX-MBs (red) under optimized conditions. The peak *m/z* values of potential markers are denoted in the diagram. The *m/z* of the peptides that match the theoretically predicted peptide masses are also denoted in the diagram. The black color labeled peaks in (C) to (F) are the identified mass spectral peak markers. The other colored peaks are the identified biomarkers and their corresponding digested peptides. The proteins on MBs pellets were eluted and digested for analysis by MALDI-TOF MS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Numbers of peaks with signal-to-noise ratio (SNR) ≥ 3 in MS spectra of fish muscle exudates with and without using WCX-MBs.

fish species	No. of peaks with S/N ≥ 3 (n = 6)	
	original exudate	exudate processed by WCX-MBs
yellow drum (*Nibea albiflora*, NA)	102 ± 4	139 ± 3 (pH 5.0)
large yellow croaker (*Larimichthys crocea*, LC)	98 ± 3	106 ± 4 (pH 5.0); 136 ± 5 (pH 4.0)
brown croaker (*Miichthys miiuy*, MM)	106 ± 6	151 ± 2 (pH 5.0)
light maigre (*Collichthys lucidus*, CL)	100 ± 5	153 ± 6 (pH 5.0)
small yellow croaker (*Larimichthys polyactis*, LP)	83 ± 8	163 ± 4 (pH 5.0)

enrichment of proteins in exudate by WCX-MBs for fish differentiation via discovering biomarkers was demonstrated, by taking exudate samples of five fish species from the family Sciaenidae as models.

To screen fish-species specific peak markers in mass spectra for fish recognition, the mass spectra for the analyzed fishes were further analyzed. In detail, for screening the peptide markers, the following conditions were taken into account. 1) VIP scores indicate the significance of the corresponding mass spectrometry signals, labeled with *m/z* values in the plot of projection VIP scores, on the separation of the different samples. MS signals with a VIP score of >1.0 are usually considered to be significant signals. MS signals with VIP scores >1.0 are considered to significantly contribute to the differentiation among samples. To identify the peaks responsible for species differentiation, ions with VIP scores >1 were screened and further compared among species to figure out ions that can distinguish the species from each other. The total contribution of each variable to the PLS-DA model was ranked by VIP; 2) The jack-knife is a non-parametric method for estimating a sampling distribution for a statistic. The jack-knife plots show the uncertainty of each variable. Jack-knife-based confidence intervals were used as a complementary tool for biomarker discovery. Variables with a small range of confidence intervals are supposed to be more plausible for the model; 3) The fold change is calculated as the ratio of the normalized spectral count of the identified protein with its bait. Herein, the variables with fold change >1.5 or < 0.67, and $p < 0.05$ were selected.

Table 2&4 list the screened species-specific ion peaks in the MS spectra for exudate samples with and without using WCX-MBs. It can be found that the utilization of WCX-MBs dramatically increases the number of specific ions screened for each fish to varying degrees, with the number of NA specific ions increasing from 2 to 8, LC specific ion peak markers increasing from 1 to 3, MM specific ion peak markers increasing from 1 to 2, CL specific ion peak markers increasing from 1 to 7 and LP specific ions increasing from 1 to 6.

3.6. Identification of fish-species specific protein biomarkers in mass spectra

To obtain possible fish-species specific protein biomarkers for fish fraud test, the potential biomarkers in fish muscle exudate were identified by MALDI-TOF MS and the UniProt database. MALDI-TOF mass spectrometric data and the UniProt database can assist to characterize the proteins in fish muscle exudates. Candidate proteins were selected from the UniProt database by matching the protein molecular weight to the MS peak of exudate. Then, the candidate proteins were in-silico digested by choosing trypsin as the enzyme, and the molecular weight (Mw) of the in-silico tryptic digested peptides were matched to the MS peak of the tryptic digests by using the FindMod tool on the server of the SIB (*http://lexpasy.org/*). Table 3 lists the identified potential protein biomarkers of analyzed fishes and their theoretical masses of fragments from trypsin digestion. Table 3 also lists the identified peptides derived from the potential biomarkers captured by WCX-MBs.

Herein, the masses of peptides obtained from the tryptic digestion of potential protein biomarkers were compared with the predicted masses of peptides from the theoretical digestion of proteins in the database. WCX-MBs were utilized for the extraction of proteins in fish exudate sample. Table 3 lists the identified potential protein biomarkers in exudate of fish sample. The MALDI-TOF mass spectra shown in Fig. 1F displays the digests of captured protein biomarkers and the matched nineteen theoretically predicted peptide masses from CL; of which, three peptides from vacuolar protein sorting-associated protein 11 originating from LC, and sixteen peptides matches from cytochrome *c* oxidase subunit, interleukin-8, nance-Horan syndrome protein and chemokine interleukin-8-like domain-containing protein originating from CL, are highlighted.

The elucidation of these peptides allows the successful identification of cytochrome *c* oxidase subunit, interleukin-8, nance-Horan syndrome protein and chemokine interleukin-8-like domain-containing protein as potential protein biomarkers for CL, and vacuolar protein sorting-associated protein 11 as a potential protein biomarker for LC that is, by the characterization of the exudate proteins and their tryptic digests using MALDI-TOF MS, five proteins are identified. The number of identified proteins is small which is probably due to the very limited number of proteins in the analyzed fishes available in the UniProt database.

Table 3 lists the identified proteins by directly analyzing the exudate sample and performing analysis assisted by WCX-MBs. It can be found that only one protein can be identified in the CL fish when directly analyzing the exudate sample. However, when analyzing the extracted proteins from fish muscle exudate samples by WCX-MBs, four proteins can be identified from fish CL muscle exudate, and one from fish LC. The findings show that the utilization of WCX-MBs may play important roles in the purification of fish muscle exudate sample, and provide improved proteomic pattern, and enhanced average peak numbers and peak intensities. WCX-MBs shows excellent capturing ability of low abundance proteins or peptides in the analyzed fish exudate samples. The utilization of WCX-MBs enhances the discovery of biomarkers, and shows great

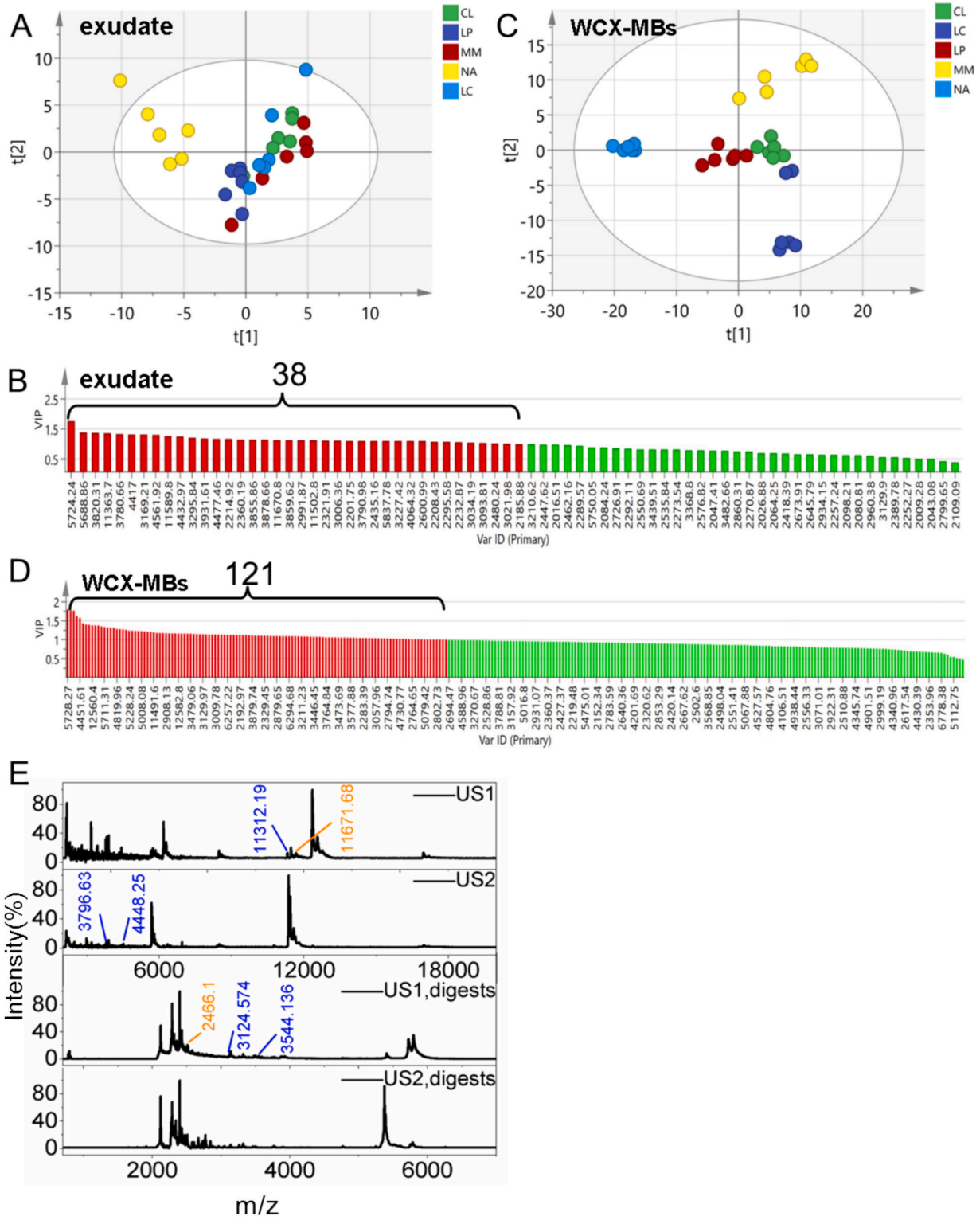

Fig. 2. (A) Plot of PCA scores for MALDI-TOF mass spectra of muscle exudates that flowed from five analyzed fish species. (B) Plot of VIP scores for MALDI-TOF mass spectra of muscle exudates collected from five analyzed fish species. Experimentally, the collected exudate samples were diluted 100 folds with DI water prior to MALDI-TOF MS analysis. (C) Plot of PCA scores for MALDI-TOF mass spectra of muscle exudates collected from five analyzed fish species under enrichment of WCX-MBs. (D) Plot of VIP scores for MALDI-TOF mass spectra of muscle exudates collected from five analyzed fish species under enrichment of WCX-MBs. The collected exudate samples were enriched with WCX-MBs after diluting the exudates 100-folds with sodium citrate buffer (50 mM, pH 5.0). (E) MALDI-TOF mass spectra for unknown sample (US) 1 and 2 and their tryptic digests for proteins. The proteins were extracted from fish exudate samples from unknown samples, US1 and US2, via WCX-MBs. Fish muscle exudates of two fish samples randomly selected from the studied fishes were collected and analyzed as single-blind samples.

Table 2
VIP, *P*-values, and fold-change values of specific peaks in mass spectra of fish muscle exudates with and without using WCX-MBs. The raw MS data were processed using MALDIquant R package and then formed using SIMCA (14.1) software for multivariate analysis.

analyzed sample	fish species	*m/z* at	theoretical mass (Da)	VIP value	P-value	fold-change value
original exudate	*Nibea albiflora*	11,965.9 ± 0.7	11966.1	1.168	0.0004284	0.3734
		11,659.3 ± 0.6	11659.7	1.137	3.170E-06	9.2820
	Larimichthys crocea	3616.3 ± 0.4	3616.6	1.340	0.0004084	0.3747
	Miichthys miiuy	12171.5 ± 2.0	12172.2	1.026	0.0041380	0.6253
	Collichthys lucidus	11309.0 ± 0.7	11308.3	1.296	0.0022050	0.1681
	Larimichthys polyactis	12158.1 ± 0.4	12157.1	1.331	0.0413000	0.3508
exudate processed by WCX-MBs	*Nibea albiflora*	2241.7 ± 1.1	2241.1	1.085	6.900E-06	0.1072
		2655.5 ± 0.8	2655.6	1.142	0.0006650	0.2621
		2842.7 ± 0.9	2841.3	1.294	0.0234100	0.4175
		2928.3 ± 1.2	2929.5	1.257	6.910E-09	0.1619
		3025.5 ± 0.8	3024.9	1.231	0.0041560	0.3230
		3081.4 ± 1.1	3081.9	1.204	0.0056270	0.3779
		4357.4 ± 0.9	4357.3	1.083	6.370E-05	0.1274
		4762.7 ± 0.8	4763.0	1.036	0.0068000	0.2347
		3732.5 ± 0.8	3732.2	1.539	0.0365700	0.6572
	Larimichthys crocea	4555.9 ± 1.1	4557.3	1.434	0.0172500	0.5908
		6227.4 ± 2.0	6227.1	1.346	0.0113000	0.6467
	Miichthys miiuy	2995.6 ± 2.0	2996.8	1.049	0.0368600	0.6251
		3297.8 ± 0.8	3297.6	1.268	0.0101500	0.3101
		3413.9 ± 0.8	3414.1	1.222	2.202E-05	0.2607
		4429.9 ± 0.8	4430.0	1.027	2.802E-05	0.6253
		5655.8 ± 1.0	5656.9	1.289	2.302E-05	0.4729
	Collichthys lucidus	11,311.6 ± 0.5	11311.7	1.501	1.894E-06	0.5607
		11,309.5 ± 1.3	11308.0	1.501	1.894E-06	0.5607
		11671.2 ± 1.7	11671.8	1.461	0.0000601	0.2544
		11952.2 ± 0.9	11954.2	1.621	7.513E-06	0.4040
	Larimichthys polyactis	2873.3 ± 2.7	2872.8	1.397	0.0418100	0.3608
		2292.7 ± 0.7	2291.5	1.337	0.0309000	0.5875
		3645.9 ± 0.4	3645.3	1.277	0.0492100	0.2805
		3687.4 ± 0.7	3687.7	1.539	0.0003727	0.1582
		3796.4 ± 1.1	3796.0	1.270	0.0262600	0.3930
		4447.4 ± 1.1	4448.4	1.874	0.0371400	0.4366

promise for the detection of biomarkers from fish muscle exudate for fish species recognition. These findings keep consistent with the findings from previous reports (Fiedler et al., 2007).

Specifically, three peptides from cytochrome c oxidase subunit originating from fish CL were affirmed as biomarkers for the authentication of CL with a molecular weight of 11,312 Da. Cytochrome c oxidase (COX) is a useful endogenous metabolic marker for neurons, and catalyzes the terminal oxidation reaction in the electron transfer chain (ETC) of aerobic respiratory systems (Broadley, Brown, Cakmak, Rengel, & Zhao, 2012). The structure of cytochrome *c* oxidase isolated from heart and liver of rainbow trout (*Salmo gairdnerii*), a cold-blooded animal (fish), were identified by SDS-PAGE and N-terminal sequencing of subunits isolated from gel bands (Arnold et al., 1997). Hu et al. established an optimal method for the measurement of maximal COX activity in fish gills, since COX activity is an important indicator for the evaluation of energy production by aerobic respiration in various tissues (Hu, Chung, & Lee, 2018).

Three peptides were derived from interleukin-8 and six peptides were derived from chemokine interleukin-8-like domain-containing protein, originating from CL. The molecular weights of these two proteins are 11,308 Da and 11,950 Da, respectively. Interleukin-8 (IL-8) is a CXC-type chemokine and it's a key regulator in acute inflammation through the recruiting and activation of neutrophils in mammals. IL-8 is one of the first few CXC chemokines discovered in fish (Kendrick et al., 2014). Wang et al. investigated the function of IL-8 in inflammation, and found that IL-8 is a functional CXC chemokine in mandarin fish (*Siniperca chuasti*), and plays a key role in the inflammatory responses towards bacterial infection (G. L. Wang et al., 2017).

The molecular ion peak at $m/z = 11{,}671$ in mass spectrum of CL can be assigned to nance-horan syndrome protein. The molecular ion peak at $m/z = 6227$ in mass spectrum of LC can be assigned to vacuolar protein sorting-associated protein 11. It was reported that in humans nance-horan syndrome protein plays a role in actin remodelling coordination and maintenance of cell morphology (Brooks et al., 2010). The function of vacuolar protein sorting-associated protein 11 is unknown to the best of the authors' knowledge.

Table 4 compares the number of proteins in the database and identified proteins of fishes analyzed in the present study. It can be found that the number of proteins for some fish species in UniProtKB database are very limited, making the biomarker identification hard. This may be the main reason why biomarkers cannot be identified in fishes, LP, NA and MM. It can be envisaged that the peak markers can assist the recognition of all the analyzed fish, however, the protein biomarkers can only be potentially utilized for recognition of the fishes, LC and CL.

3.7. Application of WCX-MBs for recognition of unknown fish samples

The application of the presently developed approach for the recognition of fish species was demonstrated. The proteins in fish muscle exudate combined with WCX-MBs extraction from unknown fish were analyzed. During the experiment, two randomly selected exudate samples from fish muscle of the analyzed model fishes were analyzed as single-blind samples. Fig. 2E shows the MALDI-TOF mass spectra of the unknown fish samples, US1 and US2. In the obtained mass spectra for sample US1, the molecular ion peaks belonging to CL's specific peak markers $m/z = 11{,}312$ and $m/z = 11{,}671$ are presented, indicating that the single blind fish species of US1 may be from fish species CL. The molecular ion peaks for species-specific ion peak markers belonging to LP ($m/z = 3796$ and $m/z = 4448$) were present in the obtained mass spectra for sample US2, suggesting that the single blind fish species may be from fish species LP. The results keep consistent with the fact that the fish species CL and LP were used to prepare exudate samples US1 and US2, respectively, which was kept unknown to the examiner. As shown in Fig. 2E, and as listed in Table 4, the fish species CL can be recognized

Table 3
Information on the identified potential protein biomarkers in exudate of fish sample by enzymatic protein digestion. WCX-MBs were utilized for the extraction of proteins in fish exudate samples.

fish exudate	fish species	protein	entry name	ions with *m/z* at	theoretical mass (Da)	identified peptides (calculated)	theoretical mass (Da)	detected mass (Da)	position	function of the identified protein
direct analysis	*Collichthys lucidus*	Interleukin-8	A0A4U5VW79	11309.0 ± 0.7	11308	LIHFVLERQLHVGGALPKN	2141.2	2142.8 ± 0.4	84–102	extracellular space; chemokine activity; immune response; inflammatory response
						IVPEGPHCPDTEVIAGLVSG ER	2275.1	2275.7 ± 0.8	50–71	
						IVPEGPHCPDTEVIAGLVSG ERVCLNPRSSWVK	3544.8	3545.1 ± 2.0	50–82	
enriched by WCX-MBs	*Collichthys lucidus*	Cytochrome c oxidase subunit	A0A4U5VRX4	11,311.6 ± 0.5	11312	MSLSTAALATRRVLAAASHS SHEGGARTWK	3123.6	3123.4 ± 1.3	1–30	energy metabolism; oxidative phosphorylation.
						TKKFPWGDGNHSMFHNPHTN ALPDGYESSHH	3545.5	3544.6 ± 0.2	72–102	
						KFPWGDGNHSMFHNPHTNAL PDGYESSHH	3316.4	3317.3 ± 0.5	74–102	
		Interleukin-8	A0A4U5VW79	11,309.5 ± 1.3	11308	LIHFVLER	1026.6	1026.8 ± 0.8	84–91	extracellular space; chemokine activity; immune response; inflammatory response
						LIHFVLERQLHVGGALPKN	2141.2	2142.5 ± 0.4	84–102	
						IVPEGPHCPDTEVIAGLVSG ER	2275.1	2275.3 ± 1.6	50–71	
		Nance-Horan syndrome protein	A0A4V6AMQ8	11671.2 ± 1.7	11671	VWVLQNKIGQIQQTASALDP K	2337.2	2338.1 ± 2.1	69–89	actin remodelling co-ordination and maintenance of cell morphology
		Chemokine interleukin-8-like domain-containing protein	A0A4U5UXC5	11952.2 ± 0.9	11950	NLPCVRAVIFETTNGEVCSH WR	2531.2	2530.4 ± 0.7	54–75	extracellular space; chemokine activity; immune response
						CCTEVSVTNVNATILGYRIQ RK	2468.2	2469.2 ± 1.2	32–53	
						CCTEVSVTNVNATILGYRIQ R				
						KNLPCVR	2340.1	2340.0 ± 1.0	32–52	
						AKKNITSAPATK	829.4	829.2 ± 0.4	53–59	
						CCTEVSVTNVNATILGYRIQ RKNLPCVR	1229.7	1229.0 ± 1.2	92–103	
							3150.6	3153.0 ± 0.8	32–59	
	Larimichthys crocea	Vacuolar protein sorting-associated protein 11	A0A0F8AUT4	6227.4 ± 2.0	6227	KFVFFDK	930.5	930.5 ± 0.2	9–15	N/A
						FVFFDKDTVK	1245.6	1245.7 ± 0.6	10–19	
						DPGDSGKNFALPGGISACDS GR	2120.9	2120.9 ± 1.7	24–45	

Table 4
Number of proteins recorded in UniProtKB database (*www.uniprot.org*) for the organisms (fishes) analyzed in the present study, and number of proteins identified. The data was compiled in Oct. 2023.

fish species	No. of proteins	No. of proteins with molecular mass < 20,000	No. of peak markers identified		No. of biomarker identified	
			by direct analysis of fish muscle exudate sample	by assistance of WCX-MBs	by direct analysis of fish muscle exudate sample	by assistance of WCX-MBs
Larimichthys crocea (LC)	30,532	3185	1	3	0	1
Larimichthys polyactis (LP)	124	33	1	6	0	0
Nibea albiflora (NA)	70	16	2	8	0	0
Collichthys lucidus (CL)	27,270	3584	1	7	1	4
Miichthys miiuy (MM)	253	35	1	2	0	0

by using both peak markers and protein biomarkers. However, the protein biomarkers related to LP were not identified. LP can only be identified by using the information of peak markers. It can be concluded that peak markers can potentially assist to identify all the analyzed fish species, but the protein biomarkers can only assist to identify the fish species where protein biomarkers were identified, that is, LC and CL in the present study. The findings show that it is promising to perform fish recognition using the developed strategy.

4. Conclusions

In the present study, the enrichment and analysis of proteins in fish muscle exudate was carried out by using surface-modified MBs and MALDI-TOF MS. The exudate samples of fish species including yellow drum (*Nibea albiflora*, NA), large yellow croaker (*Larimichthys crocea,* LC), brown croaker (*Miichthys miiuy*, MM), light maigre (*Collichthys lucidus*, CL), and small yellow croaker (*Larimichthys polyactis*, LP) were analyzed as fish models. The optimal conditions were explored to improve the efficiency of protein extraction. Buffers with pH ranging from 4.0 to 9.0 can provide an environment for protein and peptides in fish muscle exudate to bind to the utilized WCX-MBs. The optimal enrichment can be achieved when the exudate samples are diluted 100-folds. Chemometric tools were executed on the obtained mass spectra for revealing the species-specific markers. More potential peak markers can be found after enrichment and/or purification of proteins from fish muscle exudate using WCX-MBs. Species-specific peak markers and protein markers in mass spectra can be identified. The number of ions with VIP values>1.0 that can be considered as peak markers that differentiate the analyzed fishes, increases from 38 to 121 when using WCX-MBs to isolate peptides/protein in fish muscle exudate. Specifically, eight (8) peak markers in mass spectra are assigned to be specific to NA, 3 peak markers specific to LC, 2 ion peaks specific to MM, 7 peak markers specific to CL and 6 peak markers specific to LP. Furthermore, five proteins were identified based on the characterization of tryptic peptides that can act as biomarkers of which, 4 proteins were specific to CL and 1 to LC. Comparatively, when directly analyzing the fish muscle exudate, only one protein could be identified, specific to CL. Clearly, the utilization of WCX-MBs can improve the discovery of fish species-specific biomarkers in fish muscle exudate samples. The single-blind samples analysis demonstrated that these species-specific peak markers and identified protein biomarkers can be utilized for fish recognition. It's promising to discover more biomarkers by assistance of MBs in fish muscle exudate for efficient fish species recognition and food fraud test.

CRediT authorship contribution statement

Weijiao Chen: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Winnie C. Soko:** Formal analysis, Visualization, Writing – review & editing. **Jing Xie:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Writing – review & editing. **Hongyan Bi:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101509.

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