Evaluation of Extraction Buffers for Protein Identification in Fish Paste with Chicken Blood Plasma (CBP) Spikes: A Preliminary Mass Spectrometry Study

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Abstract: *Blood plasma is commonly used as a protease inhibitor in surimi production to improve product quality and stability during processing. However, its use in food is prohibited in Islam and classified as* najs al-mutawasitah*, necessitating the detection of blood plasma adulteration to uphold halal standards and food safety. This study compared the effectiveness of different extraction buffers—ultrapure water, 0.05 M Tris-HCl, 0.05 M Tris-Urea, and ultrapure water followed by acetone precipitation—on protein and peptide yield from chicken blood plasma (CBP) using liquid chromatography–quadrupole linear ion trap mass spectrometry (LC-QTRAP-MS). Ultrapure water and acetone precipitation yielded the highest protein content, prompting further proteome profiling of CBP, fish paste, and surimi spiked with CBP (0.5, 1, and 1.5%) via liquid chromatography–quadrupole time-of-flight mass spectrometry (LC–QTOF-MS). Apolipoprotein AI (Apo AI) and fibrinogens emerged as key proteins in CBP. Apo AI was detected in all spiked surimi samples, demonstrating its potential as a marker for blood plasma contamination. The proposed method enhances extraction and detection protocols, using mass spectrometry to provide a reliable tool for addressing halal compliance and mitigating food safety risks associated with blood-derived adulterants in surimi products.*

Keywords: *mass spectrometry; chicken blood plasma; spiked sample; Apolipoprotein AI; fibrinogen*

■ INTRODUCTION

Surimi is made of fish tissue that has been cleaned, finely chopped, deboned, dewatered, and refined, and with sugar and phosphate supplements to retain the protein during cold storage [1]. The demand for surimi has risen in recent years owing to its convenience, availability of high protein, low lipid content, and great taste with established products, such as fish balls, crab meat imitation, and cakes, which were first introduced in Japan. Various factors including fish species, freshness, handling, protein structure, and heating methods, influence surimi production. Different fish species have distinct protein compositions, affecting the quality of surimi. Marine fishes, such as Pacific whiting (*Merluccius productus*), Alaska pollock (*Gadus chalcogrammus*), threadfin bream (*Nemipterus virgatus*), and other freshwater species, such as tilapia, are frequently used as raw materials in manufacturing surimi. Protein gelation is a crucial property of surimi,

determining its texture during production. However, certain species exhibit compromised gelation, necessitating additives to enhance gelation, texture, and water-holding capacity [2].

Blood plasma has gained attention as a cost-effective alternative to egg whites [3] and starch, offering comparable or superior functional properties. As a natural protease inhibitor with excellent gelling characteristics [4], blood plasma significantly improves the textural qualities of surimi-based products. In general, blood has long been recognized as a rich source of proteins [5], essential minerals, and vitamins. These components make blood a versatile ingredient in food, commonly utilized as an emulsifier, stabilizer, color enhancer, binding agent, and functional additive [6]. For example, previous work highlighted the utilization of bovine plasma to strengthen heat-induced myosin gels to encourage gelling activities [7]. It has also been noted that salmon plasma also provides good functional properties to inhibit proteolytic degradation in Pacific whiting surimi and salmon mince [3]. Beyond surimi and sausages, blood is widely used in blood sausages, bakery items, lysine supplements, pet food, and feedstuff [8]. Given its versatile functionality, blood plasma has emerged as a refined alternative to whole blood in food applications. Researchers and industries have developed food-grade proteins from blood plasma, which are now patented under brand names like *Fibrimex*, *Harimix P*, *Plasma Powder FG*, and *Vepro Globin* [9]. Improvements in acquiring and processing animal blood have increased the usage of blood plasma products in pet and human foods, positively affecting the demand for these goods. Consequently, the market for animal blood plasma products is expanding significantly and is expected to do so in the future [10].

The rising incorporation of blood plasma in food products presents a significant challenge in detecting its adulteration in surimi. The concern is not only related to food safety but also to religious dietary restrictions, as blood and blood plasma are prohibited from being consumed in certain cultures, such as among Muslims and Jews [11]. To ensure consumer safety and regulatory compliance, reliable methods for detecting blood plasma in surimi are essential. Contemporary techniques based on mass spectrometry (MS) have successfully analyzed complex and processed meat products, identifying species in bolognese sauce [12], processed meat products [13], and diagnosing disorders like cardiovascular [14-15], liver [16], and neurodegenerative diseases [15]. While commonly employed, applied immunochemical and DNA-based techniques have drawbacks when examining food matrices containing multiple components that have undergone heat processing.

A critical gap in the current research is the lack of suitable extraction buffers capable of isolating plasma proteins, such as Apo AI, from complex food matrices for reliable detection. Previous studies on plasma markers mainly were reported from the medical and veterinary fields, which were used to detect plasma amyloid in Alzheimer's disease [17] and multiple organ dysfunction syndrome (MODS) as well as systemic inflammatory response syndrome (SIRS) in canine [18]. However, the potential of MS to identify protein markers for blood plasma adulteration in food, particularly surimi, has not been explored. Moreover, a suitable proteome extraction buffer for isolating plasma proteins in food matrices like surimi has not been established. Hence, this study aims to compare the yield of different extraction buffers in isolating protein markers from chicken blood plasma (CBP) incorporated into fish paste mimicking the surimi. The primary focus is identifying Apo AI as a potential marker for blood plasma adulteration. By optimizing the extraction process and identifying protein markers, the study seeks to enhance the reliability of detection methods for blood plasma in surimi. The results of this study could offer significant advancements in food safety, particularly in ensuring compliance with regulatory standards and addressing consumer concerns related to food adulteration.

■ EXPERIMENTAL SECTION

Materials

The MS grade formic acid (FA) and acetonitrile (ACN) were provided by Fisher Chemical (Fair Lawn, New Jersey, USA). Meanwhile, dithiothreitol (DTT),

electrophoresis-grade iodoacetamide (IAA), thiourea, tris-HCl, urea, and EDTA were sourced from Bio-Rad (Shanghai, China). Bradford protein assay kits were obtained from Bio-Rad Laboratories in the USA, while ultrapure water was prepared using a Sartorius Stedim Arium®611VF system (Goettingen, Germany). Sequencing-grade trypsin was purchased from Promega (Madison, Wisconsin, USA). The standard chemical kits with high and low concentrations of polypropylene glycol (PPG, PN 4406127) for calibrating AB SCIEX instruments were utilized from AB Sciex Pte. Ltd. (Framingham, Massachusetts, USA).

Instrumentation

The instrumentation was divided based on the type analysis. For LC-QTRAP-MS acquisition, chromatographic separation and tandem MS detection were conducted using a high-performance liquid chromatography (HPLC) system (Agilent 1200 Series, Waldbronn, Germany) equipped with an autosampler (G1316A) and coupled to an AB SCIEX 4000 QTrap MS (Singapore). For LC-QTOF-MS acquisition, chromatographic separation and MS analysis were carried out using an HPLC system (Agilent 1200 Series, Waldbronn, Germany) coupled to an Agilent 6520 Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA, USA) with a dual ESI ionization source. Vortex (IKA) and centrifuge (Eppendorf model 5417 r, Germany) were also used. The software used following the analysis included Petunia Trans-Proteomic Pipeline (TPP) version 6.3.3 and Mass Hunter Workstation Software (MHW) Qualitative Analysis version B.06.00.

Procedure

Pooled CBP was prepared from slaughtered chicken and investigated in this study. A fish paste sample was also included as a negative control to validate the proteins in CBP against fish proteins.

CBP collection

The blood samples were collected from the commercial slaughterhouse around Selangor, Malaysia. Individual live chickens were weighed and bled via slaughtering at the neck area, and samples of blood were taken in and collected into K1-sodium citrate-containing Vacutainer tubes (BDT Vacutainer Science). As a precautionary measure, the blood was collected within 2–5 s after the neck cutting to ensure the purity and integrity of the samples. The tube was stored at 4 °C before being transported to the laboratory.

Preparation of CBP

Blood samples were centrifuged at 4.00 g for 10 min at 4 °C to extract plasma. The collected plasma was pooled, aliquoted into a 1 mL tube, and kept at −40 °C until further analysis [19].

Preparation of protein extracts from CBP

Four extraction buffers were evaluated with modifications, i.e., (1) ultrapure water [20], (2) 0.05 M Tris-hydrochloric acid (Tris-HCl), (3) 0.05 M Tris-urea (Tris-Urea) [21], and (4) ultrapure water followed by cold acetone precipitation [22]. Extraction involved applying 1 mL of extraction solvent to 500 µL of CBP, vortexing for 10 min to guarantee complete uniformity. Afterward, the mixtures were centrifuged at 4 °C for 30 min at 10,000 × g [19]. For solvent (4), the mixture was combined with 4 mL of cold acetone and left overnight at −20 °C. Two phases were generated, and the pellet was collected and reconstituted in 1 mL ultrapure water. It was kept in a freezer at −20 °C until further analysis. Protein concentrations were subsequently measured utilizing the Bradford protein assay, which performed the extraction process in triplicates.

Preparation of fish paste samples and spiked fish paste samples

To minimize variation, the fish paste was sourced from one of the largest surimi manufacturers in Malaysia, located in Teluk Intan, Perak Darul Ridzuan. The fish paste samples were prepared following the procedure outlined by [23] with minor modifications to suit experimental requirements. To control variations in composition, the fish paste was thawed at 4 °C for 4 h, minced, and mixed with CBP, sorbitol, 2% w/w NaCl, 4% w/w sucrose, and 0.3% sodium tripolyphosphate [19]. Moisture content was adjusted to 80–85% using crushed ice, ensuring a consistent and homogenous slurry, as per the method described by [3]. The samples

were then portioned into 2 cm × 2 cm × 2 cm ice cube molds, carefully wrapped, and frozen at −18 °C for a minimum of 12 h. For spiked samples, blank fish tissue (50 g) was spiked in triplicate with CBP at 500 mg/kg concentration, using three spiking levels (0.5, 1.0, and 1.5%). Frozen cubes were boiled at 90 °C for 20 min, cooled to room temperature, and analyzed. Specific handling conditions, such as temperature and spiking criteria, were rigorously maintained to ensure reproducibility and accuracy in protein marker identification [24].

Protein digestion

All samples, including CBP, fish paste, and spiked samples, as well as Bovine Serum Albumin (BSA), were digested according to the instructions provided by the manufacturer outlined in the analysis certificate with regards to Promega Trypsin Gold, MS Grade, Part No. V5280. The digestion was achieved by adding 4 µL of 1 g/L Trypsin Gold in 50 mM acetic acid at a temperature of 37 °C for 24 h. Next, the samples were decreased by 100 mM DTT for 3 h at 37 °C and subsequently alkylated employing IAA in the dark for another 3 h at room temperature. The digestion step was terminated with the addition of 1 µL of FA, and each sample was done in triplicate.

LC-QTRAP-MS acquisition

The CBP samples extracted through four types of solvents have proceeded with LC-QTRAP-MS injection following the methodology described by [25]. Chromatographic separation and tandem MS detection of all samples were carried out using HPLC. Samples were injected at 45 °C with a flow rate of 300 µL/min over 25 min onto a Phenomenex Kinetex Core-shell C18 reversed-phase column (Torrance, CA; 100 × 2.1 mm, 2.6 µm, 100 Å). The mobile phases A and B comprise 0.1% FA and 100% ACN, respectively. The column was equilibrated to ten times its volume prior to sample injection. The original concentration with regards to mobile phase A was 97% for 1 min and decreased gradually to 71.6% for 22 min. Subsequently, this was followed by the gradual increase of eluent B until 28 min and consistently maintained at 0% at 28 to 29 min. At 29 min, the concentration of mobile phase A was returned to its initial concentration of 97% and sustained until min 35. Finally, 10 µL of each sample was inserted into the system in triplicate.

LC-QTOF-MS acquisition

The method was conducted with certain adjustments following prior investigations [26]. The chromatographic separation of all five samples (CBP, fish paste, 0, 0.5, and 1.5% spiked fish paste) was performed using an HPLC system. Each sample was injected three times onto a reversed-phase Phenomenex Kinetex Core-shell C18 column (100 × 2.1 mm, 2.6 µm, 100 Å) at 45 °C. The flow rate was maintained at 300 µL/min for 25 min. Separation was achieved using gradient elution, with eluent A being ultrapure water with 1% FA, and eluent B being pure ACN. At first, eluent B was set at a concentration of 3% for 1 min. This was followed by the gradual increase of eluent B concentration of 98% for 20 min. Finally, the concentration remained constant for 1 min before being returned to the original condition for 3 min. The samples separated via chromatography were examined employing the Agilent 6520 Q-TOF LC/MS. The Dual ESI ionization source was set for electrospray ionization with the following parameters, i.e., a temperature of 325 °C, source gas pressures of 40 psi for gas 1 and 30 psi for gas 2, nebulizer voltage at 5200 V with a pressure of 40 psi, a declustering potential of 50 V, a curtain gas pressure of 30 psi, as well as a scan rate of 1000 Da/s. MS and targeted tandem mass spectrometry (MS/MS) were performed utilizing enhanced mass spectrometry (EMS), with a scan range of 200–2000 *m/z* for both MS and MS/MS. The evaluations were performed in a positive mode.

Data analysis

Raw data were processed using the Petunia Trans-Proteomic Pipeline [26-27], and raw data based on chromatographic separation were processed, including normalization and alignment. Peaks were detected with a noise threshold of at least 3000 counts per second (cps) between 2 and the minimum retention time. The raw data were aligned using a retention time tolerance of 0.1 min and a mass tolerance of 0.5 Da. Any peaks with

less than 90% confidence in a specific set of samples were discarded to avoid false-positive peaks, provided the number of peaks exceeded 3000 cps. The study used a reference from [28] focusing on the Gallus gallus taxonomy to compare the acquired data with known sequences for proteome analysis and marker detection. This reference served as a decoy, with a randomized and concatenated database (SwissProt.2016.6.9.6 random.concan) containing 60,570 protein entries. The precursor mass tolerance was established to 0.5 Da, and carbamidomethylation of cysteine and oxidation of methionine were decided to be fixed and variable modification, accordingly. PeptideProphet™ and ProteinProphet™ were used to analyze the generated pepXML files, with a false discovery rate (FDR) of 1%.

■ RESULTS AND DISCUSSION

Evaluation of Protein Extraction Buffer

This section aims to evaluate the efficacy of different extraction buffers on extracting proteins from CBP using ultrapure water, 0.05 M Tris-HCl, 0.05 M Tris-Urea, and a combination of ultrapure water with cold acetone. Initially, the mixtures were subjected to a thorough analysis using LC-QTRAP-MS, and 189, 133, 340, and 537 proteins were detected (Table 1). Notably, 0.05 M Tris-HCl yields the poorest protein number discovered, which was supported by quantitatively fewer spectra, while the combination of ultrapure water followed by cold acetone protein precipitation increases total spectra, proteins, and peptides in complex sample matrices, resulting in a fivefold increase in protein and peptide content. It is encouraging to compare this figure with that found by previous work [21], who found that Tris-HCl yielded the lowest number of identifications of proteins and peptides. Tris-urea was able to yield more protein identifications and was almost comparable to ultrapure with acetone precipitation. Fig. S1(a-d) provides a total ion chromatogram (TIC) for each extraction buffer with the separation of CBP in variations of retention time distribution, signal intensity, peak complexity, and the removal of contaminants, varying the effectiveness of each buffer for protein extraction. The retention time distribution analysis revealed that the TIC from ultrapure water displayed a broad and poorly resolved peak, indicating limited protein recovery, particularly for hydrophobic or membrane-bound proteins. Conversely, distinct and concentrated peaks are observed in the retention time of Tris-HCl (Fig. S1(b)) and Tris-Urea (Fig. S1(c)), indicating their capacity for solubilization and protein recovery. Tris-HCl serves as a standard buffer that effectively maintains pH stability, while Tris-Urea incorporates urea, which functions as a denaturing agent, facilitating the unfolding of proteins to improve their solubility. This makes Tris-Urea particularly advantageous for extracting highly folded or membrane proteins that may pose challenges for solubilization with Tris-HCl alone [29]. Analysis of the TICs for ultrapure water (Fig. S1(a)) and ultrapure with acetone (Fig. S1(d)) revealed reduced peak intensities in comparison to Tris-based buffers. Ultrapure water exhibited inadequate protein recovery and ionization, whereas acetone enhanced protein concentration but was less effective than Tris-HCl or Tris-Urea. Tris-HCl and Tris-Urea exhibited increased signal intensities, indicating their capacity to stabilize proteins and improve ionization efficiency in MS. Therefore, 50–100 mM of a neutral pH buffer (pH 7.5–8.5) combined with a potent denaturant is frequently utilized for global proteomics investigations when increasing the number of protein or peptide identifications is the aim [30]. This highlights the established function of Tris buffers in maintaining protein structure during extraction, thereby enhancing sensitivity in subsequent analyses.

A more detailed examination of peak complexity was demonstrated by the most complex TIC profile from

Table 1. Number of proteins detected from CBP

Samples	tSpectra*	Peptides	Proteins
Ultrapure water	193	192	189
0.05 M Tris-HCl	136	134	133
0.05 M Tris-Urea	561	344	340
Ultrapure water + cold acetone	583	583	537
**Ultrapure water + cold acetone	686	559	409
Control (BSA)	754	573	513

*tSpectra-total spectra in the file; Peptides-number of identified peptides; Proteins number of identified proteins
*samples were analyzed using LC-QTOF-MS

Tris-Urea, which had a wide range of peaks distributed over a long retention time. This suggests its efficacy in recovering both hydrophilic and hydrophobic proteins. The chaotropic effect of urea disrupts hydrogen bonds, thereby enhancing the solubilization of proteins embedded in plasma matrices, including lipoproteins. It also induces carbamylation with limited when heating samples [31]. In contrast, ultrapure water and ultrapure with acetone yielded fewer peaks, suggesting limited protein recovery. These findings are consistent with the literature, highlighting urea-containing buffers' effectiveness in achieving comprehensive protein solubilization [30]. The selection of an extraction buffer significantly influences protein recovery and sample quality in proteomics. Tris-Urea was the most effective for comprehensive protein profiling, particularly for biomarker discovery, due to its ability to recover hydrophilic and hydrophobic proteins. However, its use in downstream MS is hindered by residual urea, which suppresses ionization efficiency and interferes with enzymatic digestion during trypsinization [30]. These limitations may restrict its utility in workflows requiring precise quantification or post-translational modification analysis.

While less effective in recovering a wide range of proteins, ultrapure water with acetone provided cleaner chromatograms with minimal background noise and low-intensity peaks, indicating effective contaminant removal. This makes it a superior choice for workflows prioritizing purity and reproducibility, such as quantitative proteomics and MS-based assays. Its ability to deliver high-quality, low-contaminant samples enhances the reliability and accuracy of downstream analysis, compensating for its narrower protein recovery range. Findings on the protein yield of this study highlight the advantages of ultrapure water with acetone as a reliable buffer for MS workflows requiring high sample purity. Tailoring buffer selection to specific analytical goals will ensure optimized performance in proteomic studies involving complex biological matrices given by CBP.

The findings determined that the best strategy for extracting CBP from complex matrices requires a stepwise approach. This involves ultrapure water extraction followed by acetone precipitation, yielding the optimal CBP recovery method. The following section addresses the significance of aqueous solutions, which have gained prominence over organic solvents in protein extraction protocols [32] due to their compatibility with the native structure and functional integrity of proteins. Ultrapure water has emerged as a preferred medium, offering high purity and minimizing interference from contaminants during extraction and purification [33]. While ultrapure water's high purity could lead to protein instability, its benefits, such as low ionic strength and absence of organic contaminants, aid in preserving protein structure and enhancing solubility [33]. Acetone precipitation complements ultrapure water extraction by concentrating the protein sample and eliminating contaminants from complex matrices. This method effectively recovers proteins, decreasing solubility and enabling quick, high-yield precipitation (up to 98% recovery within 2 min) while minimizing sample loss and preserving protein integrity.

Acetone precipitation also demonstrates compatibility with salts and different ionic strengths, making it suitable for subsequent applications like MS [34]. MS studies have shown that acetone precipitation improves protein yield and enhances performance, with successful applications in proteins extracted from BSA, cytochrome C, beans, yeast, and fish (*Salmo salar*) [22,34]. The protein pellet obtained through acetone precipitation is particularly well-suited for downstream applications, including MS analysis [35].

Next, high-resolution MS or LC-QTOF-MS, was used to analyze the protein samples for MS to enhance them qualitatively and quantitatively. As expected, switching to a high-resolution MS produced more tandem mass spectra. The data produced here indicates that the extraction buffer and resolution of the instruments play a vital role in identifying the yield of spectra, peptides, and proteins, in which these limiting factors, if properly optimized, can increase the yield of proteins. High-resolution LC-QTOF-MS improves protein separation and identification [36], reduces false-positive identifications, has a wider dynamic range for analyzing proteins with different abundance levels, is

more sensitive to low-abundance proteins, and acquires data quickly.

The following section critically assesses the current approaches to using the Trans-Proteomic Pipeline (TPP) due to its pivotal role in MS-based proteomics, offering open-source access, comprehensive integration, and robust statistical validation. TPP has been continually developed and refined since the introduction of PeptideProphet and ProteinProphet two decades ago, evolving into a suite of tools for spectrum processing, search validation, protein inference, and abundance computation. The latest release incorporates machine-learning models to maximize information extraction and enhance statistical modeling, ensuring the accuracy of derived data.

TPP surpasses other platforms such as MaxQuant [37], Proteome Discoverer [38], FragPipe [39], PEAKS Studio [40], and OpenMS [41] by enabling extensive customization and cross-platform compatibility. In contrast to MaxQuant's, which relies on the Andromeda engine, TPP supports multiple search engines, including SEQUEST, Mascot, X!Tandem, and Comet, expanding its applicability. A major advantage of TPP is its statistical framework, with PeptideProphet and ProteinProphet ensuring high-confidence identifications and reduced false discovery rates (FDR), contrasting FragPipe's dependence on Percolator. TPP's versatility in label-free quantification and multiplexed methods like iTRAQ and SILAC provides broader flexibility than many proprietary tools. It also facilitates modular processing scalability for large datasets and high-throughput proteomics, addressing challenges faced by MaxQuant and Proteome Discoverer. Its versatility is also convenient as it can be deployed across personal computers, Linux clusters, and cloud environments, reinforcing its adaptability to various computational infrastructures [27]. The produced visualization and reporting tools enhance reproducibility, solidifying TPP as a critical platform for proteomic research and high-impact investigations.

On the question of the reliability of the extraction method performed on the MS system is demonstrated by quality control (QC) using BSA. BSA is frequently employed as a benchmark for quality assurance in MS analysis due to its numerous advantages. Its exceptional sequence coverage, usually above 85%, establishes it as a dependable benchmark for evaluating the efficacy of MS techniques [42]. In this study, the proteins detected from BSA gave 95.75% of sequence coverage, which is purely serum albumin from bovine, indicating that the mentioned method and instrument work excellently in the workflow. Hence, BSA functions as a helpful instrument for QC in MS analysis, owing to its well-established properties and efficacy across diverse applications.

Identification of Proteins in CBP

The sample was then proceeded using LC-QTOF-MS due to its high resolution, sensitivity, and consistent quantifications of many chemicals, including proteins, which improved protein separation and identification in complex mixtures [43]. Specific criteria have been put forth throughout the experiment, whereby to choose a marker protein specific to plasma, the candidate marker must not be present in fish paste and vice versa. The following section will compare Tables 2–4 concurrently. The analysis of the TIC of CBP alone did not provide enough information for a thorough understanding. Hence, we supplemented the TIC data with spectral analysis using tSpectra. This addition created a more solid foundation for in-depth interpretation.

The digested samples were injected and scanned using LC-QTOF-MS within an *m/z* range of 200–2000. This resulted in more than 250 MS/MS spectra. The mobile phase gradient transitioned from polar to nonpolar, facilitating the sequential elution of hydrophilic proteins, followed by hydrophobic proteins [26]. Seventeen proteins in total were found in CBP samples (Table 2). Apo A1, Fibrinogen alpha chain, Fibrinogen C-terminal domain containing-protein, Fibrinogen beta, Ovotransferrin, CN hydrolase domain-containing protein, and albumin were consistently present across all MS injections. The prominent proteins detected from CBP had medium to high sequence coverage (≥ 30%). Apo A1 is a protein with a determined molecular weight of 30,662 Da and 264 amino acid residues. It is a major protein part of high-density lipoprotein (HDL) that regulates cholesterol transport

Table 2. List of proteins detected in CBP

Identified protein	Accession no.*	Mass (kDa)	Peptide-spectrum matches (PSM)	Sequence coverage (%)
Apolipoprotein A-I	P08250	30.662	104	82.2
Fibrinogen alpha chain	P14448	82.338	42	30.6
Ovotransferrin	P02789	77.727	37	27.0
Uncharacterized protein	F1NK40	163.237	24	10.1
Uncharacterized protein	A0A1D5P9F9	198.853	7	3.5
Fibrinogen beta	Q02020	52.646	6	6.7
Albumin	P19121	69.873	3	3.4
Uncharacterized protein	A0A1L1S0P1	50.816	2	2.3
CN hydrolase domain-containing protein	A0A1D5PEU7	54.630	1	9.1
Uncharacterized protein	A0A1D5NXA6	72.456	1	2.5
SMB domain-containing protein	E1C7A7	51.619	1	2.0
Uncharacterized protein	R4GJL7	29.904	1	7.9
Uncharacterized protein	A0A3Q2TYI8	197.986	1	2.5
Uncharacterized protein	F1N8L3	35.795	1	3.2
Uncharacterized protein	A0A3Q2UA24	116.315	1	0.9
Uncharacterized protein	A0A1D5P640	63.641	1	1.2
DNA-directed RNA polymerase III subunit RPC3	A0A3Q2U0G3	41.604	1	4.6

*Accession number source: The National Center for Biotechnology Information (NCBI)

Table 3. Identification results for fish paste sample

Proteins	Accession no.*	Peptide-spectrum matches (PSM)	Sequence coverage (%)
Myosin heavy chain, fast skeletal muscle-like	A0A674BHX5	93	22.9
Myosin heavy chain, fast skeletal muscle-like	A0AIS3PLE3	87	27.4
Myosin heavy chain, fast skeletal muscle-like	A0AIS3QKG4	84	28.3
Myosin heavy chain, fast skeletal muscle-like	A0A673ZIV2	78	18.2
Myosin heavy chain, fast skeletal muscle-like	A0A060X459	69	17.6
Myosin heavy chain, fast skeletal muscle-like	A0A674DP42	64	14.5
Myosin heavy chain, fast skeletal muscle-like	A0A060X652	62	14.6
Tropomyosin alpha-1 chain isoform X1	A0AIS3PVG3	46	52.5
Tropomyosin alpha-1 chain isoform X1	A0AIS3KNT1	39	47.5
Actin alpha cardiac muscle 1	A0A060VMK5	30	43.2
Actin alpha cardiac muscle 1- like	A0A060WOR3	22	43.6
Myosin regulatory light chain 2, skeletal muscle isoform type 2-like	A0A673VIQ2	12	50.0
EF-hand domain-containing protein	A0A060XEV5	9	50.3
Uncharacterized protein	A0A060WZV7	7	28.2
Myosin light chain-1, skeletal muscle isoform like	A0A674CP61	7	18.9
Glyceraldehyde-3-phosphate dehydrogenase	A0A060WQ18	3	12.9
Tropomyosin alpha-1 chain	A0A673WGZ6	45	54.1
Myosin-6-like	A0A1S3R4R2	11	69.3
Myosin motor-domain-containing protein	A0A060WTP0	39	9.2
Uncharacterized protein	A0A060WL78	37	5.2

*Accession number source: The National Center for Biotechnology Information (NCBI)

Table 4. Identification results for spiked fish paste sample

Protein	Accession no.*	Matched peptides			Sequence coverage (%)		
		0.50%	1.00%	1.50%	0.50%	1.00%	1.50%
Myosin heavy chain, fast skeletal muscle-like	A0A674BHX5	285	205	118	12.0	9.7	9.4
Myosin 1-B	P02565	110	58	31	5.4	4.7	3.6
Myosin regulatory light chain 2	P02609	95	38	35	20.8	20.8	20.8
Apolipoprotein AI	P08250	4	3	3	12.9	3.8	3.8
Myosin light polypeptide	P02607	48	30	35	6.7	9.3	20.8
Tropomyosin alpha-1 chain-like isoform	A0A1S3MLT8	109	19	72	14.1	18.3	19.5
Actin (Fragment)	A0A385FQD3	3	7	n.d	9.0	5.9	n.d
Albumin	P19121	2	2	2	2.8	2.8	2.8

*Accession number source: The National Center for Biotechnology Information (NCBI)

and metabolism [44]. It is present in the plasma of 95% of individuals [45] and is composed of stable α-helices associated with HDL.

In our findings, the Apo AI protein was identified with good confidence scores, whereby 104 matched peptides and 82.2% protein sequence coverage were reported. Protein sequence coverage refers to the percentage of original protein sequences represented by chosen peptides. This is calculated by dividing the total number of amino acids covered by the peptides by the total length of the protein sequences in the FASTA file [46]. A higher sequence coverage percentage signifies a more comprehensive identification of the protein's amino acid sequence [47]. Other proteins such as fibrinogens (alpha and beta chains) with medium to good sequence coverage score (6–30%), ovotransferrin, and some uncharacterized proteins were also detected in CBP. These types of proteins were classified as plasma proteins [48].

Identification of Proteins in Fish Paste Sample

The raw fish paste sample was made from 100% fish meat and was also used as the control group in spiked samples, accordingly, because they did not incorporate any additives or functional preparations. More than 100 proteins were discovered based on the samples that were analyzed. However, only 20 corresponding proteins were shortlisted using the target-decoy method, with the probability of correct identification being ≥ 0.95 (Table 3). The proteins were myosin heavy chain with multiple accession numbers, tropomyosin alpha-1 chain isoform X1, actin alpha cardiac muscle 1-like, myosin regulatory light chain 2, skeletal muscle isoform type 2-like, and EF-hand domain-containing protein. It is also corroborated that there are no CBP-related proteins in the samples, and additional research reveals that no proteins are derived from the fish blood or fish paste itself. The listed proteins were comparable to those of the Food and Agriculture Organization of the United Nations (FAO) [49] that fish protein could be divided into a few categories, including myofibrillar proteins (e.g., myosin, actin, tropomyosin, actomyosin), which make up 70 to 80% of the total protein contents, sarcoplasmic proteins, which include myoalbumin, globulin, and enzymes, that make up 25 to 30% of the protein content, as well as connective tissue proteins, which are 3% collagen in Teleostei and 10% in Elasmobranchii [50].

Although fish do contain blood, it is fortunate that the fish blood is not detected during the screening. It might be due to the fish blood not being present in the fish muscle as the veins supplying blood vessels are small, and the volume is lower than in other animals such as cattle and poultry. Furthermore, the absence of blood proteins in fish paste extracts might be due to various factors such as processing-induced degradation, complex matrix interference, and inadequate extraction techniques. According to Hou et al. [1], the making of surimi started with fish tissues undergoing vigorous cleaning, finely chopped, deboned, multiple dewatering, and refined, with preservation with sugar and phosphate addition during cold storage. These can result in protein breakdown during processing and reduce their visibility.

On the other hand, the extraction methods employed here have demonstrated effective extraction of blood proteins, concluding that the absence of blood proteins cannot be attributed to poor extraction methods.

Next, adding CBP to the fish paste is crucial to verify the distinction between blood proteins in CBP and proteins in fish paste. Hence, the fish paste sample was spiked with different percentages of CBP. Table 4 shows the presence of proteins in fish paste samples spiked with a known percentage of CBP. All the proteins identified, such as various types of myosin, tropomyosin, and actin, are predominant in the spiked samples. Remarkably, Apo AI was consistently detected in the samples even with lower sequence coverage (3.8–12.9%) and low-matched peptides with a probability of 1. It is suggested that the results were affected by the samples' ingredients, the samples' processing, and the instrument's detection limit. Other major proteins, like those found in muscle and skin, may mask the presence of plasma proteins from fish due to the complex matrix.

Spiked samples play a crucial role in MS experiments. According to Thermo Scientific [51], matrix spike standards are essential for evaluating the performance of analytical testing methods. They are employed to assess the method's performance, recovery efficiency, quality control, matrix interference detection, and technique optimization [51]. These samples help evaluate analytical methods' accuracy and consistency, ensuring reliable outcomes for specific sample categories or matrices. By introducing known concentrations of analytes into samples, spiked samples help assess recovery efficiency and provide insights into the method's reliability [52]. To improve the detection of common protein components present in spiked samples, it is advisable to utilize a triple quadrupole MS that is more sensitive and focused. This specialized equipment can identify and accurately quantify the protein molecules of interest, even in low concentrations. Using a triple quadrupole MS, researchers can gain important information about the composition and properties of spiked samples, which can help advance our understanding of this vital biological substance [53]. As in this case, the consistent presence of Apo AI indicates the potential and reliability of Apo AI to be a protein biomarker in surimi.

■ CONCLUSION

Several protocols comparing a few extraction methods led to the selection of the ultrapure water followed by acetone precipitation for protein enrichment for complex sample matrices. Extracted proteins were compared with BSA as QC and CBP. Meanwhile, 0.05 M Tris-HCl buffer was quantitatively less efficient in generating poorer protein as well as having the fewest distinct peptides and proteins. Total spectra, proteins, and peptides increase when using ultrapure water as the primary diluent. Remarkably, the number increases significantly after cold acetone precipitation, yielding five times higher protein and peptides. Verification using spiked samples indicated that the Apo AI was successfully detected in 1.5, 1.0, and 0.5% spiked percentages of samples even with lower protein coverage. This study also suggested that Apo AI is a potential marker for detecting adulteration of animal blood-derived additives in fish-based products. For future studies, it is suggested that the presence of Apo AI is tested in blood from other species (cattle, pork, and goat) to evaluate its potential as a plasma marker, as blood plasma could come from multiple species of origin.

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■ CONFLICT OF INTEREST

The authors state that they have no identifiable financial conflicts of interest or personal relationships

that might have influenced the work presented in this paper.

■ AUTHOR CONTRIBUTIONS

Nurhazirah Azmi conducted all the experimental works with data analysis and manuscript preparation. Siti Aimi Sarah Zainal Abidin, Low Kim Fatt, and Mohd Hafis Yuswan validated the manuscript along with Saiful Anuar Karsani with supervision. Nurkhurul Ain Zakaria and Mohd Syarafuddin Abdul Shukor revised the manuscript together with Nurhazirah Azmi. All authors agreed to the final version of this manuscript.

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