UNIVERSITI TEKNOLOGI MARA

IDENTIFICATION OF PROTEIN BIOMARKERS IN CHICKEN BLOOD PLASMA (CBP) AND DEVELOPMENT OF GOLD NANOPARTICLES-BASED ELECTROCHEMICAL IMMUNOSENSOR FOR DETECTING CBP ADULTERATION IN SURIMI-BASED PRODUCTS

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PhD

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of the requirements for the degree of
Doctor of Philosophy
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ABSTRACT

Chicken blood plasma (CBP) has been increasingly used as an undeclared functional additive in surimi-based products, raising significant concerns regarding halal authentication and regulatory compliance. Detecting CBP in thermally processed and complex food matrices remains challenging, as conventional DNA-based and immunochemical methods often exhibit reduced sensitivity and reliability. Therefore, robust protein-based analytical strategies are required for accurate CBP authentication. An optimized protein extraction protocol was established to enhance protein recovery from CBP obtained from slaughtered chickens. The combination of ultrapure water extraction followed by acetone precipitation significantly improved protein enrichment compared to alternative methods. This dual-step approach increased protein and peptide yield, facilitating reliable downstream proteomic profiling of CBP. Proteomic analysis using liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) identified apolipoprotein AI (Apo AI) and fibrinogen as candidate biomarkers based on their detectability, abundance, and analytical robustness. Bioinformatics sequence analysis further demonstrated high conservation of Apo AI across terrestrial vertebrates while revealing marked divergence from fish homologues, supporting its suitability as a broadly applicable vertebrate plasma marker with reduced risk of cross-reactivity in fish-based matrices. Gold nanoparticles (AuNPs) synthesized via sodium citrate reduction (18.0 ± 1.3 nm) were conjugated with specific antibodies and immobilized on streptavidin-coated magnetic beads to construct the electrochemical sensing interface. As electrochemical signal intensity is highly dependent on operational and conjugation parameters, key analytical variables including electrodeposition potential, deposition time, step potential, and antibody–AuNP conjugation conditions were systematically optimized to maximize sensitivity and ensure reproducible responses. Under these optimized conditions, the developed immunosensor, operating via differential pulse anodic stripping voltammetry (DPASV), demonstrated strong linearity for Apo AI across 50–1250 µg/mL (R^2 = 0.9931) and good linearity for fibrinogen within its defined working range of 500–1250 µg/mL (R^2 = 0.9886). Detection thresholds, determined using the M + 3SD statistical criterion, were established at 0.58 µA for Apo AI and 0.56 µA for fibrinogen. Application to spiked and commercial surimi samples demonstrated reliable detection performance, with three out of twelve commercial brands exceeding the established cut-off for Apo AI. The developed antibody-based AuNP electrochemical immunosensor provides a sensitive, portable, and halal-compliant platform for CBP authentication in processed food products.

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LIST OF SYMBOLS

Symbols

°C	Degree Celsius
±	Plus/Minus
Da	Dalton – Standard Unit of Atomic Mass
et al.	Et Alii
kDa	Kilodalton – Unit of Molecular Mass
m/z	Mass-To-Charge Ratio – Used in Mass Spectrometry
mg	Miligram
nm	Nanometre – Unit of Length For Wavelength
OD520	Optical Density At 520 Nm Wavelength
R^2	Coefficient of Determination – Statistical Measure For Model Fit
s	Seconds – Unit of Time
T20	Tween 20/ Polyoxyethylenesorbitan Monolaurate
V	Volt – Unit of Electric Potential
xg	Relative Centrifugal Force
µA	Microampere – Unit of Electric Current
µg/mL	Micrograms Per Millilitre – Concentration Unit

LIST OF ABBREVIATIONS

Abbreviations

ACN	Acetonitrile
Apo AI	Apolipoprotein AI
Au^0	Metallic Gold
Au^{3+}	Gold (III) Ion
AuNPs	Gold Nanoparticles
Br_2	Bromine
BSA	Bovine Serum Albumin
CBP	Chicken Blood Plasma
DNA	Deoxyribonucleic Acid
DPASV	Differential Pulse Anodic Stripping Voltammetry
DTT	Dithiothreitol
Edep	Electrodeposition potential
ESI	Electrospray Ionization
Estep	Step potential
ELISA	Enzyme-linked immunosorbent assay
FA	Formic Acid
FGA	Fibrinogen Alpha Chain
FGB	Fibrinogen Beta Chain
FGG	Fibrinogen Gamma Chain

$HAuCl_4$	Chloroauric Acid
HBr	Hydrobromic Acid
HDL	High-Density Lipoprotein
HPLC	High-Performance Liquid Chromatography
IAA	Iodoacetamide
IP	Immunoprecipitation
LC-QTOF-MS	Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry
mAb	Monoclonal Antibody
MB	Magnetic Beads
MB-S	Streptavidin-coupled magnetic bead
MS	Mass Spectrometry
m/z	Mass-to-Charge Ratio
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PSM	Peptide-Spectrum Match
RSD	Relative Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SPCE	Screen-Printed Carbon Electrode
SPE	Screen-Printed Electrode
SPR	Surface Plasmon Resonance
TEM	Transmission Electron Microscopy
tdep	Electrodeposition time

TIC	Total Ion Chromatogram
TPP	Trans-Proteomic Pipeline
UV/VIS	Ultraviolet–Visible Spectroscopy

CHAPTER 1
INTRODUCTION

1.1 Research Background

Surimi, a highly versatile processed fish product, is primarily composed of myofibrillar proteins such as myosin, actin, and tropomyosin. Its manufacturing process encompasses a series of stages including cleaning, deboning, fine chopping, dewatering, and refining of fish tissue, followed by the incorporation of cryoprotectants such as sugar and phosphate to preserve protein stability during frozen storage (Hou et al., 2019). Owing to its ability to emulate the texture and appearance of real seafood, surimi is extensively utilized in the production of imitation crab sticks, fish balls, and kamaboko. Its high protein content and firm, elastic gel-forming capacity upon cooking have established surimi as a valuable food ingredient, contributing to both nutritional value and product functionality. Furthermore, surimi production aids in promoting global food sustainability by utilizing underexploited fish species, thereby reducing fishery waste (Mangi et al., 2023)

Several factors critically influence the quality of surimi, including the fish species employed, freshness, handling protocols, inherent protein structures, and heating methods. Species-specific variations in protein composition markedly affect surimi's gelation ability, a critical determinant of texture. Commonly utilized species include Pacific whiting (*Merluccius productus*), Alaska pollock (*Gadus chalcogrammus*), threadfin bream (*Nemipterus virgatus*), and freshwater species such as tilapia (Hou et al., 2019). Some species exhibit compromised gelation, necessitating additives to enhance gel strength, water-holding capacity, and overall textural attributes (Zhou et al., 2019).

Blood plasma has emerged as an economically viable and functionally potent additive, serving as a natural protease inhibitor with superior gelling characteristics (Nopianti et al., 2019). Its substitution for traditional additives like egg whites and starch has shown promise in enhancing the textural properties of surimi-based products (Fowler & Park, 2015). Blood, in general, is rich in proteins, essential minerals, and vitamins (Sahilah et al., 2016), and is commonly utilized as an emulsifier, stabilizer,

color enhancer, binding agent, and functional additive in a variety of food applications (Ofori and Hsieh, 2014).

Beyond food safety considerations, the use of blood-derived ingredients also raises important religious and ethical implications. Although blood plasma has gained acceptance as a functional food additive due to its techno-functional properties, its permissibility differs when evaluated from a religious perspective. In Islamic and Jewish dietary laws, the consumption of blood and its derivatives is explicitly prohibited, irrespective of regulatory acceptance or technological function, thereby rendering food products containing blood plasma, including chicken blood plasma (CBP) non-compliant with halal and kosher requirements (Lubis et al., 2014).

Notably, studies have demonstrated the use of bovine plasma to strengthen heat-induced myosin gels (Nunez-Flores et al., 2018) and the application of salmon plasma to inhibit proteolytic degradation in surimi (Fowler & Park, 2015). Commercial developments such as Fibrimex, Harimix P, Plasma Powder FG, and Vepro Globin illustrate the growing industry reliance on processed blood plasma (Siti Jamilah et al., 2021). Improved acquisition and processing of animal blood have significantly increased the adoption of plasma products in pet and human food sectors (TMR Research, 2020).

However, the increasing use of CBP in surimi production raises critical concerns over food authenticity and consumer protection. Some manufacturers may adulterate surimi with CBP to lower production costs, as CBP enhances texture and appearance. This fraudulent practice deceives consumers and poses significant halal compliance issues, given that Islamic dietary laws prohibit the consumption of blood (*najis*) (Rosele et al., 2025).

Current detection methods such as enzyme-linked immunosorbent assay (ELISA) and DNA-based tests face limitations in processed food matrices due to the degradation of biomolecules under thermal and chemical treatments. Proteomics, particularly mass spectrometry (MS), provides a robust alternative by enabling the identification of specific protein biomarkers, including apolipoprotein AI (Apo AI) and fibrinogen, even in highly processed surimi products. Among the major plasma proteins, Apo AI and fibrinogen are of particular interest due to their abundance and biological relevance in the blood. Apo AI functions primarily in lipid transport, whereas fibrinogen plays a critical role in blood coagulation cascade. Both proteins are structurally conserved within species yet exhibit interspecies sequence variations,

making them potentially informative molecular targets for distinguishing chicken-derived plasma components in complex food matrices.

The study by Husna et al. (2023) is referenced in this work not for its biomedical application, but for its demonstration of advanced electrochemical biosensor performance in detecting low-abundance protein biomarkers with high sensitivity and selectivity. Their findings highlight how optimized electrochemical sensing platforms can achieve detection limits significantly superior to conventional ELISA, thereby establishing a useful analytical benchmark for protein-based detection in complex matrices. Such sensor performance characteristics are directly relevant to food adulteration analysis, where target proteins may be present at low concentrations and within highly interferent sample environments.

Building on this analytical premise, the present study adopts a similar electrochemical sensing strategy for the detection of specific plasma-derived proteins, using a c blood plasma from chicken as a model. In the context of food adulteration and authenticity assessment, the presence of species-specific plasma proteins serves as a reliable indicator of undeclared additives. Mass spectrometry was employed as the reference technique to validate biomarker identity and to evaluate the analytical performance of the developed immunosensor, thereby reinforcing the relevance of ApoA1 for ensuring food quality, authenticity, and halal compliance. The work from this research has been successfully published in academic journals (Azmi et al., 2025, Azmi et al., 2023), whereby it demonstrated the effectiveness of mass spectrometry in detecting Apo AI as a reliable marker for CBP adulteration. In addition to MS-based proteomics, electrochemical immunosensors represent a promising approach for food fraud detection. These developed platforms combine antibody-antigen specificity with the electrical signal amplification properties of gold nanoparticles (AuNPs), offering rapid, sensitive, and portable detection solutions. Ultimately, this research aspires to support better food quality assurance, protect consumer rights, and help the food industry align with halal certification standards through innovative, science-driven technologies.

1.1.1 Motivation for this Work

The idea for this research arises from growing concerns over food fraud and halal authenticity, particularly in highly processed meat products such as surimi REF.

As the global food industry expands and becomes increasingly industrialized, the risk of adulteration with undeclared or non-halal ingredients, including CBP escalates. This issue extends beyond ethical and religious considerations; it directly impacts consumer trust, food labeling integrity, and regulatory compliance. Ensuring adherence to halal standards is critically important for Muslim consumers and remains a strategic priority for Malaysia's aspirations as a global halal food hub. Although various analytical techniques, such as enzyme-linked immunosorbent assay (ELISA) and DNA-based assays, have been developed for the detection of meat adulteration, these methods exhibit significant limitations when applied to heavily processed food matrices. Heat and chemical treatments commonly used in food processing can degrade DNA and denature proteins, rendering conventional detection approaches less reliable. Consequently, the need for more robust and sensitive detection methods is paramount. Proteomics, particularly when employed through advanced mass spectrometry (MS) techniques, offers superior sensitivity and specificity for identifying unique protein biomarkers, even after extensive food processing.

Despite the potential of proteomic approaches, a significant gap persists: there is a notable absence of practical, field-deployable tools that are both rapid and sensitive enough for on-site halal testing. Electrochemical immunosensors, which offer high sensitivity, portability, rapid response times, and cost-effectiveness, have emerged as promising candidates for such applications (Mahmud et al., 2023, Low et al., 2014). Nevertheless, few studies have extended these technologies toward halal authentication in complex food systems such as processed meats and surimi.

This research is fundamentally driven by the urgent need to address these critical gaps. By integrating MS-based proteomic identification of CBP-specific proteins with the development of a highly sensitive electrochemical immunosensor, the study aims to establish a dual-platform approach for halal food diagnostics. Such innovation would not only bolster scientific rigor in halal authentication but also support the broader goals of transparent food labeling and consumer protection. Beyond its immediate technological contribution, this research aligns with global and national strategic priorities. It supports the United Nations Sustainable Development Goals (SDGs), particularly Goal 12: Responsible Consumption and Production and Goal 3: Good Health and Well-being, by promoting ethical food production practices and safeguarding consumer rights. Additionally, it directly contributes to the objectives outlined in the Malaysia Halal Industry Master Plan 2030, which emphasizes the

importance of science-based innovation to secure halal food chains. By combining cutting-edge proteomic technologies with practical biosensor applications, this study aspires to deliver timely and impactful solutions that benefit consumers, industry stakeholders, and halal certification bodies alike. The work thus not only advances academic knowledge but also addresses pressing real-world challenges at the intersection of food science, halal assurance, and public health.

1.2 Problem Statement

The increasing incorporation of blood plasma into processed food products presents a significant challenge for food authenticity verification, particularly in surimi-based formulations. Although blood plasma is valued for its techno-functional properties, the undeclared use of CBP raises serious religious, ethical, and regulatory concerns. For example, In Islamic and Judaism dietary law, the consumption of blood and its derivatives is prohibited, rendering surimi products containing CBP non-compliant with halal requirements (Lubis et al., 2016; Rosele et al., 2025). Hence, reliable analytical strategies are required to detect CBP adulteration in surimi to safeguard consumer rights, ensure halal integrity, and maintain public trust. Currently, there is no protein marker for blood plasma detection in food. Most existing knowledge on plasma biomarkers originates from medical or veterinary contexts, where markers like amyloid proteins are studied in diseases such as Alzheimer's and canine inflammatory syndromes (Beck et al., 2015; You et al., 2023). This also extends to other protein biomarkers such as Apolipoprotein AI (Apo AI) that were highly studied in early detection of cardiovascular disease (Husna et al., 2023). However, their application for food authenticity, especially CBP detection in surimi, remains largely unexplored. Furthermore, while MS offers high sensitivity in clinical diagnostics, its targeted use for plasma protein adulterants in processed foods is scarcely reported. The lack of a validated extraction method for isolating CBP proteins from surimi thus presents a critical barrier to accurate detection and quantification. Thus, this research seeks to bridge this critical void by establishing a mass spectrometry-based detection platform, coupled with the development of optimized extraction protocols for plasma proteins. Furthermore, electrochemical immunosensor development specifically targeting plasma-derived protein detection in processed food matrices remains limited. Existing electrochemical platforms have predominantly focused on pathogen or small-

molecule detection, with comparatively few studies addressing complex protein biomarkers such as blood plasma components under heat-processed conditions. Consequently, there is a lack of validated, sensitive, and portable electrochemical systems tailored for detecting plasma adulteration in surimi-based products, highlighting a clear research gap in this area.

In response to these gaps, the present study aims to identify CBP-specific protein biomarkers using MS and to develop an electrochemical immunosensor for their sensitive and selective detection. By integrating proteomic biomarker discovery with biosensor technology, this research seeks to establish a practical dual-platform approach for CBP adulteration detection, thereby advancing halal food authentication, improving food integrity monitoring, and strengthening consumer confidence in surimi-based products. The following specific objectives guide this research effort.

1.3 Objectives

This research focuses on developing a reliable method to detect CBP adulteration in surimi-based products. It aims to identify specific protein markers and to build a sensitive electrochemical immunosensor for practical food authentication applications. The specific objectives of this study are:

1. To identify candidate protein markers for the detection of CBP adulteration in surimi using mass spectrometry-based proteomic analysis.
2. To extract and enrich CBP-specific proteins using immunoprecipitation techniques.
3. To determine sequence similarities of candidate protein markers with other animal species using bioinformatics tool.
4. To develop an electrochemical immunosensor based on gold nanoparticles (AuNPs) and evaluate its performance in detecting protein markers in comparison with mass spectrometry results across different CBP spiking ratios and commercialised surimi products.

1.4 Research Questions

In addressing the challenges related to the detection of CBP adulteration in surimi-based products, this study is guided by the following research questions:

1. What specific protein biomarkers, can be identified using mass spectrometry to reliably indicate the presence of CBP adulteration in surimi?
2. How effective is extraction and immunoprecipitation technique in isolating CBP-specific proteins from complex surimi matrices?
3. How can bioinformatics tools be applied to analyze the protein sequences to assess their evolutionary conservation and species-specific variations for their suitability as reliable biomarkers in poultry authentication?
4. How does the performance of the developed electrochemical immunosensor compare to mass spectrometry-based detection methods across different CBP spiking levels in surimi?

1.5 Significance of Study

This study contributes to advancing food authentication science, with a particular focus on detecting CBP adulteration in surimi-based products. The work offers both scientific and practical significance, impacting food safety, halal verification, and consumer protection. This study focusing the application of proteomics in food science by identifying plasma-derived protein biomarkers, namely Apo AI and fibrinogen, for the detection of CBP adulteration. Using mass spectrometry-based proteomic analysis, the study advances knowledge on molecular detection methods within complex, heat-processed food matrices—an area currently underexplored. Additionally, the development of a sensitive electrochemical immunosensor, enhanced with gold nanoparticles (AuNPs), provides a portable and rapid detection tool that bridges the gap between laboratory-based analysis and real-world food testing.

From an industrial and regulatory perspective, the findings offer tangible solutions for food fraud prevention and halal assurance. The electrochemical biosensor developed in this study enables rapid detection of CBP adulteration, facilitating timely and efficient monitoring by food industries and regulatory bodies. By equipping

authorities with faster, sensitive tools, the research supports more proactive surveillance of food safety and encourages immediate preventive actions against adulteration practices, thereby strengthening public health safeguards. Moreover, the study supports Malaysia's strategic objective to enhance its role as a global halal food hub, contributing directly to the goals outlined in the Malaysia Halal Industry Master Plan 2030. It also aligns with the United Nations Sustainable Development Goals (SDGs), particularly Goal 12: Responsible Consumption and Production and Goal 3: Good Health and Well-Being, by promoting ethical production and protecting consumer rights. In summary, this research advances scientific understanding of plasma-based biomarker detection and delivers practical innovations that benefit regulatory enforcement, industrial practices, and consumer trust in processed food authenticity.

1.6 Scope and Limitation of Study

This study focuses on the identification of protein biomarkers in CBP for the detection of adulteration in surimi-based products. Specifically, it targets key plasma proteins, such as Apo AI and fibrinogen, within surimi matrices spiked with CBP. The primary scope includes proteomic biomarker identification using mass spectrometry and the development of an electrochemical immunosensor for sensitive and rapid detection.

The study is limited to CBP as the experimental model; other species are not directly tested. However, bioinformatics analyses compare protein sequences across terrestrial vertebrates and fish to evaluate Apo AI and fibrinogen as universal markers with no cross-reactivity to fish species.

The study is confined to CBP as the sole experimental model, with no direct testing of other species. Nevertheless, complementary bioinformatics analyses extend beyond the experimental scope, comparing protein sequences across a range of terrestrial vertebrates and fish to investigate the universality of Apo AI and fibrinogen as reliable biomarkers, while confirming their specificity without cross-reactivity to fish species. This research maintains a clear focus on surimi-based products spiked with CBP, deliberately excluding other processed meat products and broader species authentication efforts beyond blood plasma protein detection.

Sample collection involves the slaughtering of live chickens to obtain non-coagulated blood plasma under controlled conditions. Experimental work depends on

the availability of fresh, non-coagulated blood immediately post-slaughter to ensure sample quality and consistency. Additionally, mass spectrometry analyses rely on specialized instrumentation located at collaborating external institutions, which may impact project timelines and scheduling.

In summary, this study is confined to the identification and detection of CBP biomarkers in surimi and related processed products and does not address broader meat adulteration or proteomic profiling beyond blood plasma proteins.

1.7 Novelty and Contribution to New Knowledge

This study introduces several novel contributions to the field of food authentication, proteomics, and halal compliance, specifically in the context of detecting CBP adulteration in surimi-based products. Firstly, this study pioneers the identification of Apo AI and fibrinogen as specific plasma-derived protein biomarkers for the detection of CBP adulteration in processed food matrices. While proteomic markers have been extensively studied in clinical and veterinary sciences, their application for food authenticity verification, especially in complex and heat-processed products like surimi, remains largely unexplored. The proteomic profiling and mass spectrometry approach utilized in this study thus represent a significant advancement in the field of food science.

Secondly, a specific immunoprecipitation-based protein extraction method was developed and applied to isolate and enrich CBP-specific proteins from surimi matrices. The optimization of sample preparation using immunoprecipitation techniques offers a methodological innovation that enhances the reliability of downstream mass spectrometric detection.

Thirdly, the development of an electrochemical immunosensor enhanced with gold nanoparticles (AuNPs) for the detection of Apo AI in surimi products marks a novel technological contribution. To the best of current knowledge, this is among the first reports of a portable AuNP-based electrochemical sensor specifically designed for halal authentication related to blood plasma adulteration in processed seafood products. The sensor provides a sensitive, rapid, and low-cost alternative to laboratory-based techniques, addressing a key industry need for field-deployable detection platforms.

Fourthly, the study introduces a comparative evaluation between the developed electrochemical immunosensor and conventional mass spectrometry detection methods

across varying CBP concentrations. This direct performance comparison highlights the potential of portable biosensors to complement or partially replace complex laboratory techniques for routine food authentication and regulatory enforcement. In summary, this research contributes new knowledge by:

i) Establishing Apo AI and fibrinogen as plasma-specific biomarkers for food adulteration detection.
ii) Optimising sample preparation techniques for the isolation of target proteins from complex food matrices.
iii) Innovating portable biosensor technology for halal and food fraud authentication.
iv) Providing a dual-platform evaluation (MS and immunosensor) to validate practical applications in food safety monitoring.

1.8 Thesis Organization

This thesis is structured into five chapters, each addressing key components of the research from background foundations to conclusions and future directions. Chapter One presents the introduction to the study, detailing the research background, motivation, problem statement, objectives, research questions, significance, scope and limitations, and the novelty and contributions to new knowledge. It sets the context and justification for the research undertaken.

Chapter Two provides a comprehensive review of the relevant literature. It discusses the composition and functional properties of blood plasma proteins, their incorporation and roles in surimi products, and the challenges of detecting blood plasma adulteration. The chapter also reviews current detection methods, with a particular focus on biosensors, including their types, working principles, and the integration of nanoparticles. Special emphasis is given to the advantages of AuNPs over other nanoparticles in sensor development.

Chapter Three describes the research methodology employed throughout the study. It outlines the sample collection procedures, protein extraction and quantification techniques, proteome digestion, and protein identification using mass spectrometry. The chapter also details the fabrication and characterization processes for the electrochemical immunosensor.

Chapter Four presents the results and discussion of the study. The findings from proteomic analysis, extraction efficiency, immunoprecipitation enrichment, and electrochemical sensor performance are systematically reported and analyzed. Comparative performance assessments between mass spectrometry and the developed immunosensor are also discussed.

Chapter Five concludes the thesis by summarizing the main findings, highlighting the contributions to new knowledge, and offering recommendations for future research directions to further advance food authentication and halal compliance technologies.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Food authentication has become an increasingly important area of research in recent years, driven by rising concerns over food fraud, public health, and religious dietary compliance. Incorporating blood plasma into processed food products such as surimi, has introduced new challenges for verifying food authenticity. This is crucial in the context of halal dietary laws where blood and its derivatives are strictly prohibited. In particular, detecting the presence of blood plasma as adulterants in processed products requires sensitive and reliable analytical methods capable of overcoming the limitations posed by heat treatment and complex food matrices.

Surimi, a processed seafood product valued for its textural and nutritional properties, is highly susceptible to adulteration due to its extensive use of functional additives to enhance gelation and water holding capacity. Blood plasma, rich in proteins such as Apo AI and fibrinogen, has emerged as a potential additive that can mimic these functional attributes. However, its unauthorized addition raises serious ethical, religious, and regulatory concerns, thus necessitating the development of effective detection strategies.

Traditional analytical methods, including Enzyme-Linked Immunosorbent Assays (ELISA) and Deoxyribonucleic acid (DNA) based assays, have been widely employed for meat species authentication. Nevertheless, these approaches face significant limitations when applied to heat-processed or chemically treated foods, where DNA degradation and protein denaturation can impair detection. In this regard, Mass Spectrometry (MS)-based proteomic techniques offer a promising alternative for identifying specific protein biomarkers associated with adulterants, providing high sensitivity and specificity even in complex matrices.

In parallel, advancements in biosensor technologies have enabled the development of portable, rapid, and cost-effective platforms for food authentication. Electrochemical biosensors, particularly those enhanced with nanoparticles such as Gold Nanoparticles (AuNPs), have demonstrated superior analytical performance due

to their enhanced signal amplification, stability, and biocompatibility. AuNPs, compared to other nanomaterials, offer unique advantages in terms of conductivity, surface functionalization, and sensitivity, making them ideal candidates for biosensor applications in food safety monitoring.

Despite these technological advancements, gaps remain in the literature regarding the reliable detection of blood plasma adulteration in surimi and similar processed foods. Current studies have yet to fully integrate proteomic biomarker discovery with biosensor platform development tailored for halal food authentication. Therefore, this research seeks to bridge this critical gap by identifying CBP-specific protein markers through MS and developing an AuNP-based electrochemical immunosensor for rapid and sensitive detection.

This chapter comprehensively reviews the relevant literature, covering from biochemical properties of blood plasma to current detection techniques involved. This includes the issues surrounding food adulteration, current detection methodologies, the role of biosensors, and the integration of nanotechnology in food diagnostics. It also highlights the existing gaps in knowledge that the present study aims to address.

2.2 Overview of Blood Composition and Physiological Importance

Blood is a vital component of animal physiology, constitutes a significant proportion of total body weight, approximately 7% to 8% (Ofori and Hsieh, 2014). It is a complex fluid connective tissue composed mainly of blood cells and plasma, with plasma accounting for approximately 55% to 60% of the total blood volume (Sorapukdee & Narunatsopanon, 2017). Each plays a crucial role in maintaining physiological homeostasis. One of the important components, the plasma is a protein-rich fluid that serves as the medium for transporting nutrients, hormones, and waste products throughout the body (Mukherjee et al., 2023). Erythrocytes or the red blood cells, leukocytes (white blood cells), and platelets comprise the remaining 45% of the biological components. Most of the cells in blood are erythrocytes, whose function is mostly in charge of carrying carbon dioxide and oxygen given that they contain the iron-containing protein hemoglobin. (Mukherjee et al., 2023) (Ciannella et al., 2023). By facilitating phagocytosis and eliminating immunological complexes, these cells also contribute to immune regulation (Zhang et al., 2022). According to Mukherjee et al. (2023), leukocytes are essential components of the immune system that protect the body

from illnesses and foreign invaders. The tiniest blood cells, platelets, aid in blood clotting preventing excessive bleeding, making them essential for hemostasis (Mukherjee et al., 2023). Blood's physiological importance extends to its role in maintaining iron homeostasis, with iron being crucial for oxygen transport and immune function (Ciannella et al., 2023). The heart and blood vessels make up the circulatory system, which makes sure that blood is distributed efficiently to meet tissues' and organs' metabolic demands (Khuman et al., 2024) (Hill et al., 2022). In order to maintain the internal environment and respond to pathophysiological situations, blood serves as a barrier that controls the exchange of metabolites and medications between the bloodstream and tissues (Yk, 2024). Despite being an essential medical procedure, blood transfusion therapy needs to be carefully monitored to prevent negative effects, which emphasizes the necessity of evidence-based standards in its use (Tamai, 2024). Overall, blood's composition and functions are integral to sustaining life, facilitating nutrient transport, waste removal, immune defense, and homeostatic balance.

2.3 Preparation of Analytical Considerations of Plasma and Serum

2.3.1 Differences between Plasma and Serum

Building upon the general understanding of blood composition nd its physiological role, it is essential to distinguish between its two principal iquid derivatives, plasma and serum. Both differ in preparation and composition which affects their use in clinical and research environments. Blood treated with an anticoagulant, which prevents clotting and enables the separation of the liquid component from blood cells, is centrifuged producing plasma. On the other hand, serum is the liquid that remains after the clotting factors have been eliminated, and it is made from blood that has been allowed to clot. The process can be visualised by a schematic representation using centrifugation in Figure 2.1. Their biochemical profiles differ as a result of this basic preparation. For example, the clotting process causes serum to include more platelet-derived particles and proteins than plasma, which can change the content profiles (Hagn et al., 2024) (Zhang et al., 2022). According to Hagn et al. (2024) and Espinosa et al. (2023), plasma is frequently chosen in metabolomics and proteomics study in order to prevent interference from platelet activation and clotting-related alterations in serum. Moreover, the levels of specific metabolites and proteins may vary

between the two, with serum typically exhibiting elevated quantities owing to the 1release of cellular constituents during coagulation (Bovo et al., 2023). According to Mouna et al. (2023), these distinctions also apply to the determination of viral loads with where plasma is usually utilized for more consistent results as compared to serum which also can be used but in emergency cases. The selection of plasma versus serum might influence the reference intervals for clinical tests, evidenced by notable disparities in total protein, albumin, and globulin concentrations between the two matrices (Militello et al., 2024). In conclusion, although plasma and serum are both essential for diverse diagnostic and research applications, their unique preparation techniques and resultant compositional variations require meticulous evaluation of their applicability based on specific analytical requirements and the possibility of contributing factors.

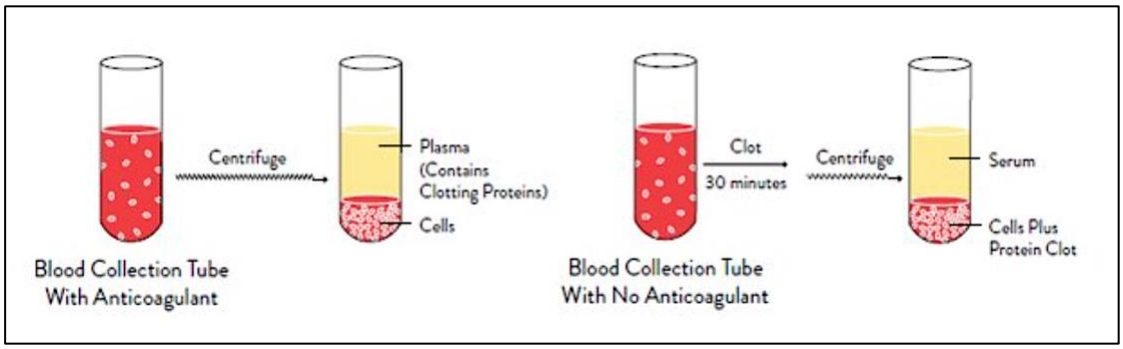


Figure 2.1 Differences between Plasma and Serum

Source: Reed & Nicholas (2025)

2.3.2 Plasma Retrieval Methods

The selection of appropriate retrieval methods that preserve the integrity of plasma for subsequent analytical applications is contingent upon an understanding of its biochemical composition and functional attributes. In both clinical and scientific settings, plasma retrieval from blood is an essential procedure that can be accomplished in a number of ways, each with unique benefits and drawbacks. Traditional methods such as plasmapheresis utilize extracorporeal techniques to extract whole blood, separating plasma from cellular components through centrifugation or filtration. This procedure is commonly employed in therapeutic contexts to eliminate pathological substances from plasma, usually conducted in clinical settings with specialized

equipment and trained professionals (García, 2024; Sala et al., 2023). Centrifugal separation is a prevalent technique in which blood is subjected to high-speed spinning to segregate its components according to density variations. This method can be improved by employing a spinning membrane separator to guarantee the elimination of cellular components from plasma (Kusters & Min, 2020). Microfluidic and passive filtration techniques provide portable and rapid solutions that are appropriate for point-of-care diagnostics. These techniques separate plasma from blood without the use of complicated equipment by using capillary forces, inertial microfluidics, or immunocapture. Microfluidic devices can attain high plasma separation efficiency through the integration of spiral inertial separation elements within syringes, facilitating plasma isolation during blood draws (Han & DeVoe, 2021; Gao et al., 2020). Passive filtration devices utilize plasma separation membranes for efficient plasma extraction, proving beneficial in resource-limited settings (Baillargeon et al., 2020; Su et al., 2020). These methods are user-friendly and cost-effective, making them suitable for rapid diagnostics and applications where traditional centrifugation is not feasible. In general, the amount of plasma required, the resources available, and the environment in which the separation is carried out all influence the choice of plasma retrieval technique.

2.3.3 Compositions and Nutritional Value of Blood Plasma

Blood plasma, the liquid component of blood, constitutes approximately 55–60% of total blood volume and plays a critical role in maintaining physiological homeostasis (Sorapukdee & Narunatsopanon, 2017). It is a complex aqueous solution comprising mainly water (~90–92%) and proteins (~6–8%), together with electrolytes, nutrients, hormones, enzymes, and metabolic waste products (Ofori & Hsieh, 2014). Plasma proteins predominantly albumins, globulins (α-, β-, and γ-globulins), and fibrinogen account for about 7.9% of plasma content (Chen et al., 2015; Sorapukdee & Narunatsopanon, 2017). Albumins, which make up roughly 56% of total plasma proteins, are particularly important for maintaining colloidal osmotic pressure, regulating blood volume, and transporting fatty acids, hormones, bilirubin, and various drugs (Fontes et al., 2015). Their structural flexibility and amphiphilic nature also enhance their functionality in food systems, especially in emulsification and gelation. Collectively, these proteins underpin plasma's diverse physiological and functional roles, making it a valuable resource for both biological systems and food applications.

Globulins represent around 40% of plasma proteins and are categorized into α-, β-, and γ-globulins (Chen et al., 2015). α- and β-globulins are mainly involved in transporting lipids, metal ions, and fat-soluble vitamins, while γ-globulins function as immunoglobulins critical for immune defense mechanisms (Fontes et al., 2015). In addition, their large, stable structures make globulins relevant to food textural enhancement when plasma is used as an additive.

Another commonly found proteins in blood plasma is fibrinogen, although present in smaller quantities (~0.6% of plasma proteins), plays an indispensable role in coagulation. During haemostasis, fibrinogen is converted enzymatically to fibrin, which forms a fibrous mesh essential for blood clot formation (Jayathilakan et al., 2012). In food applications, fibrinogen's gel-forming capability under thermal or enzymatic stimulation significantly forms strong, cohesive textures in processed meat and seafood products. In addition to proteins, plasma contains electrolytes such as sodium, potassium, calcium, and chloride, which are essential for maintaining osmotic balance, acid-base equilibrium, nerve impulse transmission, and muscle function (Ofori and Hsieh, 2014). Plasma also serves as a medium for transporting such as glucose, amino acids, and lipids to tissues and carrying carbon dioxide and urea for excretion.

Nutritionally, blood plasma is a rich source of high-quality, bioavailable proteins and essential micronutrients. It offers substantial quantities of heme iron, a highly absorbable form of iron critical for oxygen transport and energy metabolism (Chen et al., 2015; Jayathilakan et al., 2012). Other crucial minerals in plasma include zinc and copper, which are vital for enzymatic activities and immune system function. The amino acid profile of plasma proteins is well-balanced, containing all essential amino acids required for human nutrition, comparable to the profile found in egg proteins (Jayathilakan et al., 2012). Beyond its nutritional richness, blood plasma is vital in advancing sustainable food production. By utilizing plasma as a food ingredient, the food industry can valorize slaughterhouse by-products that would otherwise be wasted, contributing to environmental conservation and promoting circular economy practices (Ofori and Hsieh, 2014). Hartoyo et al. (2022) demonstrated that CBP, owing to its high soluble protein yield, can be positioned as a high-value nutritional ingredient for incorporation into animal feed and food by-products, thereby enhancing their protein content and functional quality. Estimates suggest that approximately 30% of the blood collected in slaughterhouses is repurposed for food applications, primarily for its functional protein components (Ofori and Hsieh, 2014).

Despite its numerous advantages, direct use of whole blood in food products remains limited due to its intense dark color, metallic flavor, and potential negative impact on consumer acceptance (Shen et al., 2019). In contrast, blood plasma, being colorless and having a neutral taste, circumvents these sensory barriers, offering a more adaptable ingredient for incorporation into processed foods (Jayathilakan et al., 2012). Consequently, blood plasma provides a nutritional advantage and enhances the aesthetic and sensory qualities of food products, expanding its applicability across diverse food sectors.

2.3.4 Functional Properties of Plasma in Food Systems

Blood plasma exhibits a diverse range of functional properties with significant implications for food processing and formulation. Owing to its unique physicochemical characteristics, plasma is used widely in the meat, seafood, and processed food industries for its emulsifying, gelation, water-holding, stabilization, foaming, and protease inhibitory abilities (Fontes et al., 2015; Jayathilakan et al., 2012). These properties contribute to improved product quality, stability, and sensory appeal. Hartoyo et al. (2022) further highlighted that valorization of CBP into functional protein products offers a circular economy solution that mitigates slaughterhouse waste while generating added value through sustainable bioconversion.

Emulsification is one of the most notable functions, primarily due to plasma proteins such as albumin, which possess amphiphilic structures enabling adsorption at oil–water interfaces to reduce interfacial tension and stabilize emulsions (Fontes et al., 2015). This is particularly important in products like sausages, fish paste, and surimi, where stable emulsions ensure uniform texture and cooking stability. CBP has shown superior emulsification compared to porcine and duck plasma, attributed to its higher albumin content and solubility (Shen et al., 2019).

Gelation ability is another critical property. Upon heating, plasma proteins denature and form a three-dimensional network that traps water and fat, improving texture, firmness, and sliceability (Jayathilakan et al., 2012). Fibrinogen plays a key role in network formation, and studies have demonstrated that incorporating plasma significantly increases gel strength in surimi products by both promoting protein cross-linking and inhibiting proteolytic degradation (Rawdkuen et al., 2004; Sorapukdee & Narunatsopanon, 2017). For example, CBP enhanced the breaking force and

deformation of bigeye snapper surimi gels (Rawdkuen et al., 2004) and showed better gelation than duck or porcine plasma at 70–80 °C (Shen et al., 2019). Other plasma sources, such as salmon (Fowler et al., 2015) and bovine (Seymour et al., 1997; Kang & Lanier, 1999), also improved gel strength by inhibiting proteolysis and facilitating myosin cross-linking.

Water-holding capacity (WHC) is vital for yield, juiciness, and textural stability. Plasma proteins bind and retain water within the gel network, reducing moisture loss during processing (Ofori & Hsieh, 2014; Fontes et al., 2015). In surimi, CBP and other plasma sources have been shown to enhance WHC by stabilizing the protein matrix, aided by components such as α2-macroglobulin and transglutaminase (Seymour et al., 1997). Optimization studies indicate that as little as 0.79% CBP combined with 4.68% sorbitol can significantly improve solubility, cohesiveness, and whiteness of surimi gels while minimizing sensory changes (Ain et al., 2023).

Protease inhibition is another valuable property, particularly for tropical fish surimi, which often suffers from high endogenous protease activity leading to poor gelation (Yongsawatdigul et al., 2000). Protease inhibitors in plasma, including α2-macroglobulin, prevent degradation of myofibrillar proteins, thereby preserving gel structure and improving stability during storage (Rawdkuen et al., 2004; Shen et al., 2019). Beyond surimi, plasma proteins also contribute to stabilization of complex food systems during mechanical, thermal, and storage stresses, ensuring desirable texture and appearance over shelf life (Fontes et al., 2015). While foaming is less relevant to meat and seafood, plasma's ability to form stable foams underscores its versatility across food sectors (Jayathilakan et al., 2012).

Overall, the incorporation of blood plasma, especially blood plasma into food systems leverages its multifunctional nature to address common textural and stability challenges. In surimi-based products, plasma proteins enhance gel strength, WHC, emulsification, and protease inhibition, offering technological and economic advantages for tropical and temperate species alike.

2.3.5 Studies on Plasma Applications and Halal Authentication Challenges

Recent research has highlighted growing interest in the use of blood plasma proteins as functional food ingredients, particularly in processed meat and seafood products. Numerous studies have explored their functional benefits alongside the

regulatory, ethical, and halal authentication challenges associated with plasma incorporation in food systems. Technological innovations have enhanced plasma utilization, with methods such as Response Surface Methodology (RSM) enabling precise optimization of functional performance. For example, Ain et al. (2023) demonstrated that incorporating 0.79% chicken plasma protein (CPP) with 4.68% sorbitol significantly improved protein solubility, cohesiveness, and whiteness of surimi-based products, achieving these benefits while minimizing ingredient usage. Proteomics-based techniques, particularly mass spectrometry (MS), have emerged as powerful tools for detecting plasma protein biomarkers in complex food matrices. MS enables the identification and quantification of trace plasma proteins even after food processing, providing a robust solution for detecting adulteration in highly processed products such as surimi and meatballs (Montowska & Pospiech, 2013). Advances in species-specific peptide analysis further allow differentiation between porcine, bovine, and avian plasma origins, which is critical for halal authentication (Ruzulan et al., 2021). Parallel to MS, monoclonal antibody-based enzyme immunoassays have shown high sensitivity and specificity in detecting plasma proteins, including porcine plasma in fish surimi products (Ofori & Hsieh, 2016; Raja Nhari et al., 2018). Such biosensor platforms offer advantages over MS in being faster, less equipment-intensive, and suitable for on-site halal inspection (Sahilah et al., 2016). Despite these technological advances, the incorporation of blood plasma into food presents significant halal concerns. Islamic dietary law deems blood and its derivatives najis (impure) and strictly prohibits their consumption (Anuar, 2015). The Malaysian Halal Standard MS1500:2019 explicitly states that any food containing blood or its derivatives, including plasma, is non-halal. Consequently, even trace amounts of undeclared plasma in processed foods can violate halal standards, erode consumer trust, and undermine regulatory transparency (Fadzillah et al., 2020). Emerging studies emphasize that while plasma offers technological benefits, its inclusion—especially if undeclared—could mislead consumers and compromise halal integrity (Ruzulan et al., 2021).

To address this, there is growing emphasis on sensitive halal authentication methods. In addition to proteomics and immunoassays, DNA-based detection methods such as Polymerase Chain Reaction (PCR) targeting species-specific mitochondrial genes have been explored. However, their effectiveness decreases in heat-processed foods due to DNA degradation (Lubis et al., 2016), making protein-based methods such as MS and immunoassays more reliable. Integrated halal monitoring systems—

combining proteomics, immunosensors, and certification protocols—are increasingly recommended to safeguard authenticity from production to retail (Anuar, 2015; Ofori & Hsieh, 2016). In summary, while blood plasma can deliver functional and economic advantages in food production, its use remains controversial within the halal food sector. Advanced detection methods such as MS, monoclonal antibody assays, and biosensors provide promising avenues for accurate plasma authentication. Ongoing research must continue to balance technological innovation with ethical, regulatory, and religious considerations to protect consumer rights and ensure transparency in global food markets.

2.3.6 Surimi Definition, Types, Process and Industry.

In 2022, approximately 164.6 million tonnes, representing 89 percent of the 185 million tonnes of aquatic animals produced globally, were destined for human consumption, equating to an average of 20.7 kg per person (FAO, 2024). Among these aquatic-based products, surimi occupies a significant niche as a highly versatile intermediate for value-added seafood analogues. The amount of frozen fish fillets, peeled shrimp, surimi, shucked shellfish, fish-based products such as fish balls, fish cakes, and several ready-to-eat seafood products produced has increased (Jaziri et al., 2021).

Surimi is produced primarily from white-fleshed fish, which undergoes mechanical deboning, repeated washing, and refining processes to remove lipids, pigments, and blood (Jaziri et al., 2021), thereby concentrating myofibrillar proteins essential for gel formation (Sinthusamran et al., 2023). It was then followed by the incorporation of cryoprotectants such as sugar and phosphate to preserve protein stability during frozen storage (Hou et al., 2019). This protein-rich paste serves as the base material for a wide range of processed seafood products, particularly in the Asia-Pacific markets. Originating in Japan, surimi has become a staple in the global seafood market due to its versatility and ability to mimic the texture of more expensive seafood products (Yang et al., 2025).

Examples of surimi products include imitation crab meat, fish balls, and fish cakes, which are popular in various cuisines worldwide. These products are primarily made from fish species such as threadfin bream, bigeye snapper, lizardfish, barracuda and goatfish (Figure 2.2), and some were utilised from tilapia (Hou et al., 2019) which

are processed to form a gel-like texture that is characteristic of surimi-based foods (Karbsri et al., 2024; Jaziri et al., 2021).

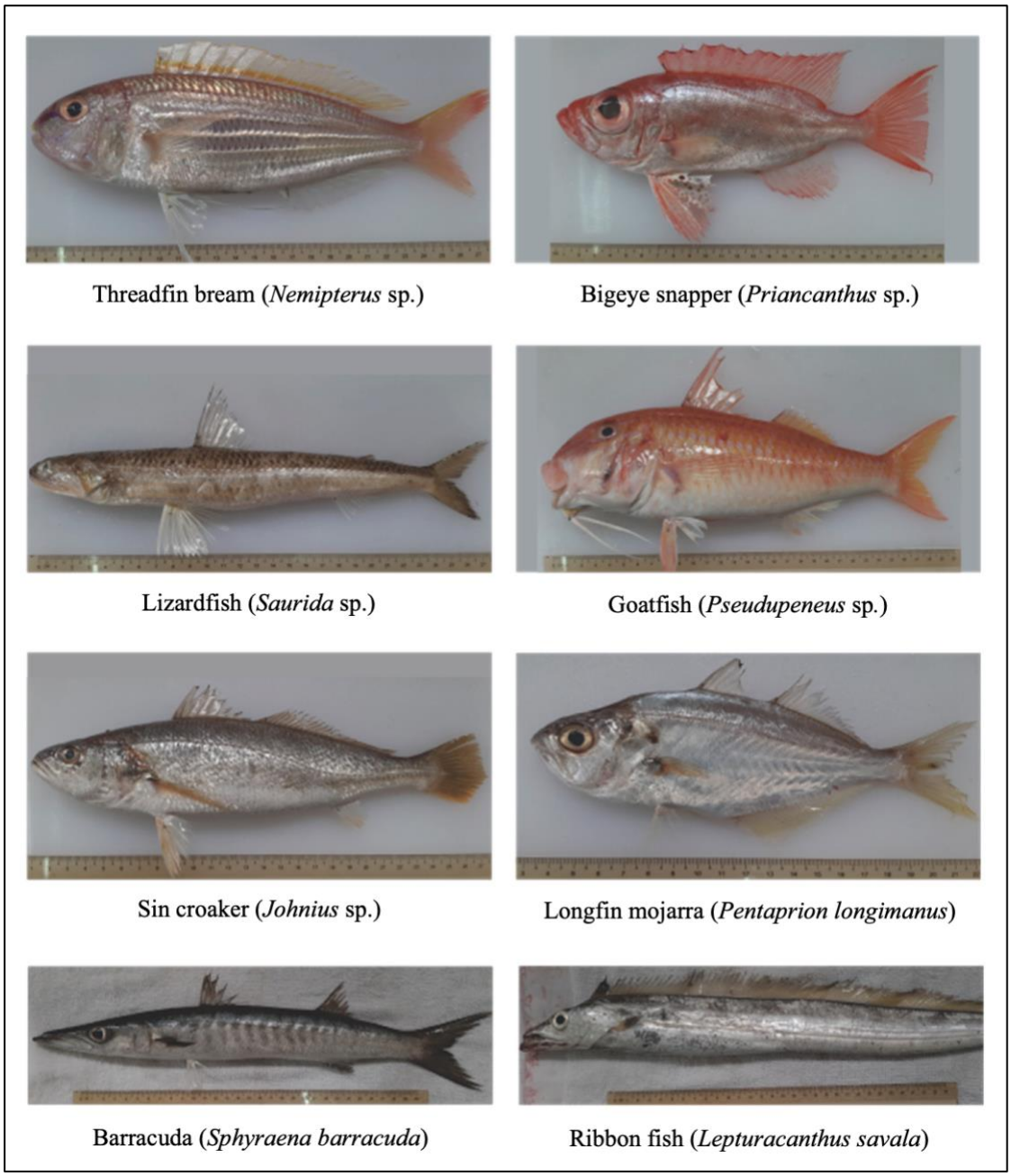


Figure 2.2 The Major Tropical Marine Fish Species used for Surimi Production

Source: Jaziri et al., 2021

The issues in producing surimi rely on the gelation process, which affects the quality of surimi products, and it can be improved by adding various additives such as proteins, lipids, and polysaccharides. A study by Lv et al., (2024) showed the improvement of texture and flavour of surimi gels by the addition of Antarctic krill oil

emulsions. Additionally, plant-based ingredients like fava bean protein isolate showed improvements of gelation quality of low grade surimi by enhancing breaking force, gel strength, and water holding capacity, while maintaining myofibrillar protein integrity and texture, at concentrations of 3-4% (w/w) ; offering a sustainable alternative to traditional fish-based surimi products (Kamani et al., 2024). The use of natural additives, such as tamarind seed polysaccharide, also provides cryoprotective effects, maintaining the quality of surimi during frozen storage by preventing texture deterioration and water loss (Xie et al., 2024; Hou et al., 2019). Furthermore, the processing methods, such as steaming, microwave heating, and air-frying, significantly affect the final quality of surimi products, influencing their texture, lipid stability, and nutrient retention (Lv et al., 2024). These innovations and processing techniques highlight the adaptability and potential for quality improvement in surimi products, catering to diverse consumer preferences and dietary needs.

The surimi industry has expanded beyond traditional fish sources, exploring alternative protein sources such as edible insects, which are eco-friendly and nutrient-rich, to create surimi-based products (Moon et al., 2024). The surimi industry continues to innovate by exploring sustainable sources and improving gel properties, ensuring its continued relevance in the global food market (Huang et al., 2024). The production process of surimi involves several key steps, beginning with the washing of minced fish to remove water-soluble proteins, blood, and fat, which enhances the gelation properties of the remaining myofibrillar proteins (Sinthusamran et al., 2023). This is followed by refining and dewatering to concentrate the proteins, after which cryoprotectants are added to prevent protein denaturation during freezing (Xie et al., 2024). The gelation process, crucial for surimi's texture, involves heat-induced protein denaturation and aggregation, forming a three-dimensional network that determines the gel's strength and elasticity (Zhao et al., 2024; Xie et al., 2024). Various additives, such as polysaccharides and proteins, are incorporated to enhance gel properties. For instance, konjac glucomannan and corn starch have been shown to improve gel strength and water retention by modifying the protein network structure (Shi et al., 2024; Jiang et al., 2024). High-pressure processing and the addition of natural additives like whey protein hydrolysate can further enhance the gel's quality by reducing protein denaturation during freeze-thaw cycles (Peng et al., 2024; Hongviangjan & Sompongse, 2024). Additionally, innovative approaches like double network strategies, which involve covalent and non-covalent interactions, have been developed to tailor the texture

properties of surimi gels, offering improved elasticity and water-holding capacity (Wang et al., 2024). These advancements in surimi production not only optimize the textural and nutritional qualities of the final product but also address challenges related to processing and storage stability, making surimi a highly adaptable ingredient in the food industry (Yang et al., 2025; Yan et al., 2024).

2.4 Applications of Marker Derived from Blood

Blood-derived markers have seen limited use in the food sector, primarily limited to proteins such as myoglobin (Ragucci et al., 2024), Apolipoprotein AI (Husna et al., 2023), and Immunoglobulin class G (IgG) (Hendrickson et al., 2022). Their application has primarily been focused on medical and clinical contexts, particularly concerning Apo AI and fibrinogen, as detailed in the subsequent section.

2.4.1 Apolipoprotein AI (Apo AI) as Clinical Biomarker

The exploration of Apolipoprotein AI (Apo AI) demonstrates a significant intersection of biochemical research, clinical diagnostics, and the increasing integration of artificial intelligence (AI) in medical practice. Apo AI serves as a pivotal biomarker primarily due to its role in lipid metabolism and cardiovascular health, while also showing potential relevance in neurodegenerative and inflammatory disorders (Castelletto et al., 2015; Kornmueller et al., 2019; Chen et al., 2021). Apo AI contributes to the formation and function of high-density lipoproteins (HDL), which are crucial for cholesterol transport and homeostasis. An increased ratio of Apolipoprotein B (Apo B) to Apo AI has been linked with a higher risk of coronary artery disease (CAD), supporting its potential as a diagnostic biomarker (Chen et al., 2020; Chen et al., 2021). Notably, the Apo B/Apo AI ratio has been identified as a robust indicator for predicting coronary disease and ischemic events, thereby establishing a direct correlation with cardiovascular risk assessments (Chen et al., 2021). Furthermore, elevated levels of Apo AI and related proteins, such as Haptoglobin, can serve as indicators of acute myocardial infarction, highlighting its relevance in clinical practice (Bakrim et al., 2018; Patel et al., 2010; N et al., 2020). In those myocardial infarction patients, Apo AI expressions differ significantly from healthy subjects, and its recurrent elevation

following myocardial infarction may reflect a physiological response to ischemia (Patel et al., 2010).

Beyond cardiovascular health, Apo AI also plays a role in inflammatory responses, suggesting potential diagnostic utility in other disease contexts (Bakrim et al., 2018; Zeng et al., 2024). In diabetes-related studies, lower Apo AI levels have been associated with increased risks of microvascular complications, further emphasizing its diagnostic value in metabolic disorders (Sasongko et al., 2012; Jenkins et al., 2013).

The implications of Apo AI extend into neurodegenerative diseases as well. Evidence indicates that Apo E, a closely related apolipoprotein, significantly influences the trajectory of Alzheimer's disease by modulating amyloid-beta aggregation and neuronal health (Risacher et al., 2015; Castellano et al., 2011). Apo AI itself may also play a role in amyloid beta clearance, with AI-enabled genetic and biochemical analysis offering opportunities for earlier detection and improved intervention strategies in Alzheimer's disease (Danter, 2024). Novel applications of Apo AI have also emerged in the form of artificial HDL nanoparticles, which can deliver therapeutics effectively while simultaneously acting as diagnostic markers (Kornmueller et al., 2019). These engineered particles not only encapsulate drugs and imaging agents but also enhance diagnosis through improved imaging techniques, suggesting innovative possibilities when combined with AI analytics (Kornmueller et al., 2019).

The integration of AI into biomarker analysis provides further opportunities to improve diagnostic precision and patient management. AI and machine learning models can process vast datasets, correlating Apo AI levels with patient outcomes to increase predictive accuracy (Kim et al., 2021; Yin et al., 2021). By leveraging these tools, healthcare providers can personalize treatment strategies based on nuanced insights from Apo AI data, supporting a paradigm shift toward personalized medicine (Filho et al., 2024; Rudnik et al., 2023). Despite these advances, challenges remain in standardizing biomarker use in clinical practice. Fluctuations in Apo AI levels under different physiological conditions highlight the need for robust assay methods capable of distinguishing normal from pathological states (Wang et al., 2021). Continued research is therefore necessary to strengthen methodological rigor while integrating AI capabilities for more comprehensive diagnostic approaches.

In summary, Apo AI is a comprehensive biomarker that revolve exclusively in cardiovascular, metabolic, inflammatory, and neurodegenerative diseases. Its established role in lipid metabolism and cardiovascular health, combined with emerging

evidence in other pathological contexts, underscores its diagnostic potential. The convergence of Apo AI research with artificial intelligence not only enhances disease prediction and management but also opens new frontiers for personalized medicine and therapeutic innovation.

2.4.2 Fibrinogen as Diagnostic and Inflammatory Marker

Alongside Apo AI, fibrinogen has also attracted considerable attention as a clinically relevant biomarker, particularly due to its central role in coagulation and inflammatory processes, thereby extending the discussion toward its potential diagnostic and food authentication applications. Fibrinogen is an essential plasma protein that plays a crucial role in the coagulation cascade. It serves as a significant biomarker for inflammation and various diseases, making it essential for both diagnostic and prognostic uses. Elevated fibrinogen levels are implicated in several clinical conditions, including cardiovascular diseases, chronic obstructive pulmonary disease (COPD), and inflammatory conditions, confirming its value as a marker in clinical diagnostics.

Fibrinogen levels are particularly important in assessing the severity of diabetic foot ulcers (DFUs). A study demonstrated that elevated fibrinogen levels correlate with increased severity in DFUs, showing better sensitivity and specificity than C-reactive protein (CRP) for this condition (Li et al., 2016). The findings indicate that high fibrinogen levels in patients with grade 3 DFU, even when CRP is not elevated, suggest that fibrinogen can act as an independent marker for disease severity, supporting its potential for routine use in clinical evaluations (Li et al., 2016). Further research also indicates a relationship between plasma fibrinogen and the severity of peripheral arterial disease (PAD) in diabetic patients, reinforcing the idea that fibrinogen serves both as an inflammatory marker and a link to vascular dysfunction, key components in ulcer pathogenesis (Khan et al., 2025). In the context of chronic inflammatory conditions such as COPD, fibrinogen is recognized as a reliable biomarker for predicting exacerbations. Elevated plasma fibrinogen levels have been established as critical indicators of exacerbation risk, qualifying it as an FDA-approved biomarker in this area (Mohan et al., 2021; Singh et al., 2021). Research shows that fibrinogen levels significantly correlate with respiratory function, demonstrating repeatability over time, and affirming their reliability in diagnosing and monitoring health outcomes related to

COPD (Dickens et al., 2011; Zemans et al., 2017). Additionally, the predictive value of fibrinogen for mortality in COPD patients underscores its role as a diagnostic marker related to disease progression (Manon-Jensen et al., 2019).

Inflammatory bowel disease (IBD) also highlights fibrinogen's significance as a marker. Its levels correlate with disease activity in IBD patients, indicating that fibrinogen can help identify active disease stages (Chen et al., 2021). This reinforces fibrinogen’s function as a systemic inflammatory marker that can facilitate timely interventions in IBD management. In orthopedic infections, particularly periprosthetic joint infections (PJI), fibrinogen exhibits diagnostic value that surpasses traditional markers like D-dimer. Studies have shown that fibrinogen provides superior diagnostic performance in identifying PJI compared to other inflammatory markers (Xu et al., 2021; Xu et al., 2021). This reflects the expanding utility of fibrinogen in surgical and postoperative contexts, emphasizing its role in monitoring and diagnosing inflammatory responses.

Fibrinogen's pro-inflammatory properties span various disease states. It acts as a marker of acute inflammation and is implicated in chronic inflammatory pathways, such as atherosclerosis. An increase in plasma fibrinogen concentration can result from ongoing inflammatory processes as well as contribute to adverse cardiovascular events (Surma & Banach, 2021). Research indicates that fibrinogen levels are closely associated with atherosclerotic cardiovascular disease (ASCVD), serving both as a risk factor and a biomarker, thus highlighting its dual role in the pathology of cardiovascular conditions (Surma & Banach, 2021).

In summary, fibrinogen is a prominent marker of inflammation across diverse clinical settings. Its utility spans predicting severity in diabetic foot ulcers, assessing exacerbation risks in COPD, and providing diagnostic potential in inflammatory conditions like IBD and orthopedic infections. The comprehensive role of fibrinogen as both a marker and mediator in inflammatory processes underscores its significance in clinical diagnostics, highlighting the need for further exploration into its multifaceted applications in healthcare.

2.5 Food Adulteration Issues in Processed Seafood Products

2.5.1 Overview of Food Adulteration Issues in Processed Seafood Products

Food adulteration in processed seafood products, particularly surimi, has raised significant concerns regarding food safety, consumer trust, and regulatory compliance. Surimi-based products, such as imitation crab sticks, fish cakes, and other seafood alternatives, are widely consumed across the globe. As the demand for surimi grows due to its affordability and versatility (Raja Nhari et al., 2018), it becomes increasingly susceptible to adulteration practices. One of the major adulterants in surimi production is blood plasma (from chicken, cow, or pig), which is added to improve texture, enhance gelation, and provide a more cost-effective solution to food manufacturers (Benjakul & Visessanguan, 2000). However, blood plasma is considered haram (forbidden) in Islam, as consuming any form of blood or blood derivatives violates halal dietary laws. Therefore, the inclusion of CBP in surimi-based products poses a unique challenge to the food industry, especially in regions with a significant Muslim population, such as Southeast Asia and the Middle East (Fischer, 2016). Furthermore, the consumption of products containing CBP compromises Halal integrity and poses ethical and religious dilemmas (Rosele et al., 2025). These adulteration practices highlight the need for robust detection methods to ensure the authenticity and safety of seafood products, thereby protecting consumers and maintaining trust in food markets.

2.5.2 Detection Methods for Food Adulteration

Traditional methods for detecting food adulteration have included techniques such as DNA-based assays and protein-based methods like ELISA (Fischer, 2016). ELISA is a widely used immunoassay that can detect the presence of specific proteins in food samples. For example, it has been employed to identify porcine blood plasma in sausages and other processed meat products (Ahmad et al., 2019). However, ELISA has limitations when it comes to detecting blood plasma in surimi due to the complex processing that surimi undergoes. This can lead to protein denaturation and loss of structural integrity (Barik et al., 2013). Similarly, Hardianto et al., 2023 studied that DNA-based assays, such as PCR are frequently used to detect species-specific for example porcine ingredients in food. However, these methods struggle with DNA

degradation during the processing and heating of surimi, reducing their effectiveness for post-processed food products (Li et al., 2019; Yap & Gam, 2019).

An emerging detection method involves the use of MS. This powerful analytical technique allows for the precise identification of proteins and other biomolecules in complex food matrices. The integration of MS into food safety practices offers an avenue for detecting even trace amounts of CBP, providing a more accurate and reliable method for ensuring food authenticity (Zhang, 2024).

2.5.3 Limitations of Current Detection Methods

While traditional detection methods, such as ELISA and PCR, offer some degree of specificity, they face significant challenges in the context of processed seafood products like surimi. One of the significant limitations is protein denaturation, which occurs during surimi processing, particularly during freezing, thawing, and heating (Hardianto et al., 2023; Zhao et al., 2024). Denaturation causes proteins to lose their native structure, making them less detectable by conventional protein-based methods (Sahilah et al., 2016).

Another limitation is the degradation of DNA during food processing, particularly in the case of surimi, where heat treatment and high moisture content lead to DNA fragmentation (Chang et al., 2019; Kumalasari et al., 2024). As a result, DNA-based methods such as PCR are often ineffective in detecting animal-derived adulterants in processed seafood products. Furthermore, these traditional methods are often time-consuming, require expensive equipment, and necessitate skilled personnel, limiting their applicability in routine quality control in food processing plants (Barik et al., 2013). Thus, there is a growing need for more efficient, cost-effective, and sensitive techniques for detecting blood plasma contamination in surimi.

2.5.4 Emergence of Proteomics in Food Authentication

Proteomics has emerged as a revolutionary field for food adulteration detection. Unlike traditional methods that focus on identifying a single target molecule, proteomics allows for comprehensive analysis of the entire protein content of a food product, providing deeper insights into the presence of specific contaminants, including blood plasma proteins. The identification of specific protein biomarkers has

significantly enhanced food adulteration detection, offering greater specificity and reliability compared to conventional analytical approaches (Hardianto et al., 2023; Zhao et al., 2024).

Mass spectrometry (MS)-based proteomic approaches provide a robust alternative for food authentication, enabling the identification of specific protein biomarkers even in highly processed matrices (Prandi et al., 2019). Despite their extensive application in biomedical and veterinary diagnostics, plasma-derived protein biomarkers such as apolipoprotein AI (Apo AI) and fibrinogen remain underexplored in the context of food authenticity verification, particularly for detecting CBP adulteration in surimi-based products. Furthermore, there is a limited availability of optimized extraction and enrichment strategies specifically designed to recover plasma proteins from thermally processed surimi matrices, thereby restricting the broader implementation of proteomics in food fraud detection (Beck et al., 2015; You et al., 2023).

Recent advances in proteomics have further enhanced the ability to detect food adulteration in processed seafood products. One of the key innovations has been the integration of nanotechnology into MS-based techniques. This novel approach offers a rapid, cost-effective, and highly sensitive method for detecting blood plasma contamination in surimi products. The use of biomarkers and proteomic tools in food safety is rapidly becoming a standard practice in regulatory agencies and the food industry. As new technologies continue to emerge, they promise to enhance the detection capabilities for food adulteration, ensuring that food products are safe an,thentic an,d comply with Halal and other regulatory standards. Among examples of biomarker used in food authentication are myoglobin, casein, tropomyosin, albumin (Guntarti et al., 2021), 13 other proteins studied by Lee and Lim (2015). Another exciting development is high-throughput proteomic profiling, which allows for the analysis of thousands of proteins in a single food sample. This method can be used to screen for a wide range of potential adulterants, including blood plasma, by comparing the protein profiles of adulterated and non-adulterated food products (Hardianto et al., 2023). Additionally, advances in data analysis algorithms, such as machine learning and Artificial Intelligence (AI), have further improved the accuracy of proteomic-based detection methods, enabling real-time analysis of food safety and quality (Tian et al., 2022).

2.6 Halal Authentication and Regulatory Changes

2.6.1 The Concept of Halal and Haram in Islamic Law

In Islamic jurisprudence, the term *halal* denotes what is permissible and lawful, whereas *haram* refers to what is forbidden. It is the foundational to guiding the moral and ethical behavior of Muslims, influencing various aspects of life, including economics, food, and social interactions. Halal, meaning "permitted," and haram, meaning "prohibited," are derived from the Qur'an and Hadith, and are interpreted by Islamic scholars to address contemporary issues (Jah & Tie, 2024) (Hussain et al., 2024). In simpler term, foods derived from pigs, carrion, blood, and intoxicants such as alcohol are classified as haram. Furthermore, permissible foods must come from animals slaughtered according to Islamic law, ensuring cleanliness, compassion, and purity.

The application of these concepts is complex nowadays especially with modern innovations in financial instruments and market developments, necessitating continuous scholarly interpretation and ijtihad (independent reasoning) to apply these rules in sharia economic transactions (Jah & Tie, 2024). Shaykh Yusuf Al-Qaradawi outlines 11 principles of fiqh that govern halal and haram, emphasizing that the determination of what is lawful and unlawful is solely the right of Allah, and that good intentions cannot justify unlawful actions (Indradi, 2023). Scientifically, the halal certification process employs various analytical and molecular methods to ensure compliance with Islamic law, reflecting the religion's adaptability to modern scientific advancements (Hussain et al., 2024). Furthermore, the ecological perspective on halal and haram highlights the importance of considering environmental sustainability and the symbiotic relationships in nature, suggesting that what is haram for human consumption may not be haram for other elements of nature, which are essential for human sustenance (Solihu, 2014). This comprehensive understanding underscores the dynamic and multifaceted nature of halal and haram, integrating religious, scientific, and ecological dimensions to guide Muslim life (Arif, 2019).

2.6.2 The Global Relevance of Halal Certification

In recent decades, the demand for halal-certified products has expanded well beyond Muslim-majority countries. Non-Muslim consumers increasingly associate halal food with higher hygiene standards, ethical sourcing, and animal welfare (Damit et al., 2019). Studies have reported that non-Muslim consumers perceive halal-certified food as cleaner, safer, and more trustworthy, motivating repeat purchases (Harshavardhan et al., 2019). This global acceptance suggests halal certification is becoming a universal standard for food quality, safety, and integrity. It is a religious requirement and a marker of ethical and sustainable consumption practices that appeal to broader audiences.

2.6.3 Challenges in Ensuring Halal Integrity in Processed Foods

Ensuring the halal integrity of processed foods presents several technical and regulatory challenges. One major concern is cross-contamination between halal and non-halal products during slaughter, processing, storage, or transportation (Riaz & Chaudry, 2018). Thus, to mitigate this risk, stringent hygiene protocols and dedicated production lines are necessary, although they can be costly for manufacturers.

Another critical challenge arises from the complexity of modern food supply chains, which often involve multiple suppliers and international sourcing. This complexity increases the risk of intentional or accidental adulteration, such as substituting high-cost halal ingredients with cheaper non-halal alternatives (Fischer, 2016). In cases involving blood or blood plasma derivatives, explicitly forbidden in Islam, the risks are particularly acute. In addition, the lack of standardization among halal certification bodies across different countries contributes to inconsistencies. While Department of Islamic Development Malaysia (JAKIM) authority maintains rigorous standards, other countries may apply looser criteria, creating consumer confusion and undermining trust in halal labels (Fischer, 2016). Calls for global harmonization of halal certification procedures are growing to ensure consistency and international trade facilitation.

2.6.4 Importance of Halal Certification for Food Safety and Consumer Trust

Halal certification systems are not merely religious requirements but also comprehensive food safety frameworks. They mandate strict hygiene standards, humane animal treatment, and careful sourcing of ingredients, thus contributing to overall food safety (Isman et al., 2024). Sanitation protocols required for halal certification often exceed standard industry practices, reducing the risk of microbial contamination, cross-contact with allergens, and chemical hazards. For example, halal slaughtering ensures that animals are healthy and properly inspected before processing, reducing the likelihood of foodborne illnesses (Kohilavani et al., 2015).

Moreover, halal certification enhances traceability and accountability across the food supply chain, from farm to fork. Regular inspections, documentation, and verification systems ensure that halal-certified products meet religious standards and broader food quality and safety benchmarks (Riaz & Chaudry, 2018). For Muslim consumers, halal certification provides spiritual assurance and ethical integrity. Meanwhile, for non-Muslim consumers, it signals a higher level of scrutiny, hygiene, and product quality (Kohilavani et al., 2015). Therefore, halal certification plays a dual role: protecting religious rights and promoting food safety and ethical standards.

2.6.5 Implications for Plasma Adulteration Detection in Surimi-Based Products

In the context of this study, halal authentication assumes critical importance due to the risk of adulteration with CBP in surimi-based products. Blood and its derivatives are categorically forbidden in Islam, regardless of their source or processing method (Hussain et al., 2016). The clandestine incorporation of CBP into food products violates halal principles, compromises consumer trust, and poses serious ethical and religious concerns. Traditional detection methods, such as DNA-based and protein-based assays, face challenges in processed foods due to protein denaturation and DNA degradation. Hence, there is an urgent need for more advanced technologies, such as proteomics and electrochemical immunosensors, to sensitively detect plasma-specific proteins even after intensive processing (Ruzulan et al., 2021; Sentandreu et al., 2010).

By identifying reliable protein markers such as Apo AI and fibrinogen, and developing a sensitive immunosensor platform, this research directly addresses a critical gap in halal authentication capabilities. It not only enhances scientific methods

for food fraud detection but also strengthens consumer confidence, supports religious compliance, and contributes to the broader goals of food integrity and transparency in global halal markets.

Table 2.1
Food Adulteration and Halal Authentication Issues in Surimi-Based Products

Components	Description	Authors
Market Context	Surimi-based products are widely consumed globally, with a market value of approximately USD 6.4 billion in 2024 and projected growth to USD 10.41 billion by 2033. Growth is driven by affordability, versatility in products such as sushi and seafood analogues, and functional protein properties.	IMARC Group, 2025; Intel Market Research, 2026
Functional Role of Plasma	Blood plasma is incorporated into surimi formulations to enhance gelation through protein–protein interactions, improve water-holding capacity and texture, and reduce production cost as an economical protein substitute.	Seymour et al., 1997; Walayat et al., 2025
Halal Prohibition of Blood	Blood and its derivatives are categorically prohibited (haram) in Islam regardless of source or processing method, as they are considered impure (najs). Their inclusion in food products violates halal dietary principles.	Hussain et al., 2016; Sukri et al., IIUM Guideline
Ethical and Consumer Impact	Undeclared incorporation of CBP in surimi undermines halal certification, erodes consumer trust, and raises serious ethical and religious concerns, particularly in Muslim-majority markets.	Rosele et al., 2023/2025; UPM Halal, 2018
Analytical Challenge in Processed Foods	Conventional DNA- and protein-based detection methods face limitations in thermally processed matrices due to DNA degradation and protein denaturation, reducing sensitivity and reliability.	Ruzulan et al., 2021; Sentandreu et al., 2010
Need for Advanced Detection Technologies	Advanced analytical approaches such as mass spectrometry-based proteomics and electrochemical immunosensors are required to sensitively detect plasma-specific protein biomarkers even after intensive processing.	Ruzulan et al., 2021; Sentandreu et al., 2010

2.6.6 Future Directions for Halal Authentication Research

The future of halal authentication research is poised to significantly enhance food safety, integrity, and regulatory compliance on a global scale. As the demand for halal products increases, both within Muslim-majority and non-Muslim-majority markets, there is a pressing need for innovative technologies that can ensure the authenticity of halal claims, particularly in complex and processed food matrices. The current literature indicates a critical gap in reliable, rapid, and cost-effective halal

testing methods, and this section highlights the emerging trends that will drive the evolution of halal authentication in the coming years.

One of the most exciting developments in the field of halal authentication is the integration of proteomics and biosensors. Proteomics, particularly through mass spectrometry (MS) and high-throughput protein profiling, has revolutionized the ability to detect even trace amounts of adulterants in food products. For instance, the application of LC-MS/MS and MALDI-TOF MS to identify species-specific markers is already gaining traction in the identification of non-halal animal proteins such as porcine and bovine derivatives in meat products (Barik et al., 2013; Montowska & Pospiech, 2013). These proteomic techniques have the potential to transform halal certification by providing highly sensitive, accurate, and comprehensive testing capabilities that can be applied across a wide variety of food products, from fresh meat to processed surimi. As proteomics technology continues to evolve, the identification of more specific biomarkers, such as blood plasma proteins, will further enhance the precision and reliability of halal authentication tests.

The incorporation of nanotechnology into biosensor platforms is another promising area for halal authentication. Electrochemical immunosensors, especially those integrated with gold nanoparticles (AuNPs), have shown great promise for rapid, on-site detection of specific proteins associated with halal adulterants (Hardianto et al., 2023). These biosensors are compact, cost-effective, and capable of providing real-time results, making them ideal for use in food processing facilities and retail environments where rapid testing is crucial. In particular, developing electrochemical immunosensors for blood plasma proteins offers a novel solution for detecting non-halal contamination in surimi and other processed foods. Combining antibody-antigen binding with electrochemical detection enhances the sensitivity and specificity of these sensors, allowing them to detect even minute traces of forbidden substances like blood or pork in halal food products. This innovation is not only crucial for halal food markets but could also be adapted to other food authentication challenges.

While advancements in detection technologies are promising, one of the key hurdles to the widespread adoption of these methods is the lack of standardization in halal certification practices. There is currently no universally accepted protocol for halal certification, which results in variability in what constitutes halal across different countries and regions (Isman et al., 2024). To ensure global consistency and consumer trust, future research must focus on the harmonization of halal certification processes.

This would involve collaboration between regulatory bodies, certification agencies, and scientific communities (Abdulraof, 2024; Tajuddin et al., 2023), to create unified standards for halal testing and certification. As halal markets expand globally, the need for a standardized certification (Susanty et al., 2024) framework will become increasingly important to facilitate international trade and ensure that products labeled as halal meet the same rigorous criteria, regardless of where they are produced. Future research should therefore aim to create comprehensive, scientifically validated guidelines that can be adopted internationally, promoting transparency and reducing consumer confusion.

Another cutting-edge approach that could transform halal authentication in the future is the use of blockchain technology for food traceability. Blockchain offers a secure and transparent way to track food products from farm to table, ensuring that each step of the supply chain meets halal requirements. By recording every transaction in an immutable ledger, blockchain technology could provide real-time verification of halal status, reducing the potential for fraud and contamination. This could be particularly beneficial in supply chains involving multiple suppliers and regions, where the risk of non-halal ingredients being introduced is higher. The integration of blockchain with electrochemical biosensors and other detection methods would create a powerful end-to-end system for ensuring halal integrity at every stage of food production, from raw material sourcing to final consumer consumption. This would not only enhance food safety but also help mitigate religious and ethical concerns, providing greater consumer confidence in halal-certified products.

2.7 Proteomics and Mass Spectrometry in Food Analysis

2.7.1 Introduction to Proteomics in Food Analysis

Proteomics provides an extensive analysis of proteins, which are crucial in food products like surimi and processed seafood, allowing for comprehensive identification and quantification of these proteins. Techniques like mass spectrometry (MS) offer sensitivity and specificity in food authentication, especially when adulteration occurs. Proteomics plays a pivotal role in analyzing complex food matrices, such as surimi-based products, where protein denaturation may make traditional methods, such as PCR or ELISA, less effective (Barik et al., 2013). With the rise in food fraud and the growing

demand for halal authentication, proteomics serves as a vital tool to ensure food integrity (Fischer, 2016).

The field of mass spectrometry (MS) has seen significant advancements, particularly in identifying food adulterants. Recent work has focused on developing better proteomics methods to identify blood plasma proteins like Apolipoprotein A1 (ApoA1) and fibrinogen, which are significant markers for halal authentication (Azmi et al., 2024).

2.7.2 Mass Spectrometry Techniques in Proteomics

Mass spectrometry, notably liquid chromatography-tandem mass spectrometry (LC-MS/MS), is a widely used method in food analysis for protein identification and quantification. These techniques provide detailed analysis by fragmenting proteins into smaller peptides, analyzing their mass-to-charge ratio (m/z), and identifying the protein based on these characteristics (Mishra et al., 2020). Recent studies have applied MS-based proteomics to detect adulterants such as blood plasma proteins, even in highly processed foods like surimi (Yuswan et al., 2018). Moreover, these techniques, when combined with database search engines for example Comet, X! Tandem, and ProteinProspector), enable the detection of trace amounts of adulterants in complex food matrices (Amir et al., 2021).The use of shotgun proteomics, where proteins are fragmented and identified without a prior hypothesis, has proved to be a robust method for detecting non-halal substances (Montowska & Pospiech, 2013). This approach is vital for the identification of blood proteins, such as Apolipoprotein A1 and fibrinogen, both of which have been shown to be specific markers for halal food certification (Azmi et al., 2025).

2.7.3 Shotgun Proteomics for Food Authentication

Shotgun proteomics involves the identification of proteins in a complex food sample using a non-targeted approach. This method does not require the pre-selection of protein markers, making it highly effective in identifying unknown adulterants (Beneito-Cambra et al., 2015). For instance, in surimi-based products, shotgun proteomics has been employed to identify blood plasma contamination by detecting specific biomarkers, which are otherwise difficult to identify using traditional assays

like ELISA (Barik et al., 2013). Chemometrics-assisted shotgun proteomics has also enhanced the ability to identify species-specific markers, such as myosin-2 and myosin-7, which are useful for authentication in food products (Yuswan et al., 2018). By utilizing liquid chromatography coupled with mass spectrometry (LC-MS), researchers can perform high-throughput analyses to screen for species-specific peptide markers in complex food products. The combination of mass spectrometry and chemometric analysis has led to the discovery of species-specific peptide markers for non-halal pork in beef and chicken products (Yuswan et al., 2018). This is particularly useful for halal food authentication, where such methods help ensure that pork-derived proteins are absent from meat products that claim to be halal.

2.7.4 Quantification in Mass Spectrometry

In mass spectrometry-based proteomics, quantification methods such as label-free quantification (LFQ) and isotope labeling allow for the precise measurement of protein abundances in complex food matrices. The LC-MS/MS analysis measures the intensity of peptide signals to estimate the relative abundance of proteins (Verma et al., 2021). These techniques are particularly beneficial when analyzing processed foods, where proteins might be altered due to thermal treatment or chemical processing (Tian et al., 2022). For instance, the quantification of Apolipoprotein A1, a specific biomarker for halal food, could serve as a quantitative measure of blood plasma adulteration in surimi products (Azmi et al., 2024). Isotope labeling, such as iTRAQ (Isobaric Tags for Relative and Absolute Quantification), is often used for accurate quantification by labeling peptides with isotopes to distinguish them in mass spectrometry (Verma et al., 2021). This method allows researchers to compare protein levels across multiple samples, thus enabling the identification of trace amounts of blood plasma proteins that could indicate halal fraud (Yuswan et al., 2020).

2.7.5 Trans-Proteomic Pipeline (TPP) in Proteomics Data Analysis

In addition to analytical instrumentation and quantification strategies, robust bioinformatics platforms are essential for reliable proteomic data interpretation. Mass spectrometry-based proteomics generates large and complex datasets that require robust bioinformatics platforms for accurate peptide and protein identification, statistical

validation, and quantification. Among the available open-source and proprietary tools, the Trans-Proteomic Pipeline (TPP) has emerged as a comprehensive and highly adaptable framework for proteomic data processing. Since the introduction of PeptideProphet and ProteinProphet more than two decades ago, TPP has evolved into an integrated suite of tools supporting spectrum processing, database searching validation, protein inference, and quantitative analysis.

A key strength of TPP lies in its rigorous statistical validation framework. PeptideProphet and ProteinProphet apply probabilistic modeling approaches to improve identification confidence while controlling false discovery rates (FDR), thereby enhancing data reliability in large-scale proteomic experiments. Recent developments have further incorporated machine-learning strategies to optimize information extraction and refine statistical modeling, contributing to improved sensitivity and specificity in protein identification (Deutsch et al., 2023).

Compared with other widely used proteomic platforms such as MaxQuant (Cox & Mann, 2008), Proteome Discoverer (Orsburn, 2021), FragPipe (Kong et al., 2017), PEAKS Studio (Zhang et al., 2012), and OpenMS (Röst et al., 2016), TPP offers distinct advantages in flexibility and cross-platform compatibility. While MaxQuant primarily relies on the Andromeda search engine, TPP supports multiple search engines, including SEQUEST, Mascot, X!Tandem, and Comet, enabling broader applicability across different experimental designs. In contrast to workflows that depend heavily on Percolator (e.g., FragPipe), TPP integrates its own validated statistical framework, providing a unified and customizable processing environment.

Furthermore, TPP accommodates diverse quantitative strategies, including label-free quantification as well as multiplexed approaches such as iTRAQ and SILAC. Its modular architecture supports scalable processing of large datasets and high-throughput proteomics applications. The platform can be deployed across personal computers, Linux-based clusters, and cloud infrastructures, demonstrating adaptability to varying computational environments (Deutsch et al., 2023). Integrated visualization and reporting tools further enhance transparency and reproducibility, reinforcing TPP's position as a critical platform in contemporary proteomic research.

2.7.6 Applications of Proteomics in Halal Authentication

The application of proteomics in halal food authentication is especially significant as it enables the detection of blood plasma proteins, which are strictly prohibited in halal diets. Recent studies have focused on identifying species-specific markers to ensure that halal food products, such as the myosin proteins, found in muscle tissues, are stable and abundant, making them ideal targets for halal authentication (Amir et al., 2021). Moreover, the ability to detect even trace amounts of blood plasma via shotgun proteomics enhances confidence in halal certification of food products (Yuswan et al., 2018). Proteomics also plays a crucial role in the global halal food market, where the demand for reliable authentication methods is growing. By employing LC-MS/MS and shotgun proteomics, food scientists can confidently verify the presence or absence of non-halal proteins, thus supporting food integrity and consumer trust (Fischer, 2016).

2.7.7 Mass Spectrometry and Food Safety

The application of mass spectrometry extends beyond halal food authentication to overall food safety. It can be used to monitor for microbial contaminants, allergens, and toxic substances in food products, offering a holistic approach to food analysis (Benjakul & Visessanguan, 2000). For example, using LC-MS/MS, researchers can detect contaminants that may not be detectable by traditional methods, such as chemical or microbiological analysis (Beneito-Cambra et al., 2015).

2.7.8 Proteomics Approaches in Protein Biomarker Discovery

Biomarker discovery is a multifaceted field that plays a crucial role in clinical, veterinary, and food safety domains by providing insights into biological processes and disease states. Biomarkers, or biological markers, are measurable indicators of normal or abnormal biological processes, conditions, or diseases, and they can be found in body fluids, tissues, or cells (Ferreira et al., 2024; Mollarasouli et al., 2022). In clinical settings, biomarkers are pivotal for diagnosis, prognosis, and monitoring of diseases, offering a foundation for personalized medicine by tailoring treatments to individual patient profiles (Ferreira et al., 2024; Mehta et al., 2023). The discovery process

involves identifying specific molecules, such as proteins, metabolites, or nucleic acids, that correlate with disease states or therapeutic responses (Ogidi et al., 2024; Čuperlović-Culf, 2013). In veterinary medicine, biomarkers are essential for diagnosing and managing animal diseases, which can have significant implications for human health due to zoonotic diseases and food safety concerns (Moore et al., 2007; Perera et al., 2022). The use of post-genomic technologies, including transcriptomics, proteomics, and metabolomics, has advanced the identification of sensitive and specific biomarkers in both human and veterinary medicine (Moore et al., 2007; Perera et al., 2022). In the context of food safety, biomarkers help in assessing the health of livestock, thereby ensuring the safety and quality of animal-derived food products (Boehmer & Olumee-Shabon, 2011). Despite the potential of biomarkers, challenges remain in their validation and translation into clinical practice, necessitating rigorous testing and collaboration among researchers, clinicians, and funding bodies to ensure their efficacy and applicability(Perera et al., 2022). Overall, the discovery and application of biomarkers hold promise for enhancing disease diagnosis, treatment, and prevention across various fields, although the path from discovery to clinical implementation is complex and requires careful consideration of technological and methodological advancements (Davis et al., 2013; Davis et al., 2022).

2.7.9 Main Stages of Protein Biomarker in Discovery, Verification, and Validation.

The process of protein biomarker discovery is a complex, multi-stage pipeline essential for developing reliable clinical tests. The initial stage, discovery, involves identifying potential biomarker candidates using proteomic techniques such as mass spectrometry, which allows for the qualitative and quantitative analysis of proteins. This stage often employs methods like label-free quantification, metabolic labeling, and stable isotope labeling to identify differentially expressed proteins and disease-specific protein isomers (Drabovich et al., 2013; Yao et al., 2013). Following discovery, the verification stage aims to confirm the presence and relevance of these candidates in larger sample sets. Techniques such as targeted proteomics, particularly selected reaction monitoring (SRM), are employed to measure the abundance of target proteins with high specificity and sensitivity, addressing issues like sample complexity and peptide ionization (Atashpaz-Gargari et al., 2014 ; Ogata et al., 2022). The final stage,

validation, involves rigorous testing to ensure that the biomarkers are reliable and reproducible across different populations and settings. This stage often utilizes triple quadrupole mass spectrometry in SRM mode to validate biomarkers, ensuring they meet regulatory requirements and are fit for clinical use (Atashpaz-Gargari et al., 2014; Lee et al., 2006). Throughout these stages, challenges such as assay development, preanalytical planning, and the need for high-throughput and high-resolution methods are addressed to optimize the biomarker pipeline(Rifai et al., 2006; Rai, 2007). The integration of these stages into a coherent pipeline is crucial for translating biomarker discoveries into clinical applications, ultimately enhancing diagnostic and therapeutic strategies(Rifai et al., 2006; Yang et al., 2015).

2.8 Bioinformatics Tool in Protein Biomarker Discovery with Clustal Omega

Following the stages of biomarker discovery, verification, and validation, bioinformatics tools such as Clustal Omega play a crucial role in analyzing protein sequences, enabling the identification of potential markers with greater sensitivity and efficiency. Clustal Omega's efficiency in bioinformatics, particularly for identifying potential protein markers, is attributed to several key factors. Firstly, its ability to handle very large datasets, including tens of thousands of DNA/RNA or protein sequences, is facilitated by the mBed algorithm, which efficiently calculates guide trees, allowing for rapid alignment even on personal computers (Sievers and Higgins, 2021; Sievers and Higgins, 2014). This scalability is crucial in modern bioinformatics, where large-scale data is common. Additionally, Clustal Omega employs the HHalign method for aligning profile hidden Markov models, significantly improving the accuracy of alignments compared to earlier versions of the Clustal series (Sievers and Higgins, 2021; Sievers and Higgins, 2014). The program's design also allows for the integration of precomputed information from public databases like Pfam, enhancing its ability to add sequences to existing alignments and exploit existing data effectively (Sievers et al., 2011). This feature is particularly beneficial for identifying protein markers, as it leverages vast amounts of pre-existing sequence information to improve alignment quality and speed. Furthermore, Clustal Omega's command-line and online accessibility make it versatile and user-friendly, broadening its applicability across different computational environments (Sievers and Higgins, 2021; Sievers and Higgins, 2014). While Clustal Omega excels in sequence alignment, other tools like AAclust and clustQ

focus on clustering and structural prediction, respectively, offering complementary approaches to protein analysis. AAclust, for instance, optimizes amino acid scale selection for machine learning applications, which can enhance feature engineering in protein prediction tasks (Breimann & Frishman, 2024). Meanwhile, clustQ provides a superposition-free method for protein decoy clustering, improving computational efficiency in structural predictions (Alapati & Bhattacharya, 2018). These tools, alongside Clustal Omega, contribute to a comprehensive toolkit for bioinformatics, each addressing different aspects of protein analysis and marker identification.

2.9 Biosensors for Food Detection

2.9.1 Types of Biosensors for Food Authentication

Biosensors are analytical tools integrating biological recognition elements, such as antibodies, with transduction mechanisms to detect specific food contaminants. Among these, electrochemical biosensors have become a promising choice for food authentication due to their high sensitivity, cost-effectiveness, and ease of miniaturization. These sensors translate biological interactions into measurable electrochemical signals, such as changes in current, voltage, or impedance (Kumalasari et al., 2024; Wang et al., 2022).

The most common biosensors used in food safety include optical, piezoelectric, and electrochemical biosensors Electrochemical biosensors, particularly electrochemical immunosensors, are the focus of recent advancements in detecting food adulterants. These biosensors are favored due to their simplicity, rapid response time, and reliability (Hardianto et al., 2023). Furthermore, they can detect target proteins such as blood plasma proteins (Apolipoprotein A1) at sub-nanomolar concentrations, making them ideal for monitoring the integrity of processed food products like surimi (Hardianto et al., 2023). Meanwhile, electrochemical immunosensors operate based on the specific interaction between antigens and antibodies, where the binding event is transduced into a measurable electrochemical signal. In addition, the biorecognition element, typically an antibody, selectively binds to the target analyte, such as proteins or DNA markers related to food adulterants. Moreover, the transduction element (usually an electrode) converts this interaction into a measurable signal (Campuzano et al., 2016). These sensors offer several advantages, such as high sensitivity, low cost,

ease of use, and the ability to miniaturize, making them highly suitable for rapid, on-site food adulteration detection (Nath, 2024).

Recent advancements in electrochemical immunosensors have focused on improving their ability to detect multiple analytes simultaneously in complex food matrices. This multiplexing ability is particularly beneficial for detecting multiple contaminants that may be present in food products, such as pesticides, antibiotics, or animal proteins (Melo et al., 2016). In addition to their sensitivity, electrochemical immunosensors can provide real-time results, which is crucial for food safety monitoring across the global food supply chain (Nath, 2024).

2.9.2 Monoclonal and Polyclonal Antibodies as Biorecognition Elements

Antibodies are the most widely employed biorecognition elements in immunosensor platforms, where their inherent specificity and affinity toward antigens enable accurate detection. Among them, monoclonal and polyclonal antibodies exhibit distinct structural and functional properties that determine their binding behavior, selectivity, and suitability for different biosensor applications. Monoclonal and polyclonal antibodies exhibit distinct binding characteristics to antigens, primarily due to their structural and functional differences. As depicted by Figure 2.3, monoclonal antibodies (mAbs) are highly specific, binding to a single epitope on an antigen. This specificity is achieved through the precise interaction of the antibody's antigen-binding fragment with the epitope, as demonstrated by structural proteomics techniques that map these interactions at a high resolution, revealing the detailed interface between the antibody and its target epitope (Petrotchenko et al., 2023). Monoclonal antibodies bind to a specific epitope on an antigen due to their uniform structure, derived from a single clone of B cells, ensuring specificity. In contrast, polyclonal antibodies are produced by multiple B cell clones, allowing them to recognize and bind to several different epitopes on the same antigen (Goldberg & Ackerman, 2024 ; Felix and Agnes 2017).

The specificity of mAbs is further highlighted in studies where monoclonal antibodies are shown to recognize specific phosphorylated epitopes with high affinity and selectivity, driven by atomic-level interactions that enhance binding affinity (Kasahara et al., 2024). In contrast, polyclonal antibodies are a heterogeneous mixture of antibodies that can bind to multiple epitopes on the same antigen. This diversity in binding is due to the natural immune response, where different B cells produce

antibodies against various parts of the antigen, as elucidated by methods like hydrogen/deuterium exchange mass spectrometry (HDX-MS), which map the complex epitope landscape targeted by polyclonal sera (Vorauer et al., 2024). The ability of polyclonal antibodies to recognize multiple epitopes provides a broader range of antigen recognition, which can be advantageous in neutralizing pathogens with high variability.

Computational models and structural analyses have further enhanced our understanding of these interactions, allowing for the prediction and characterization of antibody-antigen interactions based on structural features (Gu et al., 2024; Lee et al., 2022). These insights are crucial for the design of vaccines and therapeutic antibodies, as they inform the selection of epitopes that can elicit a robust immune response, whether through the precise targeting of monoclonal antibodies or the broad coverage of polyclonal antibodies (Barnes et al., 2024). Understanding the binding mechanisms and recognition capabilities of monoclonal and polyclonal antibodies is therefore essential, as these molecular interactions form the basis for the signal transduction processes that underpin electrochemical immunosensors.

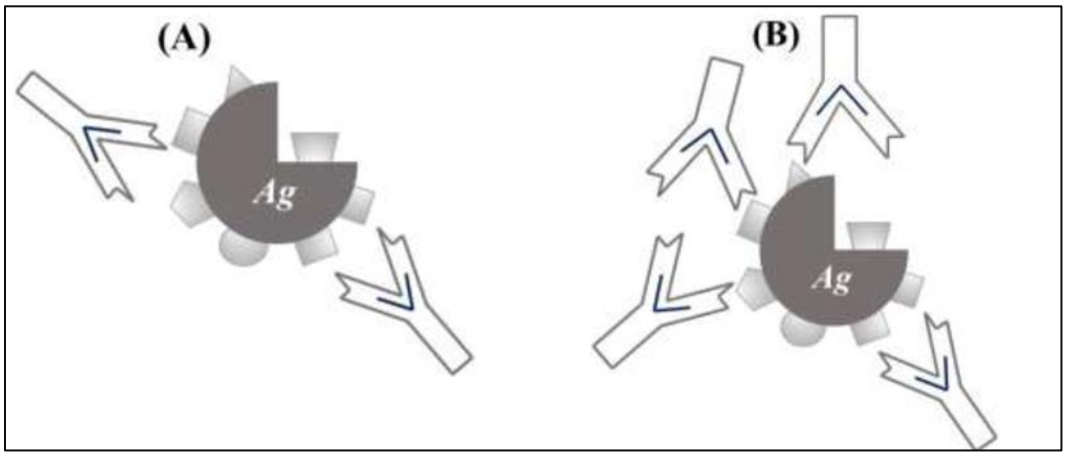


Figure 2.3 Schematic Representation of (A) Monoclonal Ab Binding a Specific Epitope on Ag and (B) Polyclonal Ab Binding to Several Epitopes on an Ag

Source : Felix and Agnes 2017

2.9.3 Working Principles of Electrochemical Immunosensors

Electrochemical immunosensors are highly efficient devices designed to detect specific biological analytes through antigen–antibody interactions. The core principle of these sensors lies in the interaction between a biorecognition element such as

antibodies, aptamers, or enzymes and a target analyte such as toxins, food contaminants or pathogens. Upon binding of the analyte to the recognition element, a measurable electrochemical response is produced in the form of changes in current, voltage, or impedance. This signal allows for quantification of the target substance with high sensitivity, even in complex food matrices (Iglesias-Mayor et al., 2019; Lim & Ahmed, 2019; Nordin et al., 2018).

Electrochemical immunosensors utilize a transducer, typically an electrode, where the immunological interaction occurs, and the resulting electrochemical signal is proportional to the concentration of the target analyte (Kokkinos & Economou, 2018; Aydın et al., 2019). Various electrochemical techniques, including cyclic voltammetry (CV), differential pulse voltammetry (DPV), amperometry, potentiometry, and impedance spectroscopy, are employed to monitor these changes (Tran & Lam, 2019; Ju, 2017).

The immobilization of antibodies or antigens onto the transducer surface is a critical step, commonly achieved through methods such as physical adsorption, covalent coupling, or self-assembled monolayers. These approaches improve the sensitivity, selectivity, and stability of the sensor (Qiao et al., 2014). To further enhance performance, nanomaterials such as gold nanoparticles (AuNPs) are commonly integrated into sensor platforms. AuNPs provide a large surface area for antibody immobilization, improve electron transfer rates, and enhance biocompatibility and stability, making them ideal for detecting low-concentration food adulterants such as blood plasma proteins (Chang et al., 2019; Kumalasari et al., 2024; Nath, 2024). Other nanomaterials, including graphene quantum dots and metal nanoparticles, have also been utilized to improve electron transfer and sensitivity (Mansuriya & Altintas, 2019; Wang et al., 2024). Signal amplification strategies, such as the use of enzyme labels for example alkaline phosphatase, further increase the sensitivity of detection (Chen et al., 2023). In addition, the development of lab-on-a-chip systems and portable devices has facilitated the miniaturization and automation of electrochemical immunosensors, enabling point-of-care testing and high-throughput analysis (Kokkinos & Economou, 2018; Scherf et al., 2015).

The use of electrochemical immunosensors in food safety monitoring offers significant advantages over conventional analytical methods, including high specificity, low cost, ease of miniaturization, and rapid response. These properties make AuNP-based immunosensors particularly suitable for addressing food adulteration, ensuring

food safety, quality, and authenticity in industrial and field-based applications (Felix & Angnes, 2018; Melo et al., 2016; Chen et al., 2023).

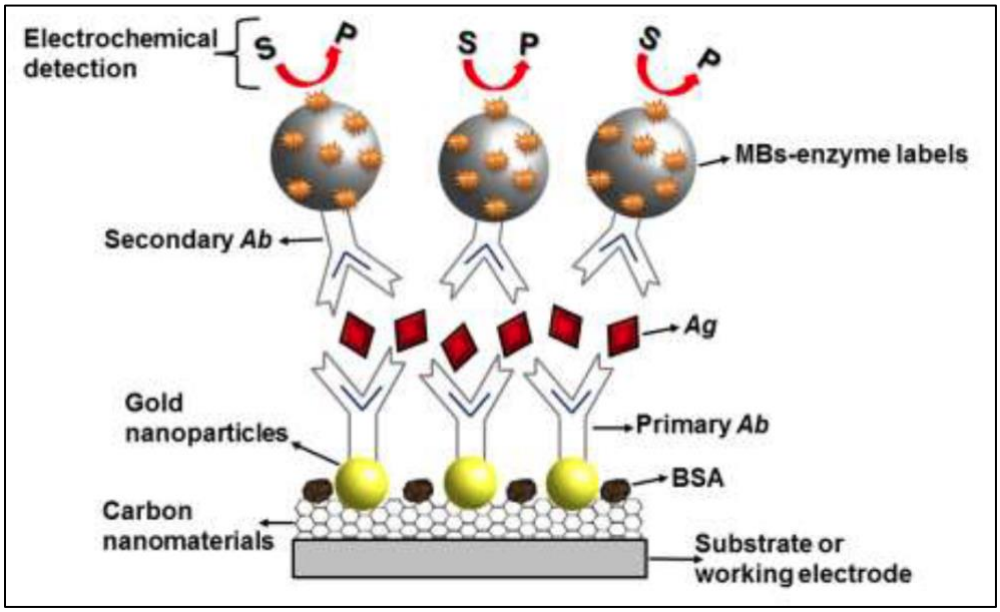


Figure 2.4 Schematic Representation of Sandwich Immunosensor Showing a Strategy for its Construction

2.9.4 Gold Nanoparticles in Electrochemical Biosensors

Gold nanoparticles (AuNPs) are widely utilized in electrochemical biosensors for food detection due to their unique optoelectronic properties, excellent biocompatibility, and low toxicity (Ghobashy et al., 2024). These properties enable AuNPs to act as effective electrochemical transducers for detecting adulterants such as blood plasma contamination in surimi products. Additionally, AuNPs can be easily functionalized with antibodies or other molecular recognition elements, making them highly effective for use in immunosensors designed to detect halal authenticity markers (Iglesias-Mayor et al., 2019).

A key attribute of AuNPs is their high surface-to-volume ratio, which allows for increased biomolecule attachment compared to traditional materials. This property makes them ideal for immobilizing large quantities of antibodies or aptamers, thereby enhancing the likelihood of efficient antigen–antibody binding and contributing directly to sensor sensitivity and selectivity (Mahmud et al., 2023; Zhang et al., 2020). Moreover, AuNPs exhibit Surface Plasmon Resonance (SPR), which amplifies electrochemical signals during antigen–antibody interactions. This amplification is

particularly crucial for detecting low-concentration analytes, such as trace levels of CBP in surimi-based products (Nath, 2024).

Beyond SPR, AuNPs enhance electron transfer between the biorecognition element and the electrode surface. Their excellent electrical conductivity improves the efficiency of electrochemical signal transduction, resulting in higher sensitivity and faster response times (Felix & Angnes, 2018). Studies have shown that variations in the size and morphology of AuNPs (e.g., nanorods, nanospheres) can be tailored during synthesis to further optimize detection performance across different food safety applications (Ghobashy et al., 2024; Mishra et al., 2020; Marrs et al., 2021).

Functionalization of AuNPs is another critical factor in ensuring specificity. Biomolecules such as antibodies, aptamers, or enzymes can be attached to AuNP surfaces via physisorption or chemisorption. Physisorption, involving non-covalent attachment, is cost-effective and straightforward, while chemisorption provides stronger covalent bonds that offer greater stability and reduce biomolecule detachment during use (Sharma et al., 2010; Zhang et al., 2020). Optimizing antibody orientation and surface coverage maximizes the availability of active binding sites, improving antigen recognition. Furthermore, blocking agents like Bovine Serum Albumin (BSA) or Polyethylene Glycol (PEG) are often employed to minimize nonspecific binding, ensuring selective responses even within complex food matrices (Zhang et al., 2020).

Taken together, the distinctive properties of AuNPs including high surface area, SPR-induced amplification, superior electron transfer, and versatile functionalization making them indispensable in the development of high-performance electrochemical immunosensors for food adulteration and halal authentication.

2.9.5 Functionalization of AuNPs for Target Analyte Detection

Gold nanoparticles (AuNPs) require functionalization to achieve selective and stable target recognition. This is achieved by attaching biomolecules such as antibodies, aptamers, or enzymes to the nanoparticle surface, enabling detection of proteins scuh as aflatoxins (Sharma et al., 2010; Zhang et al., 2020). Functionalization strategies are generally categorized into physisorption and chemisorption. Physisorption relies on non-covalent interactions such as electrostatic forces, van der Waals interactions, and hydrogen bonding. It is simple to implement but offers limited stability. In contrast, chemisorption employs covalent attachment through linkers such as thiols, carboxyl, or

amine groups, providing more robust and durable binding (Sharma et al., 2010). Chemisorption also allows better control over the orientation of immobilized biomolecules, which directly influences antigen-binding site accessibility and the sensitivity of the biosensor (Zhang et al., 2020).

To minimize non-specific binding, blocking agents such as bovine serum albumin (BSA) or polyethylene glycol (PEG) are introduced after functionalization. These agents mask unmodified AuNP surfaces, enhancing selectivity in complex food matrices like surimi (Zhang et al., 2020). Overall, functionalization ensures the attachment of biorecognition elements, stabilizes the sensor, and maintains reproducibility in real food testing scenarios (Nath, 2024; Azmi et al., 2024).

2.9.6 Complementary Nanomaterials in Sensor Technology

While AuNPs are the main nanomaterial in the sensor platform, integrating complementary nanomaterials like magnetic beads, graphene, and carbon nanotubes can further improve the performance of the electrochemical immunosensor. Magnetic beads are often combined with AuNPs to improve sensor stability, signal amplification, and analyte concentration. Magnetic beads possess superparamagnetic properties, allowing them to be manipulated using external magnetic fields. This makes them ideal for magnetic separation of the target analyte, which can concentrate the analyte and reduce interference from other components in the sample. When combined with AuNPs, magnetic beads can be used for sample enrichment and signal enhancement, improving the overall sensitivity and specificity of the sensor. The integration of magnetic beads in immunosensor platforms is well-documented in the literature, where their role in analyte capture and detection has been demonstrated to significantly enhance sensor performance (Azmi et al., 2024; Iglesias-Mayor et al., 2019; Lim & Ahmed, 2019).

Other complementary nanomaterials, such as graphene and carbon nanotubes, are also increasingly used in electrochemical sensors. These materials enhance the electrical conductivity of the sensor platform and improve electron transfer during the antigen-antibody interaction. Graphene's high surface area, mechanical strength, and excellent conductivity make it an ideal material for sensor enhancement, especially when combined with AuNPs (Felix & Angnes, 2018; Li et al., 2020). In addition to AuNPs, nanomaterials like carbon nanotubes and graphene are integrated into sensor designs to improve electron transfer and reduce background noise, which is essential

for detecting low concentrations of analytes in complex food samples (Khan et al., 2019; Nath, 2024).

By combining AuNPs with these other nanomaterials, sensor performance can be optimized to detect a broader range of food contaminants. For instance, graphene and carbon nanotubes can enhance the electrical conductivity and mechanical stability of AuNP-based sensors, making them more robust for long-term use (Nath, 2024). These hybrid nanomaterials enable the simultaneous detection of multiple contaminants, which is crucial for comprehensive food safety monitoring.

2.9.7 Applications of Electrochemical Imunosensor in Food Adulteration Detection

Recent advancements in electrochemical immunosensors have significantly improved the sensitivity, specificity, and practicality of food safety monitoring. In 2024, nanomaterial-assisted sensors incorporating conductive polymers enabled rapid, on-site assessment of food freshness and quality, reflecting the growing trend toward real-time diagnostics (Kogularasu et al., 2024). The paper focuses on the critical role in enhancing food safety, quality, and traceability within the global food supply chain. These sensors are pivotal in detecting a wide array of foodborne pathogens, chemical contaminants, and adulterants, thereby ensuring public health and economic stability (Kogularasu et al., 2024). The review highlights the integration of cutting-edge materials such as nanomaterials and conductive polymers, which have improved the sensitivity and selectivity of these sensors, allowing for real-time and on-site analysis (Kogularasu et al., 2024). This aligns with the broader trend in sensor technology, where the development of sensitive, affordable, and user-friendly sensors is crucial for food safety and security

Next is the introduction of three-dimensional electrode architectures in 2023 further enhanced analyte adsorption and electrochemical efficiency, demonstrating how structural innovations can boost detection performance for a variety of food adulterants (Zhang et al., 2023). In 2022, competitive immunosensors designed for capsaicinoid detection in edible oils employed computational modeling to optimize antibody–analyte interactions, illustrating the value of tailored sensor design for targeted adulteration detection (Sun et al., 2022). Moreover, sandwich-type immunosensors successfully detected *Salmonella Typhimurium*, highlighting their role in rapid pathogen

surveillance and reinforcing the importance of reliable detection platforms in ensuring both public health and the integrity of halal-certified food products (Sun et al., 2022).

The evolution of electrochemical immunosensor applications over the past decade illustrates a clear trend toward enhanced sensitivity, selectivity, and practicality in food safety monitoring. Table 2.1 presents a chronological summary of representative studies from 2015 to 2024, highlighting key innovations in sensor design, target analytes, and analytical performance. By situating recent advances within this broader timeline, the table underscores the growing versatility of immunosensors in detecting contaminants, pathogens, and adulterants, while also emphasizing their potential utility in specialized contexts such as halal authentication and quality assurance in food products. However, there is still the lack of research in detecting blood derived plasma proteins specifically in surimi hence the key gap is prominent by reviewing the summary presented below.

Table 2.2
Chronological Summary of Electrochemical Immunosensor Applications in Food Safety and Adulteration Detection Relevant to Halal Authentication in the recent 5 years

Year	Application Area	Details	References
2024	Food Freshness & Quality Monitoring	Electrochemical sensors using nanomaterials and conductive polymers to detect foodborne pathogens and chemical contaminants, supporting real-time freshness and quality assessment.	Kogularasu et al., 2024
2023	3D Electrochemical Sensors	3D-structured electrodes with increased surface area and active sites for improved analyte adsorption, enhancing detection efficiency for a variety of food adulterants.	Zhang et al., 2023
2022	Capsaicinoid Detection in Oils	Competitive electrochemical immunosensor using computer-aided molecular modeling to optimize antibody–capsaicinoid binding for adulteration detection in edible vegetable oils.	Sun et al., 2022
2022	Pathogen Detection – Salmonella Typhimurium	Sandwich-type electrochemical immunosensor enabling sensitive and rapid detection of S. Typhimurium in food samples, aiding in contamination control.	Sun et al., 2022
2022–2021	MIP-Based Sensors for Contaminants	Electrochemical sensors incorporating molecularly imprinted polymers (MIPs) for selective and sensitive detection of pesticides, veterinary drugs, and other food contaminants.	Elfadil et al., 2021

Year	Application Area	Details	References
2021	Dairy Product Adulteration	Silicon photonic immunosensor for rapid detection of mozzarella and feta adulteration with cow milk, ensuring authenticity and compliance.	Angelopoulou et al., 2021
2022–2020	Nanomaterial-Assisted Detection	Gold nanoparticles and carbon-based nanomaterials used to enhance electrochemical response, enabling rapid detection of illegal additives, heavy metals, and biological toxins; supports miniaturization for on-site use.	Li et al., 2020; Gu et al., 2022
2020	Allergens & Emerging Adulterants	Electrochemical affinity biosensing for identifying emerging food allergens and new adulterants, improving food quality control and safety monitoring.	Campuzano et al., 2020

2.9.8 Challenges and Future Directions

While electrochemical immunosensors hold significant promise, there are still challenges in their widespread implementation. Issues such as sensor fouling, matrix interference, and lack of standardization that remain as major obstacles that must be addressed for routine food monitoring (Hardianto et al., 2023). Moreover, while AuNPs offer excellent performance, their synthesis and functionalization require precise control over particle size and surface chemistry (Ghobashy et al., 2024). Thus, continuous advancements in nanoparticle synthesis techniques and sensor design are necessary to improve the reproducibility and scalability of these sensors for food safety applications. Research also underscores the need for improved biosensor stability, cost-effective fabrication, and integration into regulatory frameworks to facilitate widespread adoption. Some studies emphasize overcoming nonspecific interactions and enhancing aptamer screening to improve sensor reliability (Thiruvengadam et al., 2024; Rastogi et al., 2021; Tang and Tao, 2023).

Future directions include combining nanotechnology with forensic principles and advanced data analysis to address emerging adulteration threats (Thiruvengadam et al., 2024). Moreover, developing miniaturized sensors capable of real-time food safety monitoring at the point of sale or in remote areas is a promising avenue for future innovation (Nath, 2024). Additionally, emerging trends like wearable sensors and smart food safety devices are likely to play an essential role in the evolution of food adulteration detection technologies (Azmi et al., 2024). The future of electrochemical

immunosensors lies in developing multi-analyte sensors that simultaneously detect multiple food fraud markers. Additionally, integrating machine learning algorithms with electrochemical immunosensors could enable automated analysis, improving detection speed and accuracy in food testing environments (Mishra et al., 2020).

2.10 Key Summary

The literature review highlights the challenges of detecting CBP adulteration in surimi-based products, with a focus on the functional properties of CBP and its impact when incorporated into surimi products. Based on table 2.2, the summary of literature evidence highlight the current detection methods, such as ELISA, PCR, and mass spectrometry, are effective but costly and unsuitable for on-site food monitoring. The literature also reveals the potential of electrochemical immunosensors for food adulteration detection, especially incorporating gold nanoparticles (AuNPs), due to their high surface area, biocompatibility, and ability to functionalize with specific biomolecules. However, the integration of AuNPs in sensor platforms to detect CBP adulteration remains underexplored, particularly in the context of surimi-based products. While there is extensive research on sensor design for food adulteration, the development of affordable, user-friendly, and portable sensors that can effectively detect CBP in real-world food matrices is lacking. This gap in the literature highlights the need for foundational research focused on creating sensors that can detect blood plasma components in food products. This study aims to address this gap by developing an electrochemical immunosensor using AuNPs to detect CBP adulteration in surimi, laying the groundwork for more cost-effective, practical food safety monitoring systems.

To build on this foundation, Chapter 3 will detail the research methodology employed to design and develop the electrochemical immunosensor for detecting CBP adulteration in surimi-based products. This includes the detection of potential proteins in CBP, starting with mass spectrometry to identify the target proteins present in CBP for example Apolipoprotein AI and fibrinogen. The chapter will discuss the optimization of protein extraction buffers to enhance the yield and purity of the proteins of interest, followed by the use of immunoprecipitation to isolate these proteins, ensuring specificity in the sensor platform. Once the protein isolation process is optimized, attention will shift to sensor development, focusing on the selection of

materials, sensor design, experimental setup, and analytical techniques necessary to evaluate the performance of the electrochemical immunosensor. This process will demonstrate the integration of AuNPs into the sensor platform, which will enable real-time detection and monitoring of CBP adulteration in surimi-based products.

Table 2.3
Summary of Literature Evidence Highlighting the Need for Protein Biomarker in Surimi Detection Approach

Area reviewed	**Description**	**Authors**
Blood plasma intergration in surimi	CBP improves surimi texture such as solubility, cohesiveness, and whiteness; also inhibit endogenous proteolytic enzymes that otherwise degrade muscle proteins during processing	Ain et al., 2023 ; Rawdkuen et al., 2004; Shen et al., 2019
Detection method (DNA, ELISA)	DNA and ELISA degrade with heat, to lose their native structure, making them less detectable giving poor results in processed foods.	Sahilah et al., 2016 ; Barik et al., 2013
Biomarker research using proteomics	Protein markers like Apo AI can survive processing and be species-specific, offers precision.	Amir et al., 2021; Hardianto et al., 2023 ; Zhao et al., 2024.
Nanotechnology based-sensor	AuNPs increase sensitivity, rapid, reliability, allow portability to detect Apo A1 as breast cancer as biomarker; and also used in detecting other marker in human diseases and low-cost halal testing.	Lim et al., 2019., Low et al., 2014 ; Eldeeb 2020 ; Li et al. 2024; Husna et al., 2023.

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Introduction

This chapter outlines the research methodology used to identify protein biomarkers in CBP and develop an electrochemical immunosensor for detecting CBP adulteration in surimi-based products. The process begins with the preparation of CBP and surimi samples, followed by protein and proteome extraction techniques that are crucial for the identification of potential biomarkers. Mass spectrometry, specifically LC-QTOF and LC-QTRAP**,** is then used to identify these biomarkers. To ensure accuracy and reliability, control samples are carefully prepared and used as reference standards, establishing baseline specifications for comparison.

Following the identification of Apo AI and fibrinogen, the development of an electrochemical immunosensor is undertaken to detect these proteins as indicators of CBP contamination. The sensor utilizes gold nanoparticles (AuNPs) as the transducer material, improving sensitivity and specificity for detecting low concentrations of target proteins. The immunosensor works through antibody-antigen interactions**,** where biomarker-specific antibodies are conjugated to AuNPs for the capture and detection of CBP proteins. Additionally, streptavidin-coated magnetic beads (MBs) are employed as solid-phase supports, enhancing the efficiency of protein capture. The antibodies immobilized on the magnetic beads facilitate the formation of biomarker-antibody complexes, which are then detected by the AuNPs conjugated with detection antibodies. The resulting electrochemical signal is proportional to the level of CBP contamination in surimi products, providing a rapid and cost-effective detection method.

This chapter describes each step in detail, offering a comprehensive framework for the experimental design, analytical strategies, and sensor development techniques applied in this research.

3.2 Materials and Instrumentations

All chemicals, reagents, standards and solvents used in this study were of analytical grade or higher purity and were used without further purification unless otherwise stated. Ultrapure and deionized water were used for the preparation of all aqueous solutions.

Protein extraction employed ultrapure water, urea, 0.05 M Tris–HCl and cold acetone. Surimi preparation involved sodium chloride (NaCl), sorbitol, sucrose and sodium tripolyphosphate. Total protein concentration was determined using the Bradford assay (Bio-Rad, USA) with bovine serum albumin (BSA) as the calibration standard.

Proteomic digestion utilized Trypsin Gold, Mass Spectrometry Grade (Promega, USA), dithiothreitol (DTT), iodoacetamide (IAA), formic acid and acetonitrile (ACN).

SDS–PAGE reagents comprised acrylamide/bis-acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED), Tris buffers, Laemmli sample buffer and Coomassie Brilliant Blue (Sigma-Aldrich, USA).

Gold nanoparticles were synthesized using chloroauric acid ($HAuCl_4$) and sodium citrate. Electrochemical detection employed hydrobromic acid (HBr) and bromine (Br_2). For electrochemical immunosensor development and performance evaluation, immunoreagents included Dynabeads® Streptavidin (Cat. No. 65801D, Thermo Fisher Scientific, USA) and DynaMag™-Spin magnet (Thermo Fisher Scientific, USA). Primary antibodies comprised rabbit anti-fibrinogen antibody (Cat. No. ab34269, Abcam, UK) and rabbit anti-apolipoprotein AI antibody (Cat. No. ab227455, Abcam, UK). Biotinylated detection antibodies included biotin anti-fibrinogen antibody (Cat. No. ab51416, Abcam, UK) and biotin anti-apolipoprotein AI antibody (Cat. No. ab48329, Abcam, UK).

Instrumentation included refrigerated centrifuges; homogenizer and vortex mixer; spectrophotometer for protein quantification and UV–Vis analysis; HPLC systems coupled to LC–QTRAP–MS and LC–QTOF–MS (Agilent 6520 Q-TOF LC/MS, USA); transmission electron microscope (JEOL JEM-2100, Japan); and a Metrohm Autolab PGSTAT128N potentiostat/galvanostat (Metrohm, Netherlands) with screen-printed carbon electrodes for differential pulse anodic stripping voltammetry (DPASV) measurements.

Detailed operational procedures and analytical parameters are described in the subsequent subsections.

3.3 CBP Collection

Live chickens were slaughtered at a commercial slaughterhouse (Heezrad Ayam Segar, Dengkil). The location is located at Salak Tinggi, Sepang Selangor (2.78817, 101.75187). The collected blood samples were pooled into plasma tubes containing sodium citrate as an anticoagulant to prevent coagulation. To isolate the plasma, the blood samples were centrifuged at 1500× g for 15 minutes at 4°C. This centrifugation process was repeated twice to ensure the removal of blood cells and obtain a clear plasma supernatant. The resulting CBP (Figure 3.1) was stored at -35°C until further analysis, as outlined by Hou et al. (2019)



Figure 3.1 Blood Tube Containing Blood Plasma After Centrifugation, Prepared for Storage at -20ºC

3.4 Protein Extraction and Concentration Determination

Approximately 1 mL of CBP was homogenized in 1 mL of ultrapure water and the mixture was then subjected to centrifugation at 12,000 × g for 10 minutes at 4°C, and the resulting supernatant was collected for further processing.

Protein precipitation was performed by adding 100 µL of the supernatant to 400 µL of cold acetone (pre-chilled at -20°C for at least 1 hour). The mixture was vortexed and incubated overnight at -20°C. After incubation, the samples were centrifuged again at 12,000 × g for 10 minutes at 4°C. The resulting pellet was air-dried and resolubilized in 100 µL of ultrapure urea water, followed by centrifugation to remove any residual debris. The collected protein extract was stored at -20°C until further use.

To determine the total extractable protein concentration, the Bradford assay (Bradford, 1976) was performed following the Bio-Rad protocol (Bio-Rad, Hercules USA). The protein concentration was measured in triplicate for each sample, ensuring accurate and reliable results.

3.5 Proteome Extraction for Mass Spectrometry Analysis

3.5.1 Preparation of Protein Extracts from CBP

Four extraction buffers were evaluated with modifications: (1) ultrapure water (Gordeeva et al., 2023), (2) 0.05 M Tris-hydrochloric acid (Tris-HCl), (3) 0.05 M Tris-urea (Tris-Urea) (Bose et al., 2021), and (4) ultrapure water followed by cold acetone precipitation (Baghalabadi & Doucette, 2020). Extraction was carried out by applying 1 mL of extraction solvent to 500 µL of CBP and vortexing for 10 minutes to ensure uniformity. Afterward, the mixtures were centrifuged at 10,000 × g for 30 minutes at 4 °C (Ain et al., 2023). For solvent (4), the mixture was combined with 4 mL of cold acetone and incubated overnight at -20 °C. Two phases were formed, and the pellet was collected and reconstituted in 1 mL ultrapure water. The extract was stored at -20 °C until further analysis. Protein concentrations were measured using the Bradford protein assay, with the extraction process performed in triplicate.

3.5.2 Preparation of Surimi Samples

To minimize variation, surimi samples were sourced from one of the largest surimi manufacturers in Malaysia, QL Foods Sdn. Bhd. located in Teluk Intan (3.874727, 100.9396336), Perak Darul Ridzuan. The surimi was thawed at 4°C for 4 hours, minced, and then mixed with CBP, sorbitol, 2% w/w NaCl, 4% w/w sucrose, and

0.3% sodium tripolyphosphate (Ain et al., 2023). The moisture content was adjusted to 80–85% using crushed ice to ensure a consistent and homogeneous slurry, as described by Fowler and Park (2015). The surimi mixture was portioned into 2 cm × 2 cm × 2 cm ice cube molds, carefully wrapped, and frozen at -18°C for a minimum of 12 hours. After freezing, the surimi samples were boiled at 90°C for 20 minutes, cooled to room temperature at 23°C, and then prepared for subsequent protein analysis. Temperature control and processing criteria were rigorously maintained to ensure reproducibility and accuracy in protein marker identification (Moosavi-Nasab et al., 2019).

3.5.3 Protein Digestion

All samples, including CBP, surimi, and Bovine Serum Albumin (BSA), were digested according to the manufacturer's instructions outlined in the analysis certificate for Promega Trypsin Gold, Mass Spectrometry Grade (Part No. V5280). Digestion was performed by adding 4 µL of 1 g/L Trypsin Gold in 50 mM acetic acid at 37 °C for 24 hours. The samples were reduced with 100 mM DTT for 3 hours at 37 °C and alkylated using iodoacetamide (IAA) in the dark for an additional 3 hours at room temperature. The digestion was terminated with the addition of 1 µL of formic acid, and each sample was processed in triplicate.

3.5.4 LC-QTRAP-MS Acquisition

CBP samples extracted using four types of solvents were analyzed by LC-QTRAP-MS following the methodology described by Amir Hamzah et al. (2024). Chromatographic separation and tandem mass spectrometry detection were carried out using HPLC. Samples were injected at 45 °C with a flow rate of 300 µL/min over 25 minutes onto a Phenomenex Kinetex Core-shell C18 reversed-phase column (Torrance, CA; 100 × 2.1 mm, 2.6 µm, 100 Å). Mobile phases A and B consisted of 0.1% formic acid (FA) and 100% acetonitrile (ACN), respectively. The column was equilibrated to ten times its volume before sample injection. The mobile phase composition started at 97% A for 1 minute and gradually decreased to 71.6% A over 22 minutes. Eluent B increased after 22 minutes and was maintained at 0% from 28 to 29 minutes. The concentration of mobile phase A was returned to 97% at 29 minutes and sustained until 35 minutes. 10 µL of each sample was injected into the system in triplicate.

3.5.5 LC-QTOF-MS Acquisition

The method followed adjustments based on prior studies (Yuswan et al., 2018). Chromatographic separation of all samples (CBP, surimi) was carried out 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 with a flow rate of 300 µL/min for 25 minutes. Separation was achieved using gradient elution, with eluent A being ultrapure water with 1% formic acid, and eluent B being acetonitrile. Eluent B started at 3% for 1 minute, increased gradually to 98% for 20 minutes, and remained constant for 1 minute before returning to the original conditions for 3 minutes. Samples separated via chromatography were analyzed using the Agilent 6520 Q-TOF LC/MS. The Dual ESI ionization source was set for electrospray ionization with parameters as follows: 325 °C source temperature, gas pressures of 40 psi for gas 1 and 30 psi for gas 2, nebulizer voltage of 5200 V at 40 psi, a declustering potential of 50 V, and a curtain gas pressure of 30 psi. The scan rate was set at 1000 Da/s. Mass spectrometry (MS) and targeted tandem mass spectrometry (MS/MS) were conducted in enhanced mode with a scan range of 200–2000 m/z for both MS and MS/MS. The analysis was performed in positive mode.

3.5.6 Data Analysis

Raw data were processed using the Petunia Trans-Proteomic Pipeline (Deutsch et al., 2023; Yuswan et al., 2018). Chromatographic separation data were processed to include 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 with a retention time tolerance of 0.1 minutes and mass tolerance of 0.5 Da. Peaks with less than 90% confidence in a specific sample set were discarded to avoid false positives, provided the number of peaks exceeded 3000 cps. A reference from Schmidt et al. (2023) focusing on *Gallus gallus* taxonomy was used to compare acquired data with known sequences for proteome analysis and marker detection. A randomized and concatenated SwissProt database (2016.6.9.6 random.concan) containing 60,570 protein entries served as a decoy. Precursor mass tolerance was set to 0.5 Da, with carbamidomethylation of cysteine and oxidation of methionine as fixed and variable modifications, respectively. PeptideProphet™ and ProteinProphet™ were

used to analyze the generated pepXML files, with a false discovery rate (FDR) set at 1%.

3.6 Bioinformatics Approach for Protein Identification and Evolutionary Analysis

In this study, multiple sequence alignment (MSA) was employed to examine the evolutionary relationships of Apo AI and Fibrinogen across chicken (*Gallus gallus*) from CBP, few common fish species representing surimi namely Common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar*), Rainbow trout (*Oncorhynchus mykiss),* Brown trout (*Salmo trutta*), Southern catfish (*Silurus meridionalis*) and Alaska pollock (*Gadus chalcogrammus*), and various land vertebrate mammalian species pig (*Sus scrofa),* goat (*Capra hircus*), cattle (*Bos taurus),* horse (*Equus caballus),* and duck (*Anas platyrhynchos*). The goal of this analysis was to identify convergent and divergent regions within the protein sequences, which could potentially serve as biomarkers for detecting CBP adulteration in surimi-based products. The evolutionary conservation of these regions was a key factor in selecting Apo AI and Fibrinogen as candidate biomarkers.

3.6.1 Sequence Alignment Using Clustal Omega

Clustal Omega was used to perform the multiple sequence alignment of Apolipoprotein A1 and Fibrinogen protein sequences. The protein sequences for the analysis were retrieved from the NCBInr and UniprotKB databases, which provided the FASTA sequences of Apo AI and Fibrinogen from Gallus gallus, fish species, and several vertebrate mammalian species (cow, pig, goat, horse, duck). The alignment was conducted with the following settings: the Gonnet matrix was used for scoring the sequence alignment, with a gap open penalty of 10 and a gap extension penalty of 0.2. The alignment used the neighbor-joining (NJ) method for clustering the sequences, and the output format was specified as Clustal Omega per the numbers to ensure compatibility with subsequent analyses.

The alignment was performed in a Singularity containerized environment to ensure that the process was reproducible and standardized across different computational platforms. To optimize processing speed, 8 computational threads were

assigned, and 8 GB of memory were allocated to handle the computational demands of aligning large datasets. Additionally, the alignment generated a guide tree to visualize the evolutionary relationships between the protein sequences, which helped identify convergent and divergent regions crucial for determining biomarker suitability for CBP detection.

3.6.2 Phylogenetic Analysis and Evolutionary Insight

After performing the sequence alignment, a phylogenetic analysis was conducted to assess the evolutionary relationships between Apo AI and Fibrinogen across the species. This analysis revealed the convergent regions of the protein sequences, which showed high sequence similarity across species. These convergent regions were considered to be functionally important and likely contribute to the biological activity of the proteins. Additionally, divergent regions were identified, where the sequences varied significantly across species, which might indicate species-specific adaptations. These findings helped in distinguishing the evolutionary conservation of the proteins and justified their selection as biomarkers for CBP.

3.6.3 Calculation of Percentage Identity Matrix

To further assess the evolutionary conservation of Apolipoprotein A1 and Fibrinogen across species, a percentage identity matrix was generated from the sequence alignment results. The percentage identity matrix quantifies the degree of sequence similarity between the proteins across different species, providing a clearer understanding of their evolutionary relationships. The matrix was calculated by comparing the aligned sequences for Apo AI and Fibrinogen from *Gallus gallus* (chicken), fish species , and several vertebrate mammalian species (cow, pig, goat, horse, duck).

The calculation was performed using the following formula:

$$\text{Percentage Identity} = \frac{\text{Number of Identical Residues}}{\text{Total number of Residues Compared}} x\ 100 \quad (3.1)$$

This approach allowed for the identification of highly conserved regions (where sequence identity was greater than 90%) and regions with variability (where sequence identity was lower). Convergent regions were identified as those with high percentage identity across species, indicating their functional importance. These conserved regions were considered to be strong candidates for biomarker development. The resulting percentage identity matrix provided a visual representation of the evolutionary divergence and convergence of Apo AI and Fibrinogen across species, highlighting the functional regions that are highly conserved and can serve as potential biomarkers for detecting CBP adulteration in surimi-based products.

3.6.4 Data Processing and Interpretation

The alignment data were analyzed to identify key conserved motifs and variable regions in the protein sequences. The regions showing high conservation were considered for further exploration as biomarkers for CBP detection. The sequences were compared to known databases (NCBInr and UniprotKB) to verify the biological significance of the conserved regions. The percentage identity matrix and the identification of convergent and divergent regions allowed for the selection of Apo AI and Fibrinogen as biomarkers for CBP adulteration. The conserved regions, which exhibited high percentage identity across species, were found to be functionally significant and provided strong candidates for biomarker development. These conserved regions were maintained across avian, fish, and mammalian species, supporting their potential use as universal biomarkers for detecting CBP adulteration in surimi-based products.

3.7 Immunoprecipitation and Visualisation of Apo AI and Fibrinogen from CBP using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-Page)

Five milliliters of CBP was as described previously from section 3.2 and 3.3. Plasma proteins were precipitated using an appropriate buffer, such as Phosphate-Buffered Saline (PBS) to minimize contamination, and samples were stored at -80°C for later use. Immunoprecipitation (IP) was performed using Thermo Fisher's Dynabeads M-280 Streptavidin, which allowed specific binding to biotinylated

antibodies targeting Apo AI and fibrinogen. The Dynabeads M-280 Streptavidin were first incubated with biotinylated anti-Apo AI and anti-fibrinogen antibodies for 1 hour at 4°C. The beads were then washed three times with a washing buffer containing PBS and 0.1% Tween-20 to remove any non-specifically bound proteins. CBP samples were incubated with the antibody-conjugated beads for 2 hours at 4°C, allowing the antibodies to bind specifically to the target proteins, Apo AI and fibrinogen. Following incubation, the beads were washed again with the washing buffer to eliminate unbound proteins.

The antibody-bound proteins were then eluted from the magnetic beads using an elution buffer containing SDS and reducing agents for example DTT or β-mercaptoethanol) to disrupt any disulfide bonds and release the target proteins. After elution, the proteins were mixed with SDS-PAGE sample buffer and boiled for 5–10 minutes at 95°C to denature the proteins, unfolding them so they could migrate through the gel according to their molecular weight. The purified proteins were then separated by SDS-PAGE under reducing conditions, and the gel was stained with Coomassie Brilliant Blue or another suitable stain to visualize the protein bands. Negative controls, such as non-biotinylated beads or irrelevant antibodies, were included to assess any non-specific binding. This immunoprecipitation technique allowed for the successful isolation of Apo AI and fibrinogen from CBP, demonstrating the specificity and efficiency of the method (Thermo Fisher Scientific, n.d.). Final step of this section involve in the quantification of total extractable protein concentration, the Bradford assay (Bradford, 1976) was performed following the Bio-Rad protocol (Bio-Rad, Hercules USA). The protein concentration was measured in triplicate for each sample, ensuring accurate and reliable results.

3.8 Development of Electrochemical Immunosensor Using Gold Nanoparticles (AuNPs)

3.8.1 Synthesis of Gold Nanoparticles (AuNPs)

Colloidal gold nanoparticles (AuNPs) were synthesized by reducing HAuCl4 solution with sodium citrate adapted by (Low et al., 2013; Turkevich et al., 1951). A 100 mL working solution of 5 mM HAuCl4 was prepared and added to a flask containing 1800 mL of deionized water. The solution was heated on a hot plate with

continuous magnetic stirring until it began to boil. Upon reaching boiling point, 100 mL of freshly prepared 17 mM sodium citrate solution was rapidly added, and the solution was stirred vigorously. The reaction was maintained at boiling temperature with continued stirring for approximately 3–4 minutes, during which the color of the solution changed to wine red. This color change indicated the formation of AuNPs. The reflux process continued until the wine red color stabilized for 2–3 minutes. The heating was then stopped, and the solution was allowed to cool to room temperature while continuing to stir. Finally, the volume of the colloidal AuNPs was adjusted to 2000 mL with deionized water to compensate for evaporation losses. The resulting colloidal solution was stored in a glass bottle (Duran, Mainz, Germany) at 4°C until further use.

3.8.2 Characterization of AuNPs

The optical density (OD) of the colloidal AuNPs was measured to determine their concentration. The absorbance spectrum was recorded using a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan), with measurements taken from 400 nm to 600 nm at 10 nm intervals. A 400 µL aliquot of colloidal AuNPs was placed into a 10 mm pathlength quartz cuvette (Sigma, Missouri, USA) for analysis. The absorbance spectrum was used to determine the wavelength of maximum absorbance (λmax), which corresponds to the peak absorbance of the AuNPs. This wavelength was subsequently used for all further analysis of the AuNPs' concentration. The concentration of colloidal AuNPs was estimated by measuring the OD at λmax, where changes in absorbance are most sensitive to changes in nanoparticle concentration.

3.8.3 Structural Analysis of AuNPs Using TEM Imaging

3.8.3.1 Sample Preparation for TEM Imaging

For the structural analysis of gold nanoparticles (AuNPs), a small volume of the colloidal AuNP solution was prepared for Transmission Electron Microscopy (TEM) imaging. A 5 µL aliquot of colloidal AuNPs was pipetted onto a carbon-coated copper grid (300 mesh) and allowed to dry at room temperature for 30 minutes to ensure a uniform distribution of nanoparticles on the grid. The grid was then carefully transferred into a TEM sample holder for imaging.

3.8.3.2 TEM Imaging Conditions

The TEM imaging was conducted using a (brand and manufacturer, place city country) JEOL JEM-2100 transmission electron microscope (Tokyo, Japan) equipped with an accelerating voltage of 200 kV. High-resolution images of the AuNPs were captured at varying magnifications ranging from 10,000x to 100,000x to observe the morphology and size distribution of the nanoparticles. The TEM images were analyzed to determine the average particle size, shape, and distribution of the AuNPs. The particle size distribution was calculated by measuring the diameters of randomly selected nanoparticles from the captured images using ImageJ software (National Institutes of Health, Bethesda, USA).

3.8.3.3 Data Analysis

The size distribution of the AuNPs was determined by measuring the diameters of individual nanoparticles from the TEM images. At least 100 nanoparticles were analyzed from multiple images to ensure statistically significant data. The average diameter of the AuNPs and the standard deviation were calculated. The shape of the nanoparticles was classified based on visual inspection, with nanoparticles classified as spherical, rod-like, or irregular. The data were then compared to the theoretical size and shape range expected based on the synthesis conditions.

3.9 Development of Electrochemical Immunosensor

3.9.1 Principle and Instrumentation of DPASV for AuNPs Detection

In this study, differential pulse anodic stripping voltammetry (DPASV) was employed not to detect gold nanoparticles (AuNPs) as intact nanostructures, but to quantify their electrochemical signal arising from the oxidation of metallic gold. The synthesized AuNPs exist initially in the zero-valent metallic state (Au^0). During DPASV analysis, an anodic potential is applied to oxidize Au^0 to Au^{3+}, generating a characteristic oxidation current.

The measured current response corresponds to the amount of electroactive gold present on the electrode surface, which is directly related to the quantity of AuNPs

bound via the antibody–antigen interaction. Thus, AuNPs function as electroactive labels that amplify the analytical signal, while the electrochemical readout is derived from the Au^0/Au^{3+} redox process rather than direct detection of intact nanoparticles.

Electrochemical measurements were performed using an electrochemical workstation equipped with a screen-printed carbon electrode (SPCE) comprising a carbon working electrode, carbon counter electrode, and a silver (Ag) reference electrode. The anodic stripping response of electroactive gold was recorded as a distinct oxidation peak at approximately +0.65 V, which served as the analytical signal. Parameter control and voltammogram analysis were carried out using Nova software version 2.1.

3.9.2 Optimisation and Data Analysis

The DPASV parameters were systematically optimised to obtain maximum peak current with good reproducibility. Key variables included electrodeposition potential (E_{dep}) tested from +0.75 V to −0.95 V, electrodeposition time (t_{dep}) for 150–510 s, and scan rate (0.005–0.25 V/s, adjusted via step potential). Firstly, 10 µL of colloidal AuNPs was dissolved in 100 µL of HBr/Br_2 medium, electrodeposited onto the SPCE, and scanned from 0.0 to +1.0 V. A control potential of +0.50 V yielded negligible response, confirming method reliability. The optimisation was conducted sequentially, beginning with electrodeposition potential(E_{dep}), followed by electrdeposition time(t_{dep}), and finally scan rate adjustment. Peak currents were then analysed, and the most stable and intense signals with low standard deviation were selected as optimal conditions, which were subsequently applied in electrochemical immunosensor development in Section 3.9.3 to 3.9.6.

3.9.3 Bioconjugation of Antibody to Magnetic Beads-Streptavidin (MB-S)

In this study, magnetic beads were used as solid-phase supports for the immunoreactions with protein biomarkers. The platform was prepared by immobilizing biotinylated anti-biomarker capturing antibody (α-BIOMARKER/CAP) onto streptavidin-coupled magnetic beads (MB-S). To achieve this, 20 µg of MB-S was incubated with 8 µL of 0.1 mg/mL α-BIOMARKER/CAP solution in phosphate buffer (PB) containing 0.1% v/v Tween 20 (PB/T20) for 10 minutes. After incubation, the

mixture was washed with PB/T20 for magnetic separation. The resulting α-BIOMARKER/CAP-MB conjugate was stored at 4°C for further use.

3.9.4 Bioconjugation of AuNPs with Anti-Biomarker Detection Antibodies

The AuNPs were conjugated with anti-biomarker detection antibodies (α-BIOMARKER/DET) to specifically label the target antigen. The conjugation process was carried out using conventional adsorption techniques. Briefly, 1 mL of AuNPs (pH 8.0) was mixed with 0.1 mL of 0.1 mg/mL α-BIOMARKER/DET solution in phosphate buffer (PB) under continuous tilting for 45 minutes.

Following conjugation, the α-BIOMARKER/DET–AuNP conjugates were blocked with 1% (w/v) bovine serum albumin (BSA) for 30 minutes to minimize non-specific binding. Washing steps were performed using gentle centrifugation to remove unbound antibodies and excess BSA while maintaining surface adsorption stability. The conjugates were subsequently resuspended in PB containing BSA (PB/BSA) to preserve surface passivation and reduce potential desorption during storage. The final conjugates were stored at 4 °C until further use.

3.9.5 Bioconjugation of AuNPs with Anti-Biomarker Capturing Antibodies

The previously prepared α-BIOMARKER/CAP-MB conjugate was resuspended in 10 µL of variable concentrations of biomarkers in PB/T20 buffer and incubated at room temperature to allow for the formation of immunocomplexes. After incubation, unbound components were removed from the magnetic beads using magnetic separation and washing with PB/T20 buffer.

The washed biomarker-bound MB complexes were then resuspended in 10 µL of α-BIOMARKER/DET-AuNP suspension and incubated at room temperature for 10 minutes. After incubation, the excess unreacted immunocomplexes were washed off using PB/T20. The final AuNP-probed biomarker-bound MB complexes were resuspended in 50 µL of detection medium (1 M HBr/0.15 mM Br2), which dissolves the AuNP probes as aqueous Au3+ ions for subsequent electrochemical detection.

3.9.6 Electrochemical Magneto-Sensing of Apo AI and Fibrinogens

Electrochemical detection of the resulting Au^{3+} ions was performed using differential pulse anodic stripping voltammetry (DPASV) on a screen-printed electrode (SPE). The measured current signal is expected to be quantitatively proportional to the amount of target protein biomarker present in the reaction mixture. The electrochemical analysis was conducted following the schematic representation depicted in Figure 3.2, with the current signal providing a direct measure of the biomarker concentration in surimi samples, thus allowing for the detection of CBP adulteration.

3.10 Performance Comparison of Electrochemical Immunosensors and LC-QTOF Mass Spectrometry in Detecting CBP

This study was designed to evaluate and compare the performance of electrochemical immunosensors and liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) for detecting protein biomarkers in a complex food matrix contaminated with CBP. To simulate realistic levels of food adulteration, liquid CBP was spiked into the surimi matrix at concentrations of 0.50%, 1.00%, and 1.50% (v/w), defined as the volume of CBP added per weight of surimi, reflecting the experimental procedure in which liquid plasma was added by volume to a weighed surimi matrix. These spiking levels were selected to reflect practical adulteration scenarios that may occur during surimi production.

The primary target proteins for detection were Apo AI and fibrinogen, which serve as relevant indicators of CBP contamination in processed food products. LC-QTOF-MS was employed as the reference analytical technique for protein identification and served as a benchmark for assessing the detection performance of the developed electrochemical immunosensors.

For LC-QTOF-MS analysis, proteins were extracted from the CBP-spiked samples using standard extraction procedures as described in Section 3.3.4. Subsequent protein identification and analysis of Apo AI and fibrinogen were carried out following the LC-QTOF-MS acquisition and data processing protocols outlined in Section 3.4.5. Detection sensitivity across the different CBP spiking levels (0.50%, 1.00%, and 1.50% v/w) was evaluated based on sequence coverage and the number of matched peptides obtained for each target protein.

The electrochemical immunosensors specific for Apo AI and fibrinogen were prepared and calibrated prior to testing. The sensors were incubated with the CBP-spiked samples, and voltammetric responses were measured at each concentration of CBP (0.50%, 1.00%, and 1.50%). The voltage signals generated by the immunosensors were compared to pre-determined cutoff values to assess positive or negative detection. The voltammetric signals for both proteins were compared against these cutoff values to determine detection accuracy. To evaluate the repeatability and reliability of the immunosensor responses, the relative standard deviation (RSD) was calculated across the different concentrations. Additionally, a linear regression analysis was performed to evaluate the linearity of sensor responses for both proteins, and the R^2 value was calculated to assess the strength and reliability of the correlation between protein concentration and sensor output.

3.11 Performance of Electrochemical Immunosensors for commercialised surimi in Detecting Apo AI and Fibrinogen

The electrochemical immunosensors specific for Apo AI and fibrinogen were prepared and calibrated prior to testing. The sensors were then incubated with commercial surimi products sourced from multiple countries, including Malaysia, Thailand, Japan, and China, comprising a mixture of samples both with and without Halal certification as listed in Table 3.1. Voltammetric responses were measured for each sample, and the resulting current signals were evaluated against predetermined cutoff values to determine the presence or absence of target blood proteins. The voltammetric signals obtained from each surimi sample were compared against these cutoff thresholds to assess detection accuracy. To evaluate the consistency and reliability of the immunosensor responses across the international surimi panel, the relative standard deviation (RSD) was calculated.

Table 3.1
List of Samples of Commercialised Surimi

Sample	Type of surimi product	Manufacturer Origin	Gelling agent	Halal certified
COM1	Crab ball	Malaysia	Modified tapioca starch, corn starch and soy protein isolate	Yes
COM2	Fish ball	China	Modified starch and egg white	Yes
COM3	Crab ball	Malaysia	Modified tapioca starch, soy protein and egg white	Yes
COM4	Seafood ball	Malaysia	Egg white	Yes
COM5	Fish filament	Japan	Tapioca starch and albumen (egg)	Yes
COM6	Fish ball	Japan	Soy protein isolate, starch and egg albumen	Yes
COM7	Fish ball	Thailand	Egg white and tapioca starch	Yes
COM8	Fish cake	Malaysia	Egg white and starch	No
COM9	Imitation crab claw	Thailand	Corn starch, egg albumen and distarch phosphate (thickener: E1412)	No
COM10	Fish ball	Thailand	Corn starch	No
COM11	Fish ball	Japan	Corn starch	No
COM12	Fried fish fu zhu	Japan	Wheat flour and tapioca starch	No

3.12 Overall Experimental Flow

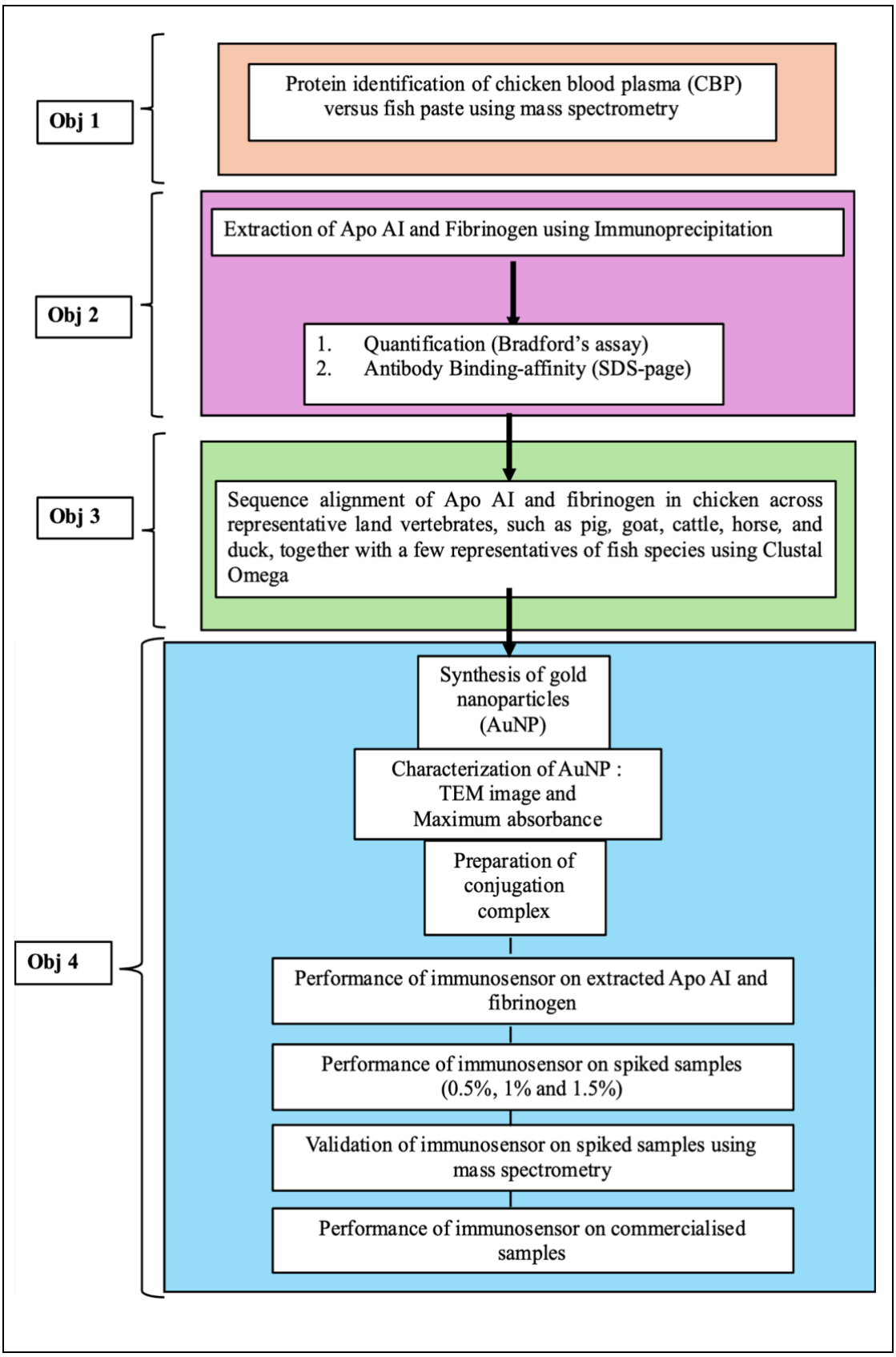


Figure 3.2 Overall Experimental Flow

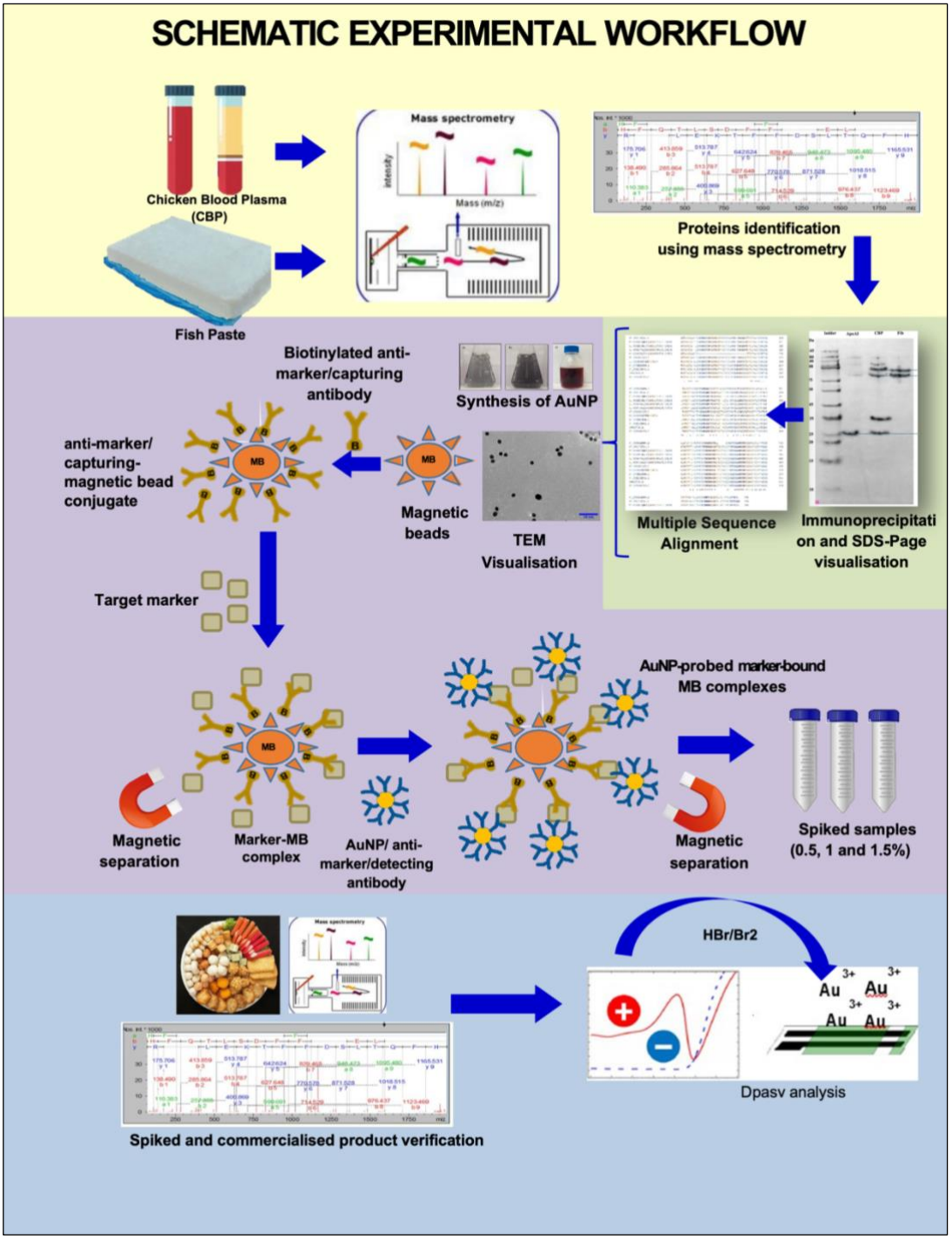


Figure 3.3 Schematic Representation of Overall Flow of Experimentation

CHAPTER 4
RESULT AND DISCUSSION

4.1 Introduction

This chapter presents started with the results and discussion from a study focused on identifying reliable protein markers for the detection of CBP in processed food matrices, specifically in fish paste products. While the incorporation of CBP offers functional and economic advantages such as improved texture and reduced production waste as its use raises significant concerns regarding food authenticity, religious dietary compliance, and consumer safety. Existing detection methods, including immunochemical and DNA-based assays, often fall short when applied to heat-treated or multi-ingredient food systems, primarily due to protein denaturation and DNA degradation. Furthermore, the lack of a standardized proteome extraction protocol poses additional challenges for accurate identification. To address these limitations, this study employs a proteomics-based strategy using mass spectrometry (MS) combined with bioinformatics analysis. Two candidate proteins namely Apo AI (ApoA-I) and fibrinogen were selected based on their abundance and potential for species-specific discrimination from the fish muscles. The study also optimises extraction protocols to maximise detection efficiency. Through sequence alignment and percent identity analysis, this research determines the uniqueness of chicken-specific peptide markers in comparison to other land vertebrates. Ultimately, these findings aim to strengthen consumer protection frameworks, support compliance with dietary and food safety standards, and contribute toward the development of more robust, label-free detection methods for animal-derived ingredients in complex food systems.

4.1.1 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 4.1). 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 Bose et al. (2021), 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. Appendix I 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.

Table 4.1
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	797	675
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

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 (Appendix I), 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 (Bose et al., 2022). Analysis of the TICs for ultrapure water and ultrapure with acetone (Appendix) I 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 mass spectrometry. 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 (Jiang et al., 2024). 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 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 (Sun et al., 2014). 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 (Jiang et al., 2024). 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 mass spectrometry is hindered by residual urea, which suppresses ionization efficiency and interferes with enzymatic digestion during trypsinization (Jiang et al., 2024). 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 mass spectrometry-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 mass spectrometry 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 (Khalid et al., 2024) 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 (Zhang et al., 2021). 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 (Zhang et al., 2021). 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 mass spectrometry (Nickerson & Doucette, 2020). 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*) (Baghalabadi & Doucette, 2020; Nickerson & Doucette, 2020). The protein pellet obtained through acetone precipitation is particularly well-suited for downstream applications, including mass spectrometry analysis (Bunk & Welch, 1997).

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 mass spectrometer 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 (Rochat,

2019), 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.

In this study, the Trans-Proteomic Pipeline (TPP) was applied to process and analyze LC-QTOF-MS proteomic data obtained from CBP and CBP-spiked samples under different extraction conditions. The use of PeptideProphet and ProteinProphet enabled high-confidence peptide and protein identification while maintaining controlled false discovery rates (FDR), which was essential for reliable comparison across complex sample matrices.

The results demonstrated clear differences in protein recovery across extraction methods. Samples extracted using ultrapure water followed by cold acetone precipitation yielded the highest number of identified peptides (583) and proteins (537), indicating superior extraction efficiency for downstream proteomic analysis. In contrast, extraction using 0.05 M Tris-HCl and 0.05 M Tris–urea resulted in lower protein identifications (133 and 340 proteins, respectively). These findings highlight the effectiveness of the applied proteomic workflow and demonstrate TPP's role in enabling sensitive discrimination of protein yields across different extraction strategies.

Furthermore, the Control (BSA) sample, which is a standard reference, yielded 573 peptides and 513 proteins. This consistency in protein identification across experimental conditions supports the reliability of TPP's statistical framework and its ability to maintain high accuracy across both complex experimental samples and controls. TPP's flexibility in processing large datasets using label-free quantification and its ability to scale across different computing environments (such as personal computers, Linux clusters, and cloud platforms) made it ideal for our study's high-throughput proteomics needs. The platform's ability to identify and quantify proteins with varying abundance, even in complex food matrices, made it an indispensable tool for ensuring the robustness and reproducibility of our results.

On the question of the reliability of the extraction method performed on the MS system, its reliability is demonstrated by quality control (QC) using BSA. BSA is frequently employed as a benchmark for quality assurance in mass spectrometry analysis due to its numerous advantages. Its exceptional sequence coverage, usually above 85%, establishes it as a dependable benchmark for evaluating the efficacy of mass spectrometry techniques (Bittremieux et al., 2017). 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 serves as a valuable tool for QC in mass spectrometry analysis, due to its well-established properties and efficacy across various applications.

4.1.2 Identification of Proteins in CBP

The sample was then analyzed 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 (Witjaksono & Alva, 2019). 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 Table 4.2 and 4.3 concurrently. The analysis of the TIC (Appendix I) 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.

Table 4.2
List of Proteins Detected in CBP

No	Identified protein	Accession no.*	Mass (kDa)	Peptide-spectrum matches (PSM)	Sequence coverage (%)
1.	Apolipoprotein A-I	P08250	30.662	104	82.2
2.	Fibrinogen alpha chain	P14448	82.338	42	30.6
3.	Ovotransferrin	P02789	77.727	37	27
4.	Fibrinogen C-terminal domain-cocontaining protein	E1BV78	26.2283	29	33.33
5.	Uncharacterized protein	F1NK40	163.237	24	10.1
6.	Uncharacterized protein	A0A1D5P9F9	198.853	7	3.5
7.	Fibrinogen beta	Q02020	52.646	6	6.7
8.	Albumin	P19121	69.873	3	3.4
9.	Uncharacterized protein	A0A1L1S0P1	50.816	2	2.3
10.	CN hydrolase domain-containing protein	A0A1D5PEU7	54.630	1	9.1
11.	Uncharacterized protein	A0A1D5NXA6	72.456	1	2.5
12.	SMB domain-containing protein	E1C7A7	51.619	1	2
13.	Uncharacterized protein	R4GJL7	29.904	1	7.9

No	Identified protein	Accession no.*	Mass (kDa)	Peptide-spectrum matches (PSM)	Sequence coverage (%)
14.	Uncharacterized protein	A0A3Q2TYI8	197.986	1	2.5
15.	Uncharacterized protein	F1N8L3	35.795	1	3.2
16.	Uncharacterized protein	A0A3Q2UA24	116.315	1	0.9
17.	Uncharacterized protein	A0A1D5P640	63.641	1	1.2
18.	DNA-directed RNA polymerase III subunit RPC3	A0A3Q2U0G3	41.604	1	4.6

Table 4.3
List of Proteins Detected in Surimi

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
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)

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 (Yuswan et al., 2018). Eighteen proteins in total were found in CBP samples (Table 4.2). 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 mass spectrometry 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 and metabolism (Tanaka et al., 2020). It is present in the plasma of 95% of individuals (Khirfan et al., 2021) and is composed of stable α-helices associated with HDL.

In this finding, 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 (Puliasis et al., 2023). A higher sequence coverage percentage signifies a more comprehensive identification of the protein's amino acid sequence (Shao et al., 2022). 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 (Pietrowska et al., 2019).

4.1.3 Identification of Proteins in Surimi Sample

The surimi sample was made from 100% fish meat and was also used as the control group in spiked samples later in section 4.9.2 , accordingly, because they did not incorporate any additives or functional preparations. The purpose of this analysis is to make sure that there are no overlapped protein between blood plasma and fish muscle. As a result, 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 4.3). 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) information website (Sampson, 2024) that fish protein could be divided into a few categories, including myofibrillar proteins for example 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 (Erbay & Yesilsu, 2021).

Although fish muscle contains residual blood components, no fish-derived blood proteins were detected during the screening analysis. 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 (ref). 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. (2019), 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.

The comparison between the proteins detected in CBP and fish paste reveals no redundancy on the proteins, demonstrating the specificity of the protein sources in each sample. Apo AI was identified exclusively in the CBP, confirming its role as a plasma-specific protein. Apolipoproteins are primarily synthesized in the liver and intestines, as observed in both mammals and birds. In chickens, significant levels of apolipoprotein B (apoB) mRNA are present in the liver and small intestine, reinforcing their roles as key sites of synthesis (Glick & Leveille, 1996; Leveille & Glick, 1980, 1993). This synthesis is essential for lipid transport within the bloodstream, as apolipoproteins are vital components of plasma lipoproteins that mediate lipid metabolism. Consequently,

the presence of apolipoproteins in CBP is consistent with their physiological role in lipid transport. Chicken plasma contains major apolipoproteins, such as Apo AI and Apo B, which are functionally similar to those found in mammals. These apolipoproteins are synthesized in the chicken's liver and intestines, rather than being derived from external sources such as dietary fish (Hermier, 2009; National Center for Biotechnology Information, n.d.; Silva & Oliveira, 2009).

In contrast, Apo AI was absent in the surimi samples. This absence can be attributed to the significantly lower blood volume in fish, which ranges from 1.5–6% of their total body weight, depending on the species. For example, research indicates that blood volume in salmonids is approximately 5 ml/100g body weight (Smith, 1966). With such a small proportion of blood, the concentration of plasma proteins, including Apo, is comparatively low, making it unlikely that sufficient levels of Apo would be detected in the fish paste sample. While there is a possibility that Apo could be present in the muscle tissue of fish, the mass spectrometry analysis did not detect it. This is likely due to the low concentration of Apo in muscle tissue compared to plasma, and the detection limits of the mass spectrometry method being optimized for plasma proteins. Thus, even if Apo were present in small amounts in the muscle, it may not have been detectable in the fish paste sample.

Tables 4.2 and 4.3 present the proteomic profiles of plasma and muscle samples, respectively, as determined by MS. The distinction between the two datasets is clear with Table 4.2 predominantly features plasma-derived proteins, whereas Table 4.3 is enriched with structural and contractile proteins typical of muscle tissue. Among the plasma proteins, Apo AI and fibrinogen alpha chain are highly abundant, as evidenced by their high peptide-spectrum matches (PSMs) and notable sequence coverage. Apo AI, with 104 PSMs and 82.2% coverage, is the most confidently identified protein in plasma. This is biologically consistent, as Apo AI is a major component of high-density lipoproteins (HDL) in plasma and is not synthesized in muscle tissue. While there is theoretical potential for Apo AI to associate with muscle under certain pathophysiological conditions for example injury or inflammation), our results show no detectable levels in the muscle proteome (Table 4.3), suggesting its absence or presence below the detection limit of the method used.

Similarly, fibrinogen alpha and beta chains, components of the clotting cascade, were detected exclusively in the plasma sample, reinforcing the conclusion that these are circulating plasma proteins (Vilar et al., 2020). Their roles are tightly regulated and

confined to vascular and hemostatic systems, making their absence in muscle biologically expected (MedlinePlus, n.d.). Fibrinogen is a plasma protein present in relatively high concentrations, typically ranging from 1.5 to 4 g/L in healthy individuals (Vilar et al., 2020). It is primarily synthesized in the liver and plays a critical role in the vascular system, making it absent in muscle tissue. A key factor influencing its detection in fish is the limited blood volume, which inherently restricts the total yield of plasma proteins. This constraint is particularly important when considering the comparatively small blood volume in fish, which significantly reduces the availability of fibrinogen and other plasma proteins in the muscle tissue (Vilar et al., 2020). Both of the blood proteins has consistent detectionCBP samples suggests that they are suitable candidates for biomarker development. Apo AI, a lipid transport protein, is recognized for its stability and high plasma concentration, while fibrinogen plays a critical role in coagulation processes. These biochemical properties make both proteins traceable and reliable indicators of CBP presence in complex food matrices. Literature indicates that fish blood volume is approximately 3–7% of total body weight (Houston, 1990) considerably less than in mammals. This low volume may contribute to lower overall protein abundance, particularly for trace plasma proteins, which could partly explain the reasons only abundant plasma proteins such as Apo AI and fibrinogen are detected, while others remain undetected.

In the existing literature, Apo AI and fibrinogen have been extensively characterized as clinical and diagnostic biomarkers, particularly in cardiovascular, metabolic, and inflammatory disorders, as detailed in Sections 2.4.1 and 2.4.2. Their detectability, high abundance, and functional relevance in plasma are therefore well established. Despite this, their exploitation as authenticity markers in food systems has been overlooked, creating an opportunity to extend their utility beyond the biomedical domain. The present findings address this gap by demonstrating their potential as novel indicators for halal food authentication, thereby establishing a conceptual link between proteomics-driven biomarker discovery and food integrity monitoring. While Apo AI and fibrinogen are readily detectable and consistently abundant in CBP, they are not unique to chicken plasma, which necessitates careful contextualization of their specificity, particularly in differentiating adulteration from the intrinsic protein background of fish-based matrices such as surimi. Nevertheless, the proteomic evidence generated in this study provides compelling support for their suitability as candidate biomarkers for halal food assurance, while also highlighting the need for further

validation across a broader range of food systems and under rigorously controlled industrial processing conditions.

By contrast, the proteomic profile of muscle samples (Table 4.3) was dominated by muscle-specific proteins including myosin heavy chains, actin, and tropomyosin, all of which are integral to contraction and structural stability. The consistent detection of multiple myosin heavy chain isoforms with high peptide spectrum match (PSM) values confirmed both the muscle origin and the analytical integrity of the dataset. Critically, no plasma proteins such as Apo AI or fibrinogen were observed in the muscle proteome, effectively excluding sample contamination or leakage from systemic circulation. This compartmentalized distribution between plasma and muscle proteins reinforces the biological specificity of Apo AI and fibrinogen to CBP. Moreover, this observation is corroborated by sequence alignment analyses (Sections 4.2 and 4.3) and further validated through electrochemical immunosensor detection (Section 4.8.1), together providing convergent lines of evidence that substantiate their role as reliable indicators of CBP adulteration in surimi-based products and affirming their translational potential in halal authentication frameworks

4.2 Extraction of Apo AI and Fibrinogen using Immunoprecipitation

This section presents the findings from the extraction and isolation of Apo AI and fibrinogen from CBP using Immunoprecipitation (IP) techniques. The objective is to evaluate the efficiency and reliability of antibody-based protein isolation in selectively capturing these target markers from complex biological matrices. Notably, the visualization of protein bands through Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) confirms the successful extraction of the proteins of interest. These findings are essential in establishing a foundation for subsequent bioinformatics analysis, which aims to explore the sequence specificity of the extracted proteins and their potential role as discriminatory markers in food systems.

4.2.1 Quantification of Immunoprecipitated Protein

As summarized in Table 4.1, the extraction yield of protein from CBP samples was quantified using the Bradford assay. The results reveal that raw CBP samples

yielded the highest concentrations of protein, with Apo AI at 0.7202 ± 0.031 mg/ml and fibrinogen at 0.3401 ± 0.027 mg/ml. This inverse relationship highlights the impact of protein solubility on extractability. It is important to note that IP was performed using Thermo Fisher's Dynabeads M-280 Streptavidin, which specifically binds biotinylated antibodies targeting Apo AI and fibrinogen. Magnetic beads-streptavidin 280 were incubated with biotinylated anti-Apo AI and anti-fibrinogen antibodies to allow efficient binding and recovery of the target proteins. The eluted samples from the IP were then quantified to assess the concentration of both Apo AI and fibrinogen.

Table 4.4
Extraction Yield of Protein (mg/ml) from the CBP Measured by Bradford Assay

	protein concentration (mg/ml)	
Proteins	**Apo AI**	**Fibrinogen**
	0.7202 ± 0.031	0.3401 ± 0.027

*The value indicates means (of samples n=10). No statistical analysis was made to show that the protein reduction is significant in the treatment.
* A constant volume of CBP, approximately 3ml, was used for the protein extraction process.

The results indicate that the IP method successfully isolated these proteins, with the concentration of Apo AI being higher compared to fibrinogen across the CBP samples. This highlights the effectiveness of the IP technique in enriching and isolating target proteins, even from complex biological samples like CBP. After elution, the protein samples proceeded to be utilized in the next section for the development of the electrochemical immunosensor. The Apo AI and fibrinogen proteins, which were isolated and purified, served as the primary targets for the sensor's recognition elements. The eluted samples were incorporated into the sensor development process, where their interaction with the sensor surface and antibody binding was carefully optimized to enhance the sensor's sensitivity and selectivity. This progression from protein isolation to sensor integration highlights the seamless workflow from IP to electrochemical detection. This demonstrates the sensor's potential for rapid protein detection in complex matrices like CBP.

These results emphasize the need for optimized protocols when isolating proteins from CBP or similar biological samples, as differences in the proteins' properties can affect how well they are extracted. The Bradford assay results also report that the efficiency of protein extraction depends on how well the proteins dissolve in the solution, indicating the need for better methods in protein quantification.

4.2.2 Molecular Weight Determination using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This section presents the results from the SDS-PAGE confirming the antibody-based extraction of Apo AI and fibrinogen from CBP via IP. *Figure 4.2* displays banding patterns for purified Apo AI (Lane 1), CBP (Lane 2), and purified fibrinogen (Lane 3), enabling assessment of target protein integrity and antibody-binding specificity. Accordingly, three prominent bands were observed: band A at approximately 30 kDa, band B approximately near 60 kDa, and band C around 75 kDa. Band A corresponds to Apo AI, consistent with its reported molecular weight of ~28 to 32 kDa, confirming successful isolation in Lane Apo AI extraction. The presence of this band, albeit less intense in pure further validates endogenous expression and supports the specificity of antibody capture.

The identification of Apo AI as a potential marker is supported by its well-documented role as a major component of High-Density Lipoprotein (HDL) and its species-specific structural characteristics. Notably, Apo AI has been extensively studied for its stability and detectability in plasma-based proteomics workflows, even under challenging sample conditions such as thermal processing or complex matrices (Huang et al., 2011; Torres-Romero et al., 2021). Studies have also highlighted the discriminatory capacity of Apo AI in differentiating animal species in meat and blood-based food products (Lecrenier et al., 2016). In essence, its relatively small size (~28 kDa) and solubility further enhance its extraction efficiency and electrophoretic resolution, making it a reliable target for both IP and downstream sequence analysis.

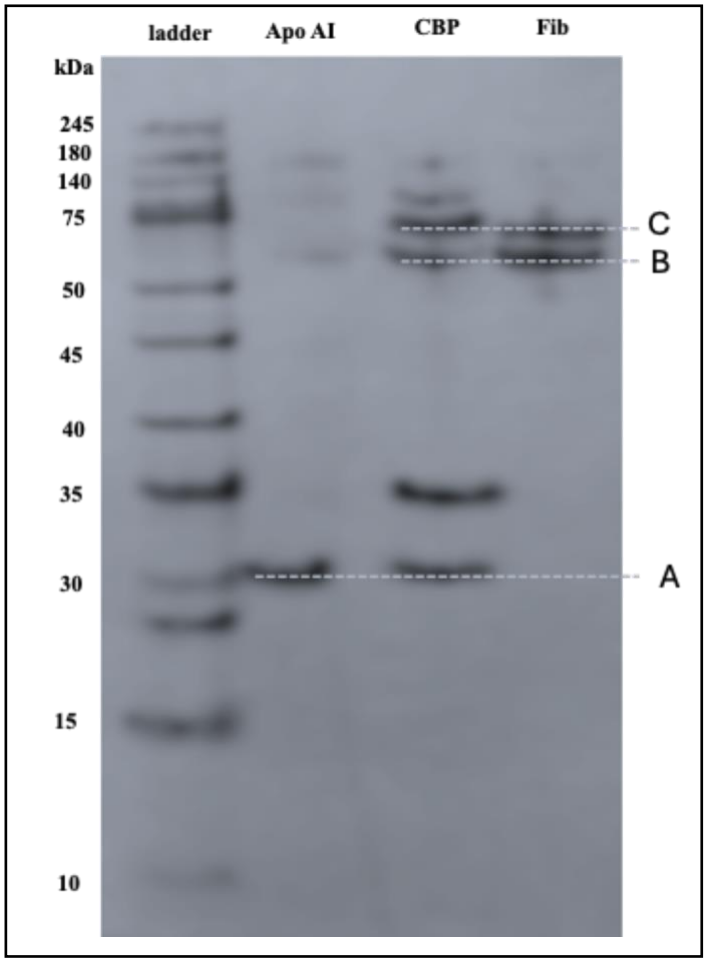


Figure 4.1 SDS Page analysis of Immunoprecipitated Samples; Denoted by Lane Apo AI, Lane pure CBP and Lane Fibrinogens

Band B and C in lane fibrinogen represent fibrinogen subunits. Under reducing conditions, SDS-PAGE disrupts quaternary and tertiary structures, separating individual polypeptide chains. The reducing agents, such as β-mercaptoethanol or Dithiothreitol (DTT), cleave disulfide bonds between chains, allowing Aα (60 kDa), Bβ (~52 kDa), and γ (~49.5 kDa) chains to migrate independently (Doolittle, 2021; Kushnir et al., 2020). This explains the multiple bands between ~45 to 100 kDa, representing fibrinogen's subunit architecture rather than its intact 340 kDa structure, which would only be observed under native gel electrophoresis (Roy & Kumar, 2014; Wang et al., 2023).

The appearance of discrete bands for both proteins confirms antibody binding and successful IP. However, faint smearing and background noise were observed in the CBP lane. Such conditions are commonly attributed to protein degradation, partial proteolysis, or residual high-abundance on the plasma proteins such as albumin or globulins that may obscure or distort target signals. In addition, incomplete

denaturation, inefficient elution, or suboptimal buffer conditions may contribute to these observations (Kurien & Scofield, 2012). Despite these minor anomalies, the immunoprecipitated lanes (Lane 1 and 3) remained well-resolved and largely contamination-free, underscoring the reliability and reproducibility of the protocol.

Following this confirmation of target protein extraction, the next analysis phase involved sequence-based evaluation using bioinformatics tools. This approach aimed to assess the universality and divergence of Apo AI and fibrinogen at the amino acid level across multiple species. By performing multiple sequence alignments and percent identity matrix analysis, this study explores their potential as universal markers, particularly in detecting chicken (*Gallus gallus*) origin in complex food matrices. The following section presents and discusses these *in silico* findings.

4.2.3 Bioinformatics Approaches to Determine Apo AI Marker Universality

The identification of a universal protein marker requires a comprehensive evaluation of its evolutionary conservation across target species and its divergence from unrelated taxa. Apo AI from chicken has been proposed as a potential universal marker due to its functional and structural conservation across terrestrial vertebrates. This study integrates sequence-based analyses with proteomic profiling data obtained from LC-QTOF MS (Section 4.1.2), providing a multifaceted approach to support the candidacy of chicken Apo AI as a universal marker.

To validate this proposition, this study assessed the sequence similarities of chicken Apo AI as candidiate protein marker across representative land vertebrates. This land vertebrates were pig (*Sus scrofa),* goat (*Capra hircus*), cattle (*Bos taurus),* horse (*Equus caballus),* and duck (*Anas platyrhynchos*). Quantitative analysis of amino acid sequence similarity among these species revealed percent identity values ranging from 27.06% to 92.42%, demonstrating strong structural and functional conservation of Apo AI across mammals and birds. At the same time, the high conservation of Apo AI across these species supports its use as a universal marker for lipid metabolism research and food authentication based on blood plasma detection.

Among the terrestrial vertebrates analyzed, duck (92.42%) presented the highest similarity with chicken Apo AI, confirming that Apo AI performs similarly in lipid metabolism across avian species. In contrast, the percent identity values for pigs (51.33%), cattle (50.57%), and horses (49.24%) were notably lower yet still substantial.

This supports the hypothesis that Apo AI from chicken could serve as a reliable marker for cross-species applications within food authentication methods. The 51.33% identity with pig Apo AI particularly bolsters the case for cross-species applications in food authentication. Meanwhile, the 50.57% identity with cattle Apo AI further supports its application in lipid metabolism studies and nutritional science. Even with horse Apo AI indicating a slightly lower identity (49.24%), it remains above the critical threshold of 70%, confirming that Apo AI from chickens can be a reliable marker in equine research.

The 92.42% identity between chicken Apo AI and duck Apo AI is the highest observed, demonstrating almost identical protein sequences between these two avian species. This high degree of conservation suggests that Apo AI performs the same function in both species, particularly in terms of lipid binding. This further reinforces its potential as a universal marker within avian species.

While concerns have been raised about potential interference from fish blood when using chicken Apo AI detection systems in fish species, the low percent identity values observed between chicken Apo AI and various fish species directly address this issue. This includes Alaskan pollock (*Gadus chalcogrammus*), Rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and Carp (*Cyprinus carpio*). Furthermore, the low identity values (ranging from 20% to 30%) confirm evolutionary divergence between avian proteins and those of aquatic vertebrates. This ensures that fish Apo AI will not interfere with chicken Apo AI detection systems. These sequence differences significantly reduce the risk of cross-reactivity and false positives in detection assays, confirming the reliability of chicken Apo AI detection even in fish species.

Table 4.5
Percent Identity Matrix of Apo AI in *Gallus gallus* with Land Vertebrates and Other Fish Species

Species	***G. gallus***	***B. taurus***	***S. scrofa***	***E. caballus***	***C. hircus***	***A. platyrhynchos***	***O. mykiss***	***S. meridionalis***	***S. trutta***	***S. salar***	***G. chalcogrammus***	***C. carpio***
G. gallus	100	50.57	51.33	49.24	27.06	92.42	27.97	27.34	27.2	25.58	25.88	23.08

Table 4.6
Percent Identity Matrix of Fibrinogen Alpha (FGA) in *Gallus gallus* with Land Vertebrates and Other Fish Species

Species	***G. gallus***	***B. taurus***	***S. scrofa***	***E. caballus***	***C. hircus***	***A. platyrhynchos***	***O. mykiss***	***S. meridionalis***	***S. trutta***	***S. salar***	***G. chalcogrammus***	***C. carpio***
G. gallus	100	32.67	16.92	32.15	27.06	83.99	NIL	NIL	NIL	43.34	41.22	25.08

S. trutta, S. meridionalis, O mykiss not available due to not present in database.

Table 4.7
Percent Identity Matrix of Fibrinogen Beta (FGB) in *Gallus gallus* with Land Vertebrates and Other Fish Species

Species	***G. gallus***	***B. taurus***	***S. scrofa***	***E. caballus***	***C. hircus***	***A. platyrhynchos***	***O. mykiss***	***S. meridionalis***	***S. trutta***	***S. salar***	***G. chalcogrammus***	***C. carpio***
G. gallus	100	65.58	67.1	68.91	65.73	29.25	55.6	55.31	55.08	55.29	54.64	52.68

Table 4.8
Percent Identity Matrix of Fibrinogen Gamma (FGG) in *Gallus gallus* with Land Vertebrates and Other Fish Species

Species	***G. gallus***	***B. taurus***	***S. scrofa***	***E. caballus***	***C. hircus***	***A. platyrhynchos***	***O. mykiss***	***S. meridionalis***	***S. trutta***	***G. chalcogrammus***	***C. carpio***
G. gallus	100	63.43	63.45	65.44	63.81	85.22	58.25	54.05	55.31	54.81	40.95

S. salar not available due to not present in database.

In conclusion, the high sequence identity between chicken Apo AI and other terrestrial vertebrates, particularly mammals and birds, strongly supports its use as a universal marker for cross-species research. The 92.42% identity with duck Apo AI and the 51.33% identity with pig Apo AI suggest that chicken Apo AI remains highly functional and structurally conserved across terrestrial vertebrates. This makes it a reliable biomarker for various applications. Additionally, the low percent identity with fish Apo AI significantly reduces the risk of cross-reactivity, ensuring that chicken Apo AI detection is not hindered by fish-specific Apo AI. These findings underscore the species-specificity of chicken Apo AI and emphasize the significance of using appropriate markers based on evolutionary conservation for accurate molecular detection across vertebrate species.

4.2.4 Bioinformatics Approaches to Determine Fibrinogen Marker Universality

The percent identity matrix for chicken fibrinogen alpha (iFGA) indicates minor degrees of conservation across different most vertebrate species. According to table 4.6, the matrix reveals highest similarity is observed with duck (*Anas platyrhynchos*), with a percent identity of 83.99%, suggesting that FGA is relatively conserved between chickens and its allies. This strong similarity makes it a potential candidate for multiplmultiple avian species comparisons.

In contrast, pig (*Sus scrofa*) presents a percent identity of 16.92%, indicating lower conservation between pigs and chickens. , respectively with goat (*Capra hircus*). These relatively low identity values suggest that FGA is not universally conserved across most studied vertebrate species, particularly in herd and sounder species. This limits its potential as a universal biomarker for the mentioned species. Despite its initial relevance, FGA demonstrates limited applicability as a universal marker across all vertebrates, particularly fish, due to its relatively low sequence identity (26% to 43%) among various species. This substantial divergence underscores its unsuitability as a primary phylogenetic or taxonomic marker in comparative studies involving diverse vertebrate taxa. In summary, the FGA protein presents varying degrees of conservation across species. The lower similarity to duck, goat, and cattle, combined with the higher similarity to horse, suggests that it may not serve as a universal biomarker for all vertebrate species. Although its potential application seems better suited for specific mammalian species, particularly the duck, it is not ideal for pigs, goats, or horse.

Consequently, the percent identity matrix for chicken Fibrinogen Beta (FBB) reveals varying degrees of conservation when compared to different vertebrate species.

In the analysis of the FBB protein identity matrix, *Gallus gallus* (chicken) demonstrated varying degrees of sequence similarity with other vertebrates, revealing its potential limitations as a universal molecular marker. The comparison showed notably low identity with fish species, ranging from approximately 29% to 56%, suggesting that FBB is not sufficiently conserved across distant vertebrate taxa to serve as a cross-species biomarker. Interestingly, despite being an avian species, *Gallus gallus* only shared 29.25% identity with *Anas platyrhynchos* (duck), highlighting a significant divergence even within the same class. Conversely, higher identity percentages were observed between *Gallus gallus* and several mammalian species, including Bos taurus (cow) at 65.58%, Sus scrofa (pig) at 67.1%, and *Equus caballus* (horse) at 68.91%. These values indicate a relatively higher conservation of FBB among selected terrestrial mammals compared to aquatic species and even other birds. Collectively, these findings suggest that FBB perform the broad phylogenetic conservation required of a universal vertebrate marker. However, its relatively distinct sequence profile in *Gallus gallus* supports its potential utility as a species-specific biomarker particularly for the detection of poultry-derived material in food products. This application is highly relevant in the context of food authenticity verification and the prevention of adulteration in processed food systems.

For fish species, although FBB was not directly compared in the matrix, it can inferred that fish would likely reveal even lower similarity due to the greater divergence between birds, mammals, and fish. Therefore, chicken FBB is likely unsuitable as a universal marker for fish species either, as it is not as conserved across all vertebrate groups. In conclusion, although FBB in chickens exhibits high conservation with horses (*Equus caballus*) and pigs (*Sus scrofa*), it demonstrates moderate conservation with goats and cattle. Its low conservation with duck and potentially with fish species suggests it may not serve as a universal biomarker across all vertebrates. While it is more appropriate for mammalian species, particularly horses and pigs, it is less for ducks, goats, or cattle and even less for fish.

The sequence identity matrix of FGG strongly supports its potential as a species-specific biomarker for *Gallus gallus*. The marked divergence between chicken and teleost fish (as low as 40.9% with *Cyprinus carpio*) suggests that the FGG sequence has undergone significant evolutionary modification across these lineages. This is

advantageous from a food authentication and blood origin-tracing perspective, as it minimizes the likelihood of false positives due to cross-reactivity with fish proteins. In contrast, the higher identity values with other birds and mammals (>60%) indicate phylogenetic conservation of core fibrinogen domains within terrestrial vertebrates. Together, this pattern underscores FGG's dual utility both as a phylogenetic marker of avian lineage and as an exclusionary biomarker for non-avian sources such as fish.

Despite the successful detection of Apo AI, FGA, and FBB through immunoprecipitation followed by SDS-PAGE visualization, fibrinogen gamma (FGG) was notably undetected in the gel electrophoresis output. This absence may be attributed to several factors, including low endogenous abundance, inefficient antibody binding, or post-translational modifications affecting solubility or electrophoretic migration. Nevertheless, FGG was confidently identified in the LC-QTOF MS-based proteomic screening, affirming its biological presence and relevance. To ensure methodological consistency and allow comparative interpretation across all shortlisted proteins Apo AI, FGA, FBB, and FGG—this protein was retained for subsequent in silico analyses. This approach aimed to evaluate its sequence conservation and evolutionary divergence across representative vertebrate species and to explore potential explanations for its inconsistent detectability in wet-lab experiments. Among terrestrial vertebrates, *Gallus gallus* exhibited the highest sequence identity with another avian species, *Anas platyrhynchos* (duck), at 85.22%, indicating strong intra-class conservation. In contrast, identity values with selected mammals were moderately conserved, with 65.44% identity observed with *Equus caballus* (horse), 63.45% with *Sus scrofa* (pig), 63.43% with *Bos taurus* (cow), and 63.81% with *Capra hircus* (goat). These values demonstrate that while FGG is moderately conserved among mammals, its conservation is significantly stronger within the avian lineage.

When compared to fish species, *Gallus gallus* displayed a more pronounced degree of sequence divergence. Identity values ranged from 40.94% with *Cyprinus carpio* (common carp), 54.81% with *Gadus chalcogrammus* (Alaska pollock), and 54.05% with *Silurus meridionalis* (catfish), to 58.25% with *Oncorhynchus mykiss* (rainbow trout) and 55.31% with *Salmo trutta* (brown trout). Among these, common carp showed the lowest similarity, while salmonid species exhibited comparatively higher identity scores, albeit still markedly below those observed in mammals or birds. These trends, visualized through a percent identity heatmap, highlight evolutionary

divergence between avian and teleost lineages, as well as partial conservation among cold-water fish species.

Collectively, the results indicate that FGG is not sufficiently conserved across all vertebrate classes to serve as a universal molecular marker. Its inconsistent experimental detectability may stem from biological and structural variability, as reflected in the bioinformatic analyses. However, its high sequence similarity within avian species, and moderate conservation in mammals, supports its potential utility as a species- or class-specific biomarker, particularly for detecting poultry-derived components in food authentication studies. While FGG may not fulfill the criteria for a broad-spectrum vertebrate biomarker, its inclusion in targeted assays aimed at poultry verification remains scientifically justified and practically valuable for traceability and halal compliance in processed food systems.

In general, the percent identity matrix for Apo AI, FGA, FBB and FGG in chicken, compared across various vertebrate species, reveals varying degrees of conservation that inform their suitability as biomarkers. In conclusion, while fibrinogen alpha and FBB demonstrate strong potential for specific species comparisons, their application as universal biomarkers is limited. FGA is better suited for studies focused on equine and certain mammalian species. However, it is less applicable for cross-species comparisons, especially in birds like ducks. Though more conserved than fibrinogen alpha, FGA is still less effective as a universal biomarker across all vertebrates, particularly in avian species. In comparison, Apo AI presents greater conservation across a broader range of species, including mammals and birds, and may serve as a more reliable universal marker across a wider spectrum of vertebrates. Therefore, future studies could focus on validating Apo AI as a cross-species biomarker while exploring additional markers to expand fibrinogen-based studies across more diverse species.

This study serves as a foundation for future research in biomarker development and species-specific studies, where Apo AI offers greater potential for universal applications. Nevertheless, fibrinogen alpha and FIB provide valuable insights for more targeted species comparisons, particularly within mammalian studies.

4.3 Development of Electrochemical Immunosensor

4.3.1 Characterization of Synthesized AuNP

This chapter started with the synthesised AuNPs by reducing a HAuCl4 solution with sodium citrate while maintaining boiling conditions. As the reaction progressed and sodium citrate was gradually added to the $HAuCl_4$ solution, the medium transitioned from a pale yellow to colorless within 10 minutes, ultimately resulting in a wine-red solution (see Fig. 4.2 (a-c)). The emergence of the wine-red color indicated the successful formation of AuNPs. It is important to note that particle aggregation influences the observed color of the solution. Zhang et al. (2016) highlighted that the hue of gold nanoparticles can be altered through the phenomenon of surface plasmon resonance (SPR). This color change was correlated with an increase in particle size, leading to a transition from dark red to dim purple. The SPR exhibited by these nanoparticles in the visible range of the electromagnetic spectrum is the primary factor responsible for this color variation (Zhang et al., 2016). SPR is essential to the optical properties of AuNPs, particularly regarding their color changes. It involves the collective oscillation of conduction electrons on the surfaces of metal nanoparticles when exposed to light at specific wavelengths, leading to enhanced light absorption and scattering that results in the distinctive colors of AuNP solutions. The SPR wavelength is influenced by the size and shape of the nanoparticles; smaller AuNPs typically show a strong absorption peak around 520 nm, producing a bright red color, while larger particles exhibit a redshift, transitioning the color to purple or blue due to increased light scattering. Additionally, aggregation of AuNPs alters SPR characteristics, further modifying the color as the delocalisation of conduction electrons occurs among neighboring particles. The sensitivity of SPR to changes in the local refractive index allows for the detection of environmental changes, making AuNPs valuable in applications such as biosensing, where colorimetric responses to target analytes provide a visual detection method. Thus, SPR is fundamental to the unique optical properties of AuNPs, facilitating their use in various scientific and technological applications (Elahi et al., 2018).

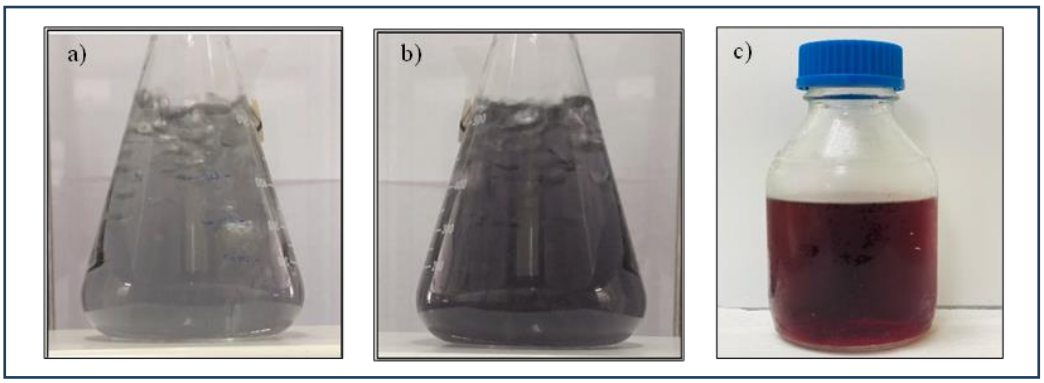


Figure 4.2 The Visual Appearance of the Change of Colour of HAuCl4 Solution After the Addition of Sodium Citrate (a) and Transitioned to (b) and (c) in Wine-red shade Gradually over 10 Minutes

The use of $HAuCl_4$ as a precursor in gold nanoparticle synthesis significantly impacts the resulting size and shape of the nanoparticles. It allows for precise control over the reduction process, enabling the formation of nanoparticles with specific sizes and shapes. Its versatility makes it compatible with various reducing agents and stabilisers, offering flexibility in nanoparticle synthesis. In addition, it also provides a clean source of gold ions, minimising impurities that could affect nanoparticle formation

4.4 Analysis of Maximum Absorbance Wavelength for AuNPs

Figure 4.3 shows the UV–Vis absorbance spectrum of the synthesised AuNPs, showing a distinct surface plasmon resonance (SPR) peak at 520 nm, which corresponds to the maximum absorbance. The presence of SPR band confirms the optimum formation and stability of the AuNPs, in agreement with previous findings reported by Low (2014) and Babaei-Afrapoli et al. (2021). Slight baseline variation observed below 300 nm was attributed to the intrinsic absorption of the glass cuvette in the UV region (not shown). The λmax at 520 nm was subsequently used for further spectrophotometric characterisation of the synthesised AuNPs.

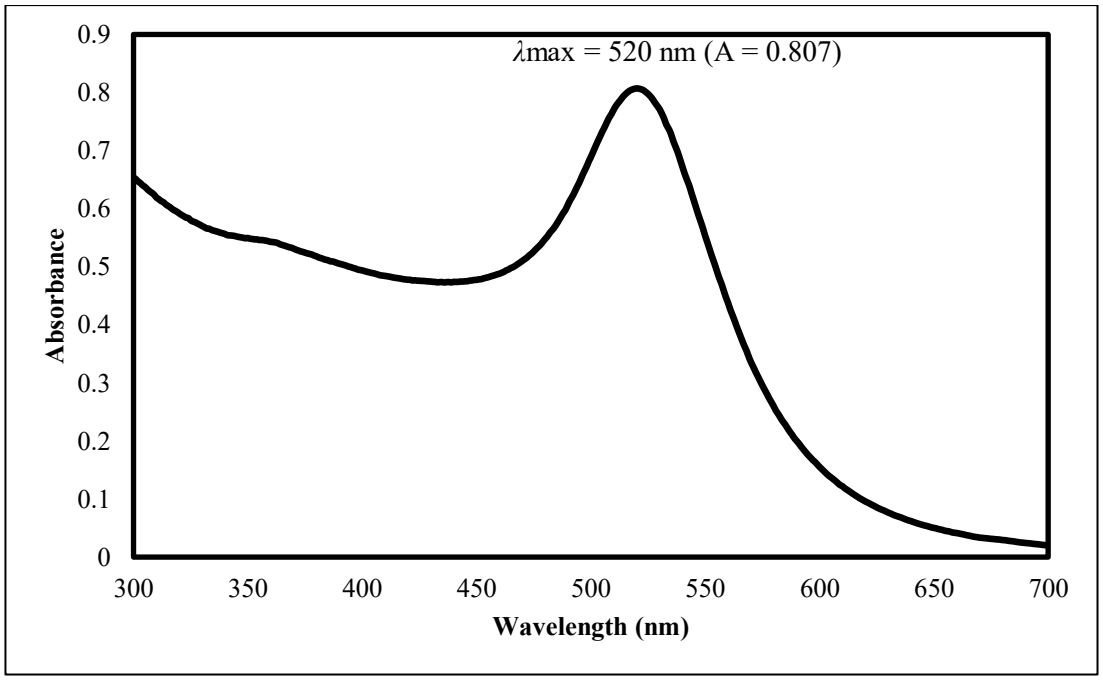


Figure 4.3 The Maximum Peaks Occurred at 520nm of Wavelength Indicating the Highest Concentration of AuNP at Absorbance of 0.8

4.5 Structural Analysis of AuNPs Using TEM Imaging

Transmission electron microscopy (TEM) analysis was performed to evaluate the morphology and size distribution of the synthesised gold nanoparticles (AuNPs). As shown in Figure 4.4, the AuNPs exhibited predominantly spherical morphology with uniform dispersion. Image analysis using calibrated TEM micrographs determined an average particle diameter of 18.0 ± 1.3 nm, corresponding to a relative standard deviation (RSD) of 7.2%. The low RSD (<10%) indicates good monodispersity, which is desirable for reproducible electrochemical performance in biosensing applications (Volkert et al., 2011).

Approximately 100 nanoparticles were measured using ImageJ, following established image-processing approaches reported by Zhang and Wang (2023). The particle size distribution (Figure 4.5) shows that approximately 80% of the particles fall within the 15–21 nm range, confirming a narrow and controlled size distribution typical of well-synthesised citrate-reduced AuNPs. The controlled particle size and uniform morphology are expected to contribute to stable surface plasmon resonance behaviour and consistent antibody conjugation efficiency, which are critical for reliable electrochemical immunosensor performance.

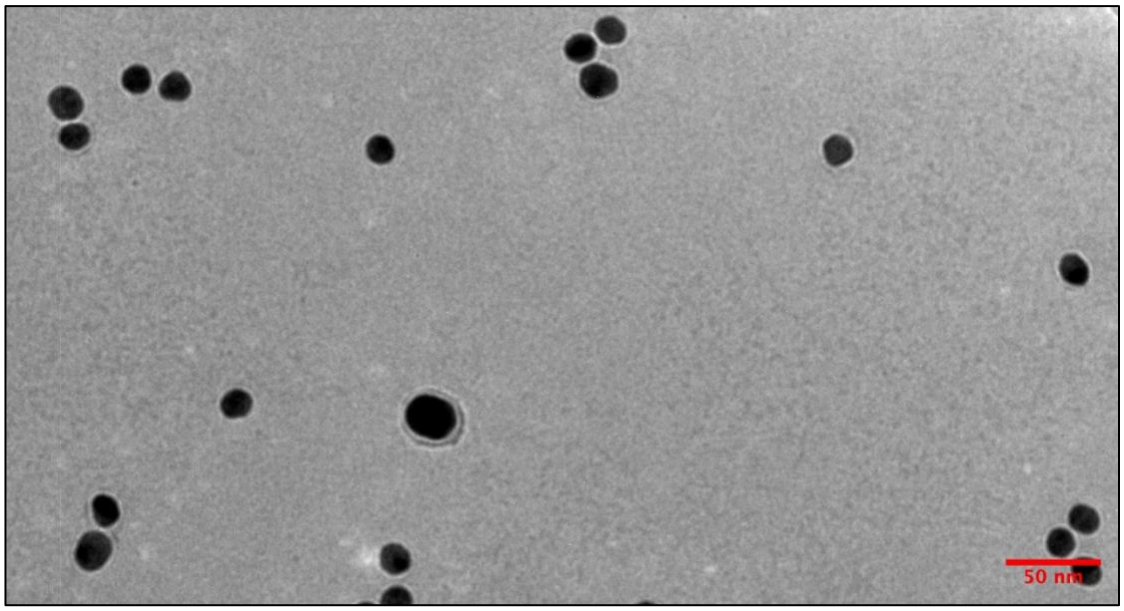


Figure 4.4 TEM images of Synthesized AuNPs using the Turkevich-Frens Method

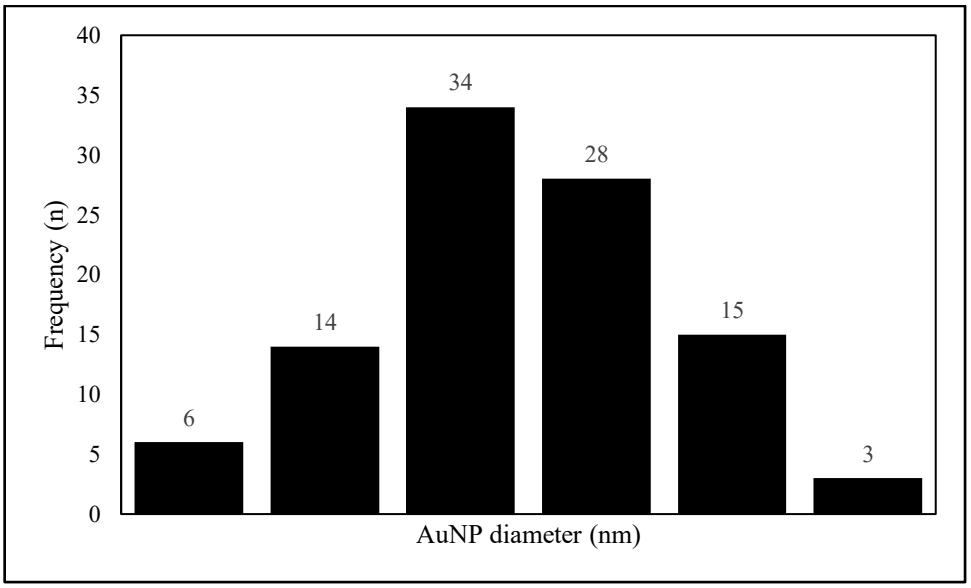


Figure 4.5 Histogram of AuNPs Sizes

4.6 Optimization of Differential Pulse Anodic Stripping Voltammetry (DPASV) Parameters

The differential pulse anodic stripping analysis (DPASV) protocol has been selected as an optimized method, as previously reported by Low's (2014) electroanalysis of AuNPs on electrode surfaces. The objective of this section was to identify the variables influencing the electroanalytical performance of AuNPs and optimise them accordingly. The parameters investigated in the DPASV protocol included

electrodeposition potential (E_{dep}), electrodeposition time (t_{dep}), step potential (E_{step}), and scan rate. In this DPASV configuration, the scan rate was a software-derived parameter administered by the selected step potential and pulse settings; therefore, optimisation was achieved indirectly through variation of the E_{step}. Electrodeposition was performed without mechanical stirring to maintain consistent mass transport conditions. This optimisation was conducted to achieve optimal efficiency in the electroanalysis of AuNPs, as detailed in Table 4.9. All experiments were performed using electrochemical-quantitative detection with 10 µL of colloidal AuNPs dissolved in 100 µL of HBr/Br_2 detection medium. The current value recorded at +0.65 V from the resulting voltammogram was used as the analytical signal without the need to determine peak height.

Table 4.9
DPASV Parameters with the Setting Values for Optimisation

DPASV parameters	**Setting values used for optimisation**
Electrodeposition potential (E_{dep})	-0.95, -0.35, 0, 0.35, 0.55, 0.75 V
Electrodeposition time (t_{dep})	150, 210, 270, 330, 390, 450, 510 s
Step potential	0.001, 0.006, 0.013, 0.01875, 0.025, 0.03125 V
Scan rate	*0.005, 0.01, 0.05, 0.1, 0.15, 0.2, 0.25 V/s
Stripping potential range	----

*Scan rate values were automatically calculated by the instrument software based on the selected step potential and pulse parameters, and were not independently adjustable.
--- concentration or setting value remained unchanged

The optimization study starts with AuNPs, which were chemically converted into Au^{3+} ions using an HBr/Br_2 detection medium. Subsequently, these Au^{3+} ions were electrodeposited onto the SPCE working electrode's surface. Various E_{dep} values ranged from +0.75 to –0.95 V for 510 s. Subsequently, the DPASV was performed to detect the preconcentrated Au atoms on the electrode surface. Figure 4.6, 4.7 and 4.8 illustrates all the recorded analytical signals obtained from this study.

For E_{dep} optimisation (Fig. 4.6), the deposition time (t_{dep}) was fixed at 270 s as a preliminary working condition based on initial screening experiments and literature guidance, while E_{dep} was varied systematically. DPASV electroanalysis was conducted with a positive E_{dep} of +0.50 V, resulted with minimal signal. However, as we shifted the E_{dep} towards the negative direction, the current signal gradually increased. Eventually, it reached a plateau at –0.55 V, indicating the optimal electrodeposition potential for subsequent studies. A slight decrease in current was observed at potentials more negative at –0.65 V which may be attributed to excessive reduction or surface instability at higher overpotentials. Lower E_{dep} was selected as to prevent potential destructive effects on the structure of the electrode surface that could arise from using higher potentials reference. For E_{dep}, a key benefit of DPASV is the ability to electrodeposit analyte onto the working electrode surface before measuring current using differential pulse stripping analysis. Preconcentration enhances detection sensitivity and improves quantification confidence for trace analytes (Hu et al., 2009). During the optimisation experiment, AuNPs were dissolved into Au^{3+} ions using 1M HBr/0.15 mM Br2 detection solution. Electrodeposition of Au^{3+} ions onto the SPCE working electrode surface was achieved by applying varying E_{dep} values from +0.50 to −1.05 V for 270 s. The preconcentrated Au atoms were then analysed by differential pulse anodic stripping. As expected, the DPASV electroanalysis with a positive E_{dep} of +0.50 V demonstrated no current signal during the control trial. As E_{dep} shifted towards negative direction, the current signal rose and plateaued at -0.55 V. To avoid damaging the electrode surface structure, a low E_{dep} was chosen for future experiments (Morrin et al., 2003).

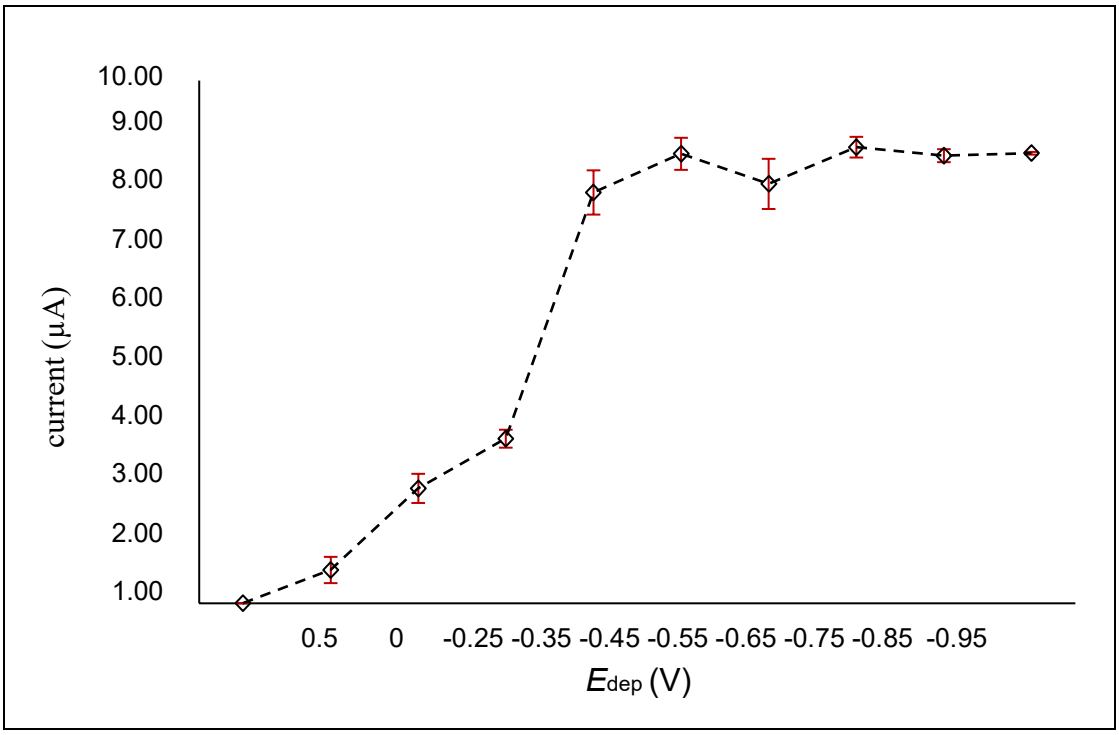


Figure 4.6 Effect of the E_{dep} on the DPASV Electroanalysis of AuNPs on the Current (µA) Electrode Surfaces

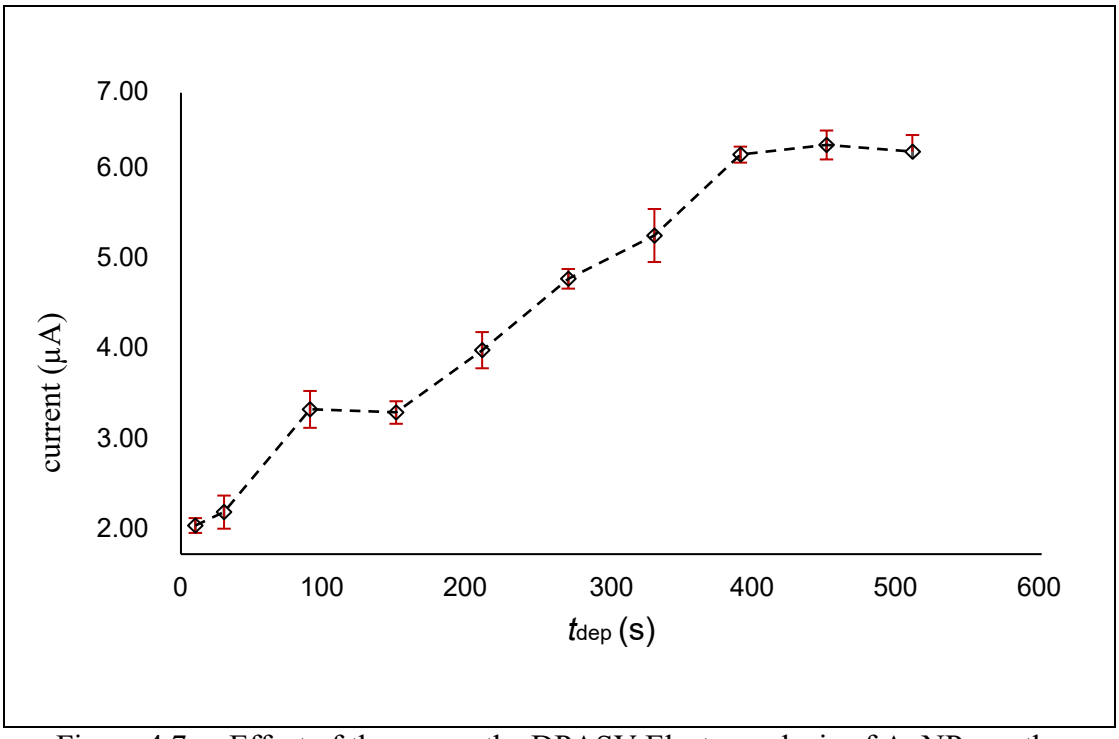


Figure 4.7 Effect of the t_{dep} on the DPASV Electroanalysis of AuNPs on the Electrode Surfaces

After determining an E_{dep} = −0.55 V, the t_{dep} was then optimised within 10 to 510 s. Jahandari et al. (2013) state that the t_{dep} is critical in determining the amount of

Au^{3+} ions preconcentrated on the electrode surface, which impacts the detection limit of the DPASV technique for electroanalysis of AuNPs on electrode surfaces. The analytical signals from this study are shown in Figure 4.7. As t_{dep} grew for Au^{3+} ions, the current signal showed almost starting to plateaued at 390 s. Optimal t_{dep} of 390 s was chosen for future investigations based on present signal intensity and reproducibility in standard deviation.

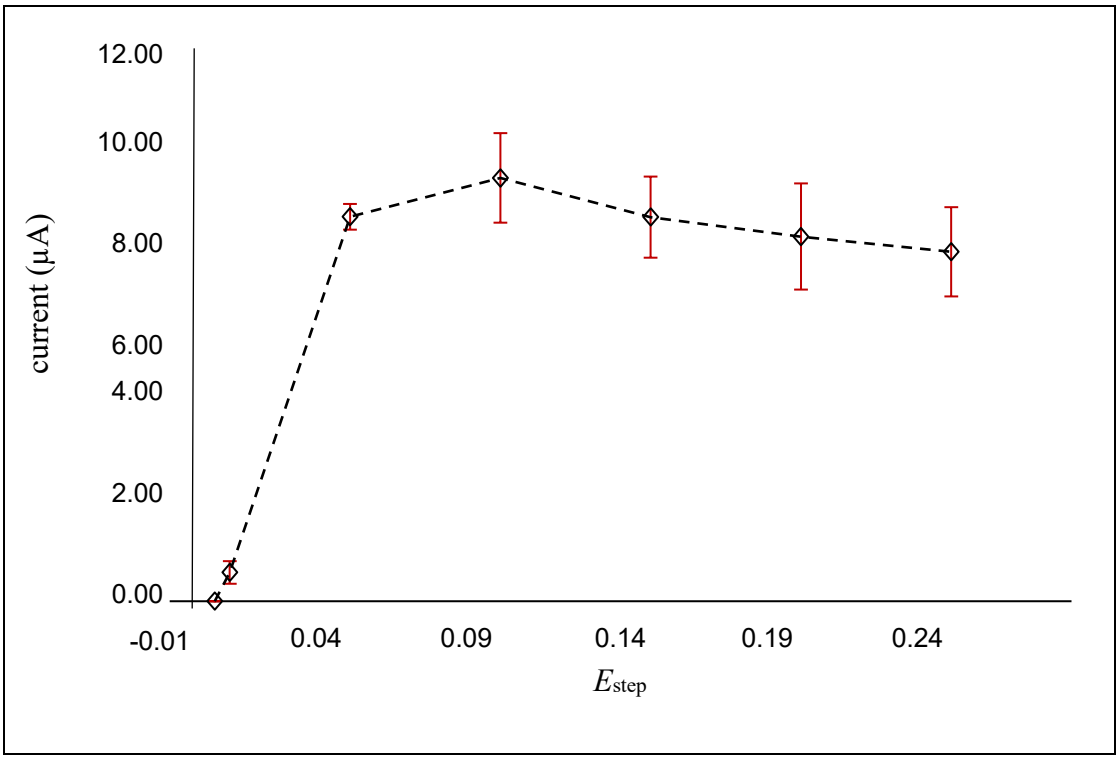


Figure 4.8 Effect of the E_{step} on the DPASV Electroanalysis of AuNPs on the Electrode Surfaces

For step potential (E_{step}) optimisation, Au^{3+} electrodeposition was performed at −0.55 V for 390 s, followed by differential pulse stripping analysis over 0.0 to +1.0 V. The scan rate was not independently adjusted but derived from the selected E_{step} values (interval time = 0.125 s). Therefore, E_{step} was treated as the primary optimisation parameter. Figure 4.8 presents the peak current plotted as a function of E_{step}. The stripping current increased with increasing E_{step} up to 0.05 V, beyond which signal stability and reproducibility decreased. Higher E_{step} values resulted in broader peaks and increased variability. Consequently, E_{step} = 0.05 V was selected as the optimal condition for subsequent analyses.

4.6.1 Conjugation Complex of AuNP to MB-S in Electrochemical Immunosensor

Optimal conjugation from nanoparticle to magnetic beads is one of critical criteria, as the antibody-gold nanoparticle (Ab-AuNP) bioconjugates should be stable while maintaining the ability of the Ab to recognize and bind its corresponding antigen. Figure 4.9 illustrates the successful conjugation of antibodies to AuNPs, as captured by TEM at 50,000× magnification. The lower panel highlights the accumulation of AuNPs around the magnetic beads (MB), confirming effective binding. The faint smearing observed in the background is attributable to the omission of washing steps, deliberately preserved to visualize the raw, unmodified conjugation process. All the same, a high coverage of antibodies on AuNPs is a key-step to build up a sensitive biosensor, but an ideal coverage requires to be perfectly balanced with the orientation and accessibility of the conjugated antibodies (Zhang et al., 2020).

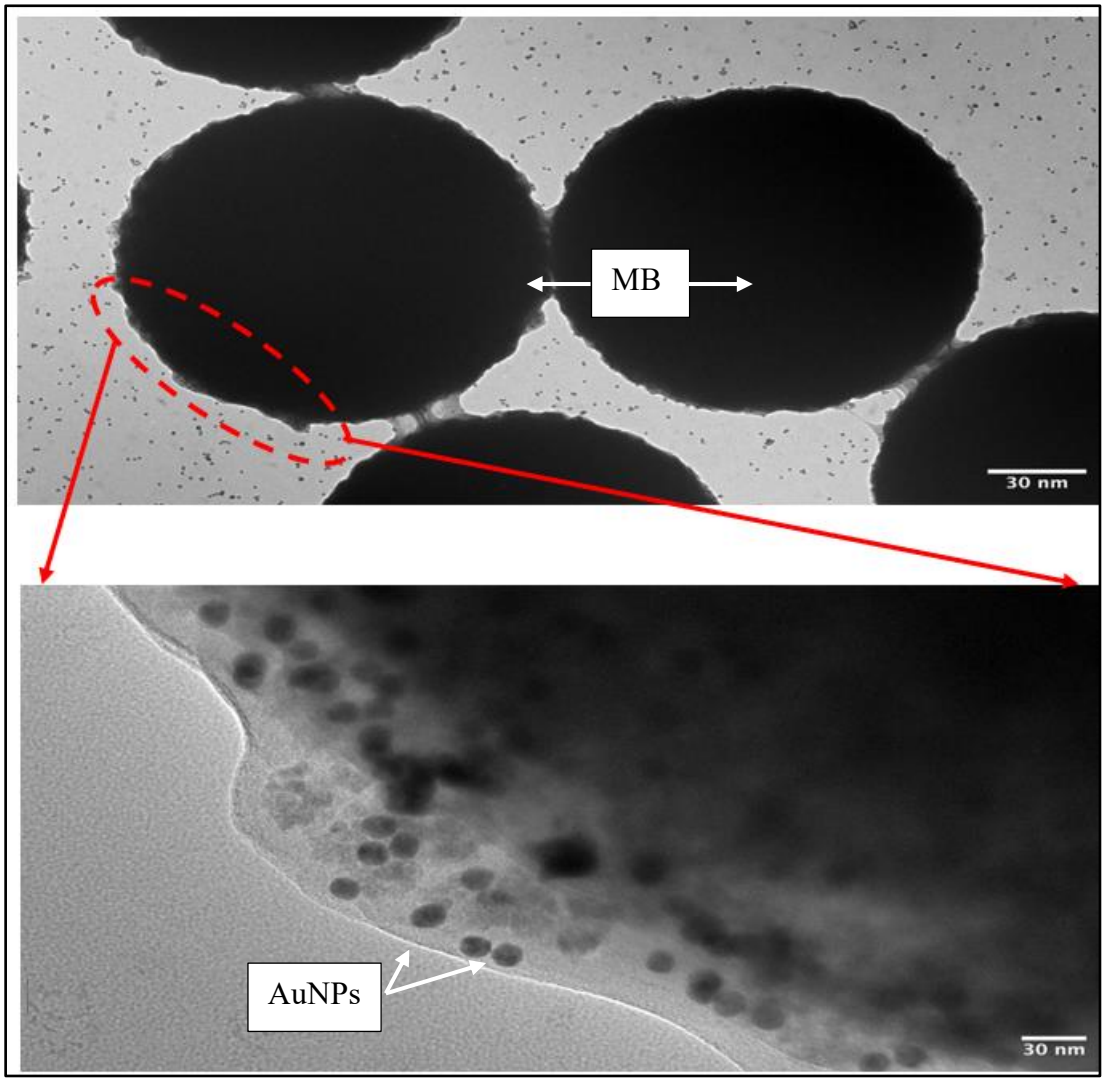


Figure 4.9 TEM Images Showing the Conjugation of AuNPs to MB at 50,000× Magnification. The Large Dark Spherical Structures Represent MB, while the Smaller Dark Nanoparticles Distributed along the Bead Surface Correspond to AuNPs. The lower Panel Presents a Magnified View Highlighting AuNP Immobilisation on the MB Surface.

4.7 Performance of the Apo AI Monoclonal Antibody (Apo AI-mAb) Immunosensor in Detecting Apo AI Protein

In this study, the analytical performance of the developed electrochemical immunosensor for Apo AI was evaluated in triplicate over a concentration range of 50–1250 µg/mL (Fig. 4.10). Detection is based on specific antigen–antibody recognition, whereby Apo AI present in diluted CBP is selectively captured by immobilized anti-Apo AI antibodies on the magnetic bead (MB)-modified electrode surface. Following

capture, AuNP-labeled secondary antibodies bind to the target protein to form a sandwich immunocomplex.

The electrochemical signal is generated indirectly via the redox activity of the gold nanoparticles. During differential pulse anodic stripping voltammetry (DPASV), metallic Au^0 is oxidized to Au^{3+}, producing an anodic current proportional to the amount of bound Apo AI. Thus, Apo AI is not detected through intrinsic electroactivity, but through direct matrix immunocapture coupled with AuNP-mediated electrochemical transduction.

The triplicate measurements demonstrated consistent electrochemical responses with relative standard deviations (RSD) ranging from 2.1% to 7.9% (n = 3), indicating good reproducibility and analytical reliability. The peak current increased progressively with increasing Apo AI concentration, reflecting concentration-dependent immunocomplex formation and effective signal amplification via AuNP labels. Blocking agents such as BSA or PEG were employed to minimize nonspecific adsorption, ensuring selective detection within the complex CBP matrix (Zhang et al., 2020).

The calibration curve, constructed from the mean values of triplicate measurements, exhibited a strong linear relationship described by Fig. 4.10. This high coefficient of determination confirms excellent linearity and demonstrates the quantitative capability of the immunosensor within the tested concentration range. The observed linear response further validates the efficiency of the bioconjugation strategy and is consistent with previous reports highlighting the signal amplification properties of AuNP-based electrochemical immunosensors in complex biological matrices (Zhong et al., 2024).

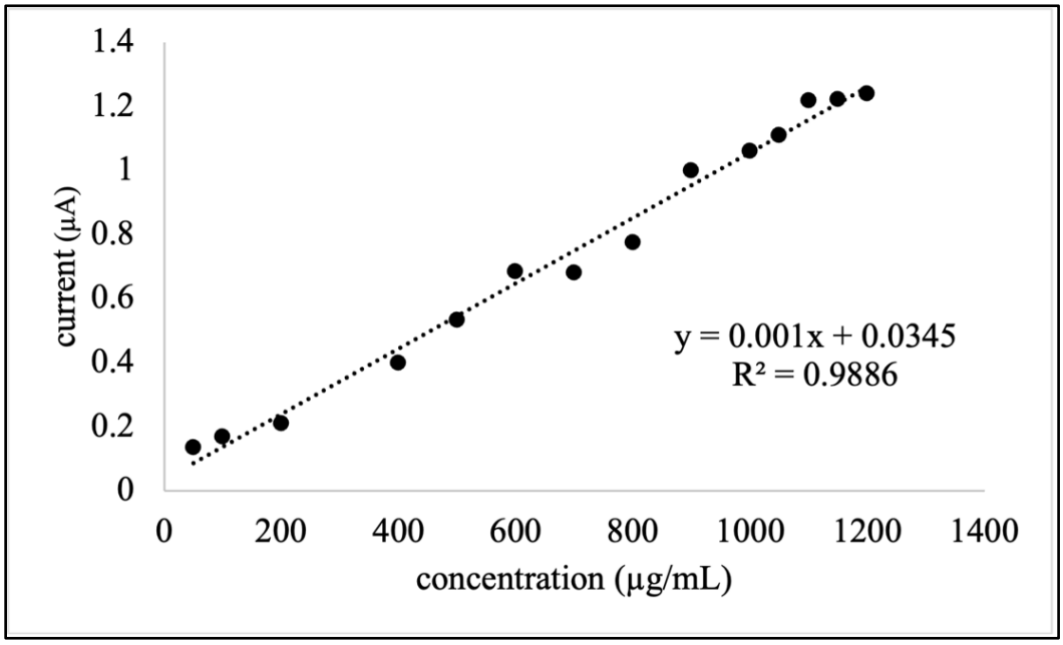


Figure 4.10 Electrochemical Calibration Curve of the Apo AI Immunosensor Against CBP Concentrations

To establish the diagnostic performance of the immunosensor, a positivity threshold was evaluated based on blank sample responses. The mean peak current of the blank measurements (M = 0.52 μA) with a standard deviation of 0.02 μA (n = 3) was first determined. Using the formula Mean + 3(Standard Deviation), the threshold value was calculated as 0.58 μA. Accordingly, samples producing a current signal above 0.58 μA were classified as positive for Apo AI, whereas signals below this value were considered negative.

The clear differentiation between the signal outputs across concentrations suggests strong specificity of the antibody-antigen interaction. This is particularly important in avoiding non-specific binding from other plasma or tissue proteins. Apo AI, with a molecular mass of 30,662 Da and composed of 264 amino acids, is primarily found in human and avian plasma but absent in teleost fish species commonly used in processed food products. This anatomical and functional distinction aligns with previous reports by Prasad et al. (2020), who highlighted the use of Apo AI as a reliable biomarker in species-specific detection. The unique electrochemical fingerprint of Apo AI bound to α-Apo AI/AuNP-MBs conjugates ensures that the system is capable of distinguishing chicken-derived proteins even in the presence of complex matrices such as surimi-based fish products. Importantly, the assay's ability to discriminate true positives from background signals minimizes the risk of cross-reactivity with functional proteins typically present in fish paste, such as actin, tropomyosin, and myosin.

Furthermore, the sensor's performance offers promising application in food quality control and halal authentication, where the detection of adulterants in processed fish-based products is critical. The high reproducibility and sensitivity observed in this assay fulfil technical benchmarks set by the Global Food Safety Initiative (GFSI) and the Malaysian Halal Standard (MS1500:2019), reinforcing the sensor's practical utility in real-world settings.

4.8 Performance of the Fibrinogen Monoclonal Antibody (Fib-mAb) Immunosensor in Detecting Fibrinogen Protein

This section highlights the the electrochemical immunosensor's capability to detect fibrinogen derived from CBP, evaluated over a concentration range of 10–1300 µg/mL using triplicate measurements. The biosensing platform was constructed using magnetic beads conjugated with AuNP-labeled anti-fibrinogen antibodies, with electrochemical detection performed via DPASV.

A concentration-dependent increase in current signal was observed with increasing CBP concentration, indicating successful antigen–antibody interaction on the sensor surface. While the response was assessed across the full tested range (10–1300 µg/mL), optimal linearity was observed within the defined working linear range of 500–1250 µg/mL. Within this range, the calibration curve yielded a coefficient of determination $R^2 = 0.9886$ (Fig. 4.11), demonstrating good linearity and supporting the quantitative applicability of the fibrinogen immunosensor. The relative standard deviation (RSD) across triplicate measurements ranged from 2.5% to 9.2%, confirming acceptable precision and reproducibility under controlled experimental conditions.

The observed increase in current corresponds to enhanced formation of the fibrinogen-bound immunocomplex, which facilitated more extensive labeling with AuNP-conjugated signaling probes. During DPASV measurement, oxidation of Au^0 to Au^{3+} generated a current proportional to the amount of captured fibrinogen. This mechanism aligns with earlier findings by Usman et al. (2025) and Zhang et al. (2025), who demonstrated signal amplification through nanogold-based electrochemical transduction.

A detection threshold was established using the standard statistical criterion M + 3SD, based on the blank sample's mean signal (M = 0.50 µA; SD = 0.02 µA), yielding a cut-off value of 0.56 µA. Signals ≥ 0.56 µA were considered positive, while signals

below this threshold were categorized as negative. This threshold corresponded approximately to concentrations ≥ 500 µg/mL, aligning with the defined working linear range and ensuring analytical consistency between qualitative discrimination and quantitative calibration.

From a protein science perspective, fibrinogen is a high molecular weight glycoprotein involved in coagulation, and its species-specific isoforms allow discriminative detection. In previous sequence-based analyses, *Gallus gallus* fibrinogen displayed unique C-terminal variations and threonine-rich regions not present in teleost species such as *Cyprinus carpio*, *Oncorhynchus mykiss*, or *Salmo salar*. These structural distinctions support the specificity of the immunosensor and minimize cross-reactivity with fish-derived proteins commonly found in commercial surimi products.

This finding reinforces the observations of Prychitko and Moore (2003) and O'Toole et al. (1994), who reported that avian fibrinogen exhibits divergent structural motifs, particularly within peripheral domains that differ significantly from conserved fish homologues. Consequently, the present fibrinogen immunosensor is unlikely to produce false positives in fish-based products, enhancing its reliability as an analytical tool for detecting poultry-derived contamination in seafood or vegetarian-labelled foods.

The practical implications of this system extend to halal compliance testing, meat adulteration investigation, and product label verification. Its robustness, coupled with good linearity within the defined working range ($R^2 = 0.9886$) and acceptable triplicate variance, supports its applicability in food safety diagnostics and supply chain authentication workflows.

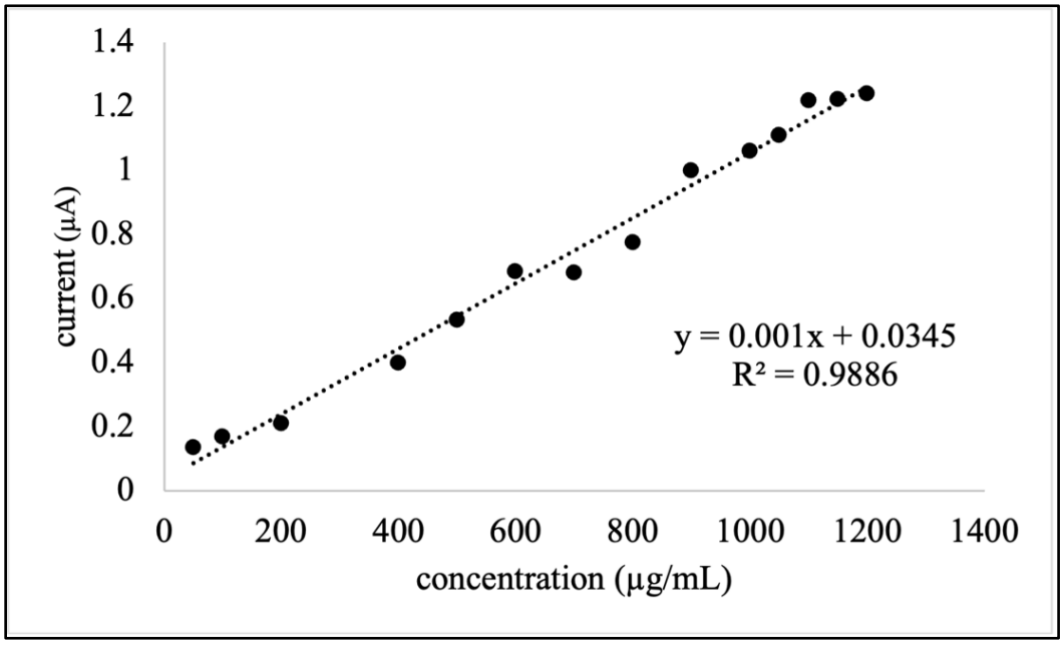


Figure 4.11 Electrochemical Detection Profile of the Fibrinogen Immunosensor Against CBP Concentrations

4.9 Preliminary Comparative Insights and Transition to Real Sample Analysis

The preceding sections (4.7 and 4.8) established the analytical performance of two electrochemical immunosensors developed for CBP detection, targeting Apo AI and fibrinogen as complementary biomarkers. Under optimized buffer conditions, both sensors produced concentration-dependent electrochemical responses, confirming successful antigen–antibody recognition and AuNP-mediated signal transduction.

The Apo AI immunosensor exhibited strong linearity across the evaluated working range of 50–1250 µg/mL (R^2 = 0.9931), establishing it as the primary quantitative calibration platform in this study. In contrast, the fibrinogen immunosensor demonstrated good linearity within its defined working range of 500–1250 µg/mL (R^2 = 0.9886). Although fibrinogen showed a narrower linear window, its response remained reproducible (RSD < 10%) and analytically reliable above the established positivity threshold (0.56 µA). Collectively, these results indicate that Apo AI provides broader quantitative coverage, while fibrinogen serves as a confirmatory biomarker within higher concentration ranges.

The observed electrochemical amplification in both systems is attributed to AuNP-labeled antibody conjugates, where oxidation of Au^0 during DPASV generates a signal proportional to immunocomplex formation. The reproducibility and linearity

achieved in this study are comparable to previously reported nanogold-enhanced electrochemical immunosensors (Song et al., 2020; Sun, 2015), supporting the robustness of the signal transduction mechanism employed here.

While the buffer-based validation confirms analytical feasibility, these experiments were conducted under controlled conditions that do not fully replicate the complexity of processed food matrices. In practical applications, surimi-based products contain diverse proteins, salts, and heat-induced aggregates that may influence binding kinetics or electrochemical background noise. Therefore, despite the strong calibration performance observed ($R^2 \geq 0.988$; RSD < 10%), further validation under matrix conditions is required to assess selectivity, recovery, and diagnostic reliability.

To address this, the subsequent phase of this study evaluates both immunosensors using CBP-spiked matrices and commercial surimi products. This step enables assessment of matrix effects, comparison with LC-MS/MS-based quantification, and evaluation of real-world applicability for CBP adulteration screening. By transitioning from buffer validation to food matrix analysis, the developed immunosensors are examined not only as analytical devices but as practical diagnostic tools for halal authentication and food fraud detection.

4.9.1 Performance Comparison of Electrochemical Immunosensors and LC-QTOF Mass Spectrometry in Detecting CBP

The performance of the electrochemical immunosensors was evaluated for detecting Apo AI and fibrinogen in spiked surimi samples, which were used to mimic real-world contamination levels typically encountered in food safety testing. Different concentrations of CBP were spiked into the surimi at levels of 0.50%, 1.00%, and 1.50%, enabling an assessment of the sensors' ability to detect low concentrations of these proteins in a complex food matrix. The LC-QTOF mass spectrometry, recognized as the gold standard in proteomic analysis, was employed to provide an initial reference for detecting Apo AI and fibrinogen, establishing a baseline for the performance of the immunosensors. It should be stated that the spike levels (0.50–1.50%) represent matrix-level CBP adulteration in surimi samples. In contrast, the calibration curves presented earlier in Figures 4.10 and 4.11 were constructed using extracted proteins retrieved from CBP which were expressed in µg/mL to evaluate the analytical performance of the

immunosensor. These concentration formats correspond to different experimental objectives and are therefore not directly comparable.

These concentrations are consistent with established protocols in the literature for spiked sample analysis, which commonly use such levels to evaluate detection sensitivity and accuracy. For instance, spiking levels around 0.2% to 2.0% have been employed in various adulterant detection methods to assess adulterant residues in milk products using ATR-FTIR spectroscopy (Julmohammad et al., 2024). Similarly, amino acid fingerprinting studies of milk powders have used spiking levels of 0.25%, 0.5%, 1%, and 1.5% to evaluate detection of protein adulterants (Q. Yang et al., 2022). In paprika adulteration research, spiking levels ranged from 0.1% to 5% for dye adulterants to validate classification models (Sun et al., 2022). DNA metabarcoding assays for meat species detection also typically use around 1% spiking to mimic minor species adulteration (Abdullahi et al., 2017). These examples illustrate the common practice of using approximately 0.5% to 1.5% spiking levels to simulate realistic adulteration scenarios for method validation, which is also a standard approach in Halal gelatin detection**.** In the case of gelatin adulteration, Shabani et al. (2015) used spiking levels around 1% to 2% to evaluate the sensitivity and accuracy of Halal verification methods**,** ensuring reliable detection of porcine gelatin in bovine gelatin to maintain Halal integrity in food products.

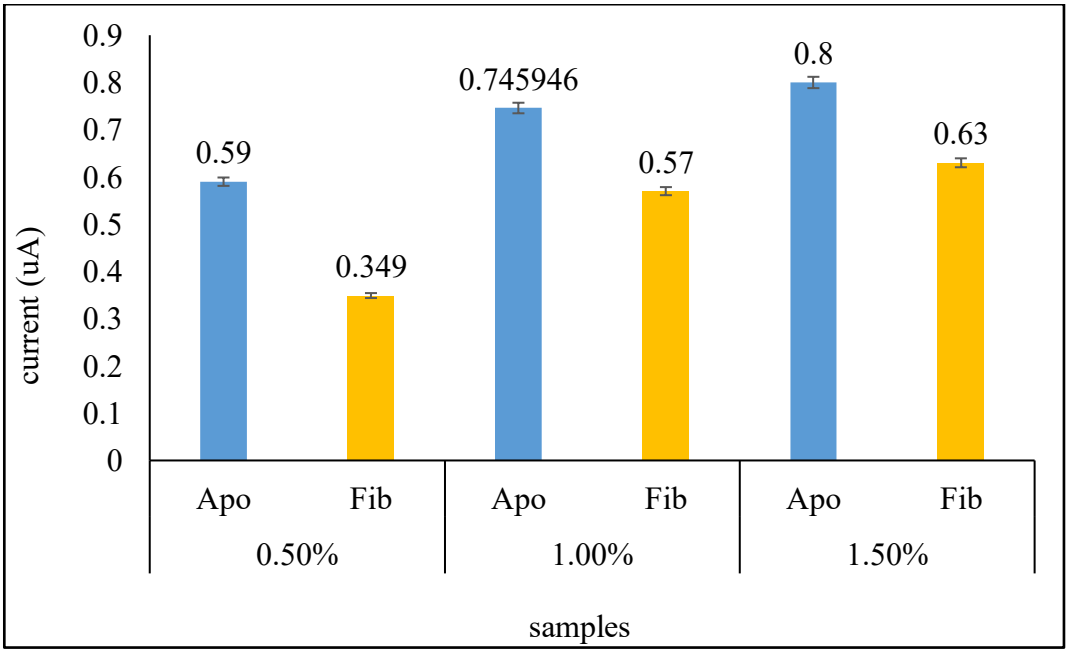


Figure 4.12 Detection of Apo AI and Fibrinogen in Spiked Samples using the Electrochemical Immunosensor

As shown in Figure 4.12, the voltammetric signals recorded by the immunosensors for Apo AI and fibrinogen were 0.8 µA and 0.63 µA, respectively at 1.50% CBP, both of which were well above the cutoff values of 0.58 µA for Apo AI and 0.56 µA for fibrinogen. These results indicated positive detection, confirming the sensors' robust performance at high contamination levels.

At 1.00% CBP, the immunosensors showed reliable detection as well, with voltammetric signals of 0.745946 µA for Apo AI and 0.57 µA for fibrinogen, both exceeding their respective cutoff values. These values further demonstrate the reliability of the immunosensors for detecting these proteins at moderate contamination levels, which was supported by LC-QTOF-MS that successfully detected both proteins at this concentration. The consistency of the immunosensor findings at these contamination levels highlights the sensors' potential for use in real-world food safety applications. However, at the lower concentration of 0.50% CBP, the immunosensors faced limitations. While the signal for Apo AI was still detectable, with a value of 0.59 µA above the cutoff of 0.58 µA, it was weaker compared to the signals at higher concentrations. Fibrinogen, on the other hand, was not detected at 0.50% CBP, which suggests that matrix interference from other proteins in the surimi, such as myosin and albumin, may be limiting the sensor’s sensitivity at lower concentrations.

4.9.2 Validating Spiking Immunosensor Performance using LC-QTOF Mass Spectrometry in Detecting CBP

To validate the immunosensor results, LC-QTOF-MS was used to identify the performance of the immunosensor in CBP spiking levels in fish paste, mimicking potential matrix interference. Myosin heavy chain, a major protein in surimi, was detected at sequence coverages of 12.0%, 9.7%, and 9.4% at 0.50%, 1.00%, and 1.50% CBP concentrations, respectively. However, Apo AI remains steadily detected even at low sequence coverage of 2.9%, 3.8% and 13.8% at 0.50%, 1.00%, and 1.50% CBP concentrations, respectively. The successive detection of Apo AI in increasing spiking percentage does not followed by the detection of fibrinogens beta chain where it only can be detected at very low sequence coverage of 3.2% and 5.9 % at 1.00%, and 1.50% CBP concentrations, respectively. This suggests that myosin may be competing with the Apo AI and fibrinogen for detection, especially at lower concentrations. Additionally, tropomyosin alpha-1 chain-like isoform and albumin, both of which are

abundant proteins in surimi, were detected with sequence coverages ranging from 14.1% to 19.5% and 2.8%, respectively. These findings indicate that these proteins could contribute to non-specific binding and matrix interference, further complicating the immunosensors' ability to accurately detect the target proteins at lower spiking concentrations.

While LC-QTOF-MS remains the gold standard for high-sensitivity protein detection, particularly in complex food matrices, the electrochemical immunosensors offer a promising, rapid, and cost-effective alternative for detecting Apo AI and fibrinogen, particularly at higher contamination levels (1.00% and 1.50% CBP). The immunosensors demonstrated strong performance at these concentrations, which was consistent with the LC-QTOF-MS results, confirming their potential for real-time food safety screening applications. However, the immunosensors also faced challenges at lower concentrations, particularly with fibrinogen, where matrix effects caused a weaker signal and higher variability. This highlights the need for further optimization of the immunosensors to address matrix interference and improve detection sensitivity at lower contamination levels. Future work should focus on enhancing sensor design, minimizing non-specific binding, and improving the overall stability and reliability of the immunosensors for practical use in food safety testing, particularly for detecting low levels of proteins in complex food matrices.

Table 4.10
List of Proteins Detected in three Different Spiking Percentage of CBP in Surimi

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	11	19	2.9	3.8	13.8
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
Fibrinogen beta-chain	Q02020	n.d	7	9	n.d	3.2	5.9
Albumin	P19121	2	2	2	2.8	2.8	2.8

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

4.9.3 Performance of the Electrochemical Immunosensor Using Commercial Surimi Samples

Following the promising results obtained from spiked surimi samples in Section 4.8.1, this section evaluates the performance of the developed electrochemical immunosensor in detecting Apo AI and fibrinogen in commercial surimi products sourced from both local and international markets. These commercial samples, representing complex food matrices, offer a more stringent test of the immunosensor's reliability, selectivity, and practical applicability, especially in the context of Halal food authentication and safety.

Based on Figure 4.1.3, the immunosensor successfully detected Apo AI in Commercialised (COMM) samples 2, 3, and 11, with measured current values exceeding the established detection threshold of 0.58 μA (denoted by blue dotted horizontal line). Specifically, COMM 2 showed a current of 0.77 ± 0.011 μA, COMM 3 exhibited 1.25 ± 0.024 μA, and COMM 11 showed 0.59 ± 0.037 μA. These values confirm the sensor's capability to reliably identify Apo AI in the presence of interfering components from the food matrix. Despite the heavy background noise from abundant non-target proteins, such as myosin and albumin, the immunosensor demonstrated remarkable selectivity by consistently detecting Apo AI in the presence of these interfering components. Selectivity in this context is demonstrated by the sensor's ability to identify Apo AI with high confidence, even when the signal is surrounded by complex protein mixtures, without cross-reacting with other abundant proteins that could interfere with detection. This underscores the importance of optimizing the immunosensor to mitigate the effects of abundant non-target proteins in real-world food matrices, such as surimi, which can impact the detection efficiency of specific biomarkers.

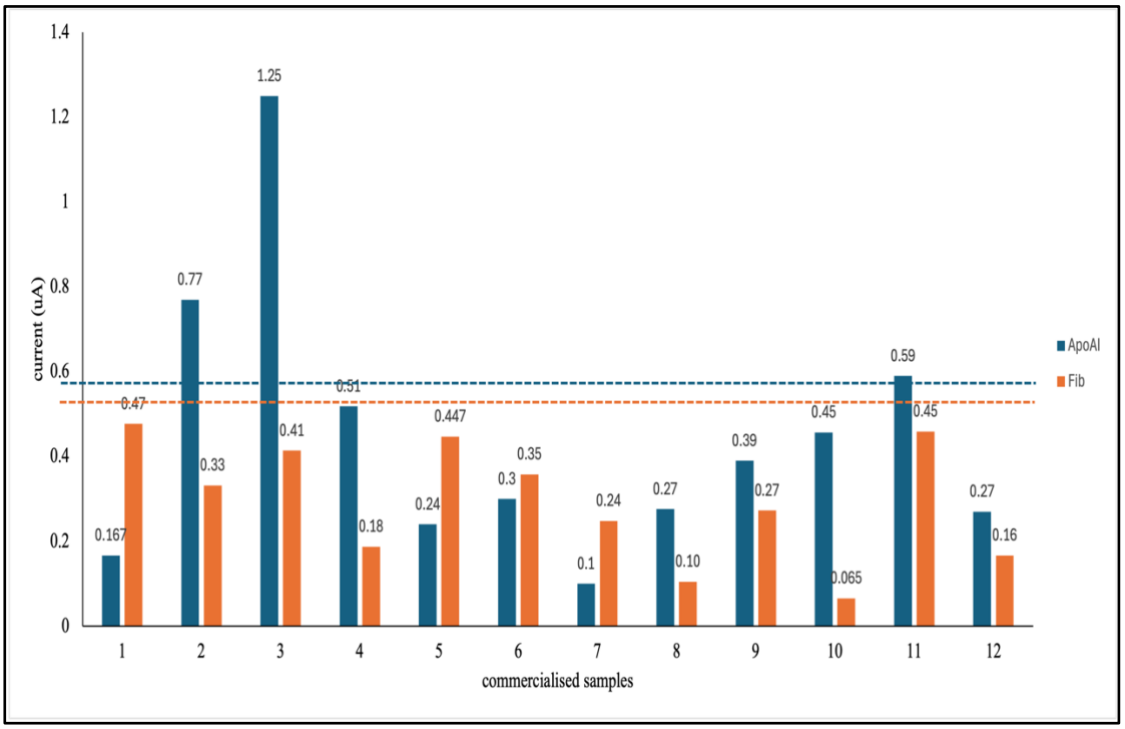


Figure 4.13 Detection of Apo AI and Fibrinogen in Commercialized Samples using the Electrochemical Immunosensor

In contrast, fibrinogen was not detected in any of the commercial surimi samples, with the current values failing to exceed the 0.56 µA threshold. This lack of detection suggests that the current version of the immunosensor may require further optimization to enhance sensitivity for fibrinogen, particularly in food matrices with high background noise. The absence of fibrinogen detection could also reflect the inherent challenges of detecting low-concentration plasma proteins in complex food products, where proteins such as myosin and albumin may obscure the target protein.

Importantly, the performance of the immunosensor can be considered strongly comparable to enzyme-linked immunosorbent assay (ELISA), the current gold standard in food protein detection. Both approaches share the same fundamental concept of antigen–antibody recognition using monoclonal antibodies, which ensures high specificity for the target protein. ELISA has been successfully validated in detecting proteins such as almond allergens in processed foods (Liu et al., 2017) and *Listeria monocytogenes* in complex food matrices (Portanti et al., 2011). In a similar manner, the electrochemical immunosensor in this study demonstrated reliable selectivity and robustness against matrix interference, even in surimi samples rich in contractile proteins. The comparable antibody-based recognition principle therefore reinforces the validity of the sensor's results, underscoring its potential to serve as a credible and field-

deployable alternative to ELISA for detecting plasma-derived proteins in food adulteration. Unlike ELISA, however, this immunosensor offers portability and rapid analysis, making it especially suitable for on-site applications such as halal authentication of surimi-based products.

4.9.4 General Discussion

From the perspective of Islamic dietary law, the detection of blood-derived proteins such as Apo AI and fibrinogen in food products carries significant implications. According to Islamic jurisprudence, the ingestion of blood or its derivatives is strictly Haram (forbidden), regardless of the species origin, including poultry. As stated in Quranic verses such as Al-Baqarah 2:173 and Al-Ma'idah 5:3, and supported by fatwas from recognized Islamic authorities like the Islamic Fiqh Academy and JAKIM (Malaysia), the presence of these proteins in food products renders them non-compliant with Halal dietary requirements.

The study detected proteins such as myosin heavy chain, actin, tropomyosin, and paramyosin in surimi samples using LC-MS/MS techniques, highlighting the presence of abundant contractile proteins that can contribute to matrix interference and reduce immunoassay detection efficiency (Kominami et al., 2020; Lee et al., 2020). However, the electrochemical immunosensor demonstrated remarkable selectivity despite these matrix challenges. While such proteins could potentially interfere with the immunoassay, the sensor's design ensures that target epitopes are still detectable, and matrix interference is minimized (Kricka, 2000). This success underscores the robustness and reliability of the immunosensor in real-world food matrices like surimi, reinforcing its capability to detect specific biomarkers, even in the presence of complex background proteins.

The presence of Apo AI in some commercial surimi samples, therefore, raises concerns about potential contamination or adulteration with CBP-derived ingredients, which are commonly used to enhance certain physicochemical properties like gelling strength, water-binding capacity, or emulsification. While such additives may be permitted under general food safety regulations, they are incompatible with Halal principles and raise ethical concerns regarding food integrity and consumer trust.

In this context, Apo AI emerges as a promising biomarker for detecting CBP-derived contamination in processed food matrices. Its detectability via the developed

immunosensor demonstrates the potential of molecular-level biosensing tools in strengthening Halal verification protocols. While the sensor did not detect fibrinogen, this limitation reflects the current threshold of detection and underscores the necessity for further optimization. Possible improvements include signal amplification, surface functionalization, and matrix-deconvolution techniques to reduce non-specific interference, which would increase the sensor's sensitivity and specificity.

Husna et al. (2023) developed an electrochemical aptasensor for Apo AI detection using gold nanorods (AuNRs) and gold nanowires (AuNWs) as nanomaterials, demonstrating sensitive detection in human serum samples. Although their sensor employed different nanostructures and targeted human serum rather than surimi or CBP, the study corroborates the feasibility and high sensitivity of nanomaterial-enhanced electrochemical sensors for Apo AI detection across diverse complex biological matrices. This agreement supports the robustness of using various gold-based nanomaterials for reliable Apo AI biosensing despite differences in sample type and sensor design.

The study by Sun et al. (2024) shows that using special materials like metal-organic frameworks combined with gold nanoparticles and small antibody fragments (nanobodies) can make sensors more sensitive and better at detecting Apo AI, even in complex samples like human serum. This supports the idea that improving sensor design with advanced materials can help overcome interference from other proteins in complex food samples like surimi, making detection more reliable.

The presence of Apo AI in some commercial surimi samples, therefore, raises concerns about potential contamination or adulteration with CBP-derived ingredients, which are commonly used to enhance certain physicochemical properties like gelling strength, water-binding capacity, or emulsification. While such additives may be permitted under general food safety regulations, they are incompatible with Halal principles and raise ethical concerns regarding food integrity and consumer trust.

Commercial surimi products often contain a variety of protein-based additives, and the use of CBP is not uncommon in formulations aimed at improving texture and shelf-life. This makes it difficult to determine Halal status based solely on labeling or supply chain documentation. Therefore, the ability of the immunosensor to detect Apo AI directly in finished commercial products provides a critical advantage for point-of-need screening applications. Its portability, cost-effectiveness, and rapid analysis time make it particularly suitable for use in Halal inspection points, manufacturing audits, or

border control scenarios. When combined with confirmatory methods such as LC-QTOF-MS, the immunosensor could serve as an efficient front-line tool, expediting the verification process and ensuring consumer confidence in the authenticity and safety of food products.

In conclusion, the evaluation of the electrochemical immunosensor in commercial surimi samples underscores its potential as a rapid, reliable, and scalable solution for food safety and Halal compliance testing. The successful detection of Apo AI confirms the sensor's robustness under complex, matrix-intensive conditions and highlights its potential in uncovering illicit or undeclared CBP-derived adulterants. While the sensor's fibrinogen sensitivity remains a limitation, the insights gained from this study provide a solid foundation for further optimization. With continued refinement, this immunosensor could evolve into a comprehensive, multi-target platform for real-time food authentication, playing a significant role in consumer protection and maintaining ethical integrity in global food supply chains.

CHAPTER 5
CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 General Conclusions

This study successfully established an integrated analytical framework for the detection of CBP adulteration in surimi-based products through proteomic biomarker discovery and electrochemical immunosensor development.

To ensure reliable downstream analysis, protein extraction conditions were first optimized. The combination of ultrapure water extraction followed by acetone precipitation significantly enhanced protein enrichment from raw CBP. This dual-step strategy improved protein and peptide recovery compared to alternative protocols, ensuring sufficient analytical depth for subsequent mass spectrometry analysis and biomarker identification.

In fulfilment of the first objective, LC-QTOF-MS–based proteomic profiling identified Apo AI and fibrinogen as candidate protein biomarkers for CBP detection. Apo AI demonstrated strong detectability and analytical robustness, while fibrinogens were the supporting biomarker which gave optimum suitability for adulteration screening in food systems.

Addressing the third objective, bioinformatics sequence analysis was conducted to evaluate evolutionary conservation and discriminative capability. Apo AI exhibited high sequence identity with other terrestrial vertebrates, particularly avian species such as duck (92.42%) and moderate conservation with mammals such as pig (51.33%), supporting its suitability as a broadly applicable vertebrate plasma biomarker. Importantly, substantially lower sequence identity was observed between chicken and fish Apo AI homologues. This evolutionary divergence is critical in surimi-based matrices, as it minimizes potential cross-reactivity with endogenous fish proteins and reduces the risk of false-positive detection.

Comparative analysis of fibrinogen subunits (FGA, FGB, and FGG) revealed variable conservation across vertebrates. Although fibrinogen remains a valuable complementary marker, its conservation pattern is more heterogeneous than Apo AI, indicating comparatively narrower universality. Collectively, the bioinformatics

findings demonstrate that Apo AI provides a favourable balance between cross-vertebrate applicability and reliable discrimination from teleost species commonly used in surimi production.

In line with the second objective, immunoprecipitation techniques were successfully employed to extract and enrich Apo AI and fibrinogen from CBP. The proteins were recovered at concentrations of 0.7202 mg/mL and 0.3400 mg/mL, respectively, providing purified fractions for immunosensor construction and analytical validation.

Subsequently, an electrochemical immunosensor based on gold nanoparticles (AuNPs) was developed and evaluated. AuNPs synthesized via sodium citrate reduction produced nanoparticles with an average diameter of 18.0 ± 1.3 nm, as confirmed by TEM analysis. Their high surface area and favourable electrochemical properties enabled efficient antibody conjugation and signal amplification. The developed immunosensor, operating via differential pulse anodic stripping voltammetry (DPASV), demonstrated strong linearity for Apo AI across 50–1250 µg/mL (R^2 = 0.9931) and good linearity for fibrinogen within its defined working range of 500–1250 µg/mL (R^2 = 0.9886). Detection thresholds were statistically defined using the M + 3SD criterion, yielding cut-off values of 0.58 µA for Apo AI and 0.56 µA for fibrinogen.

Performance evaluation using spiked surimi samples confirmed reliable detection at CBP incorporation levels of 1.5%, 1%, and 0.5%. Comparative validation with LC-QTOF-MS further substantiated the analytical credibility of the developed platform. Screening of twelve commercial surimi products revealed three samples exceeding the Apo AI threshold, indicating potential CBP presence, while none surpassed the fibrinogen cut-off value.

Overall, this research demonstrates the successful integration of optimized protein extraction, proteomic biomarker discovery, bioinformatics validation, immunoprecipitation enrichment, and AuNP-based electrochemical sensing for CBP authentication. The developed immunosensor provides a rapid, sensitive, and portable alternative to conventional mass spectrometry approaches, offering a scientifically robust tool for food authenticity verification, halal compliance monitoring, and anti-adulteration enforcement.

5.2 Future Recommendation

Looking ahead, future studies should expand the scope to explore the presence of Apo AI in blood plasma from other species, such as cattle, pork, and goat, to evaluate its broader applicability as a plasma marker for detecting adulteration from various animal sources. Moreover, future research should prioritize the optimization of immunosensor design to improve sensitivity and reduce matrix effects in complex food matrices. Matrix interference remains a significant challenge, especially at lower concentrations of adulterants, and future work should aim to engineer more robust sensor surfaces to minimize non-specific binding. Additionally, the scalability and portability of the immunosensor should be prioritized to ensure its widespread adoption for real-time, on-site testing. Advances in sensor miniaturization and cost-effective manufacturing, including techniques such as inkjet printing or roll-to-roll fabrication, will facilitate global adoption across food industries.

Further research should also integrate epitope-informed antibody design to enhance the immunosensor's specificity and sensitivity. Complementary analytical platforms, including Western blot and targeted MRM proteomics, can provide orthogonal validation of target biomarkers and support a rigorous correlation between mass spectrometry-based discovery and immunosensor detection. In parallel, the development of multiplex electrochemical immunosensors could enable simultaneous detection of multiple plasma-derived markers, improving throughput and robustness in complex food matrices. Establishing a comprehensive validation pipeline linking these platforms will ensure consistency and reliability across detection methods.

Finally, in light of ethical, regulatory, and commercial considerations, affordability and accessibility must remain central to the development of this technology, ensuring that it is available to both large-scale producers and small-scale manufacturers, especially in developing regions. The environmental sustainability of these technologies must also be addressed, with an emphasis on green nanotechnology for AuNP synthesis and exploring the use of biodegradable sensor components to minimize environmental impact.

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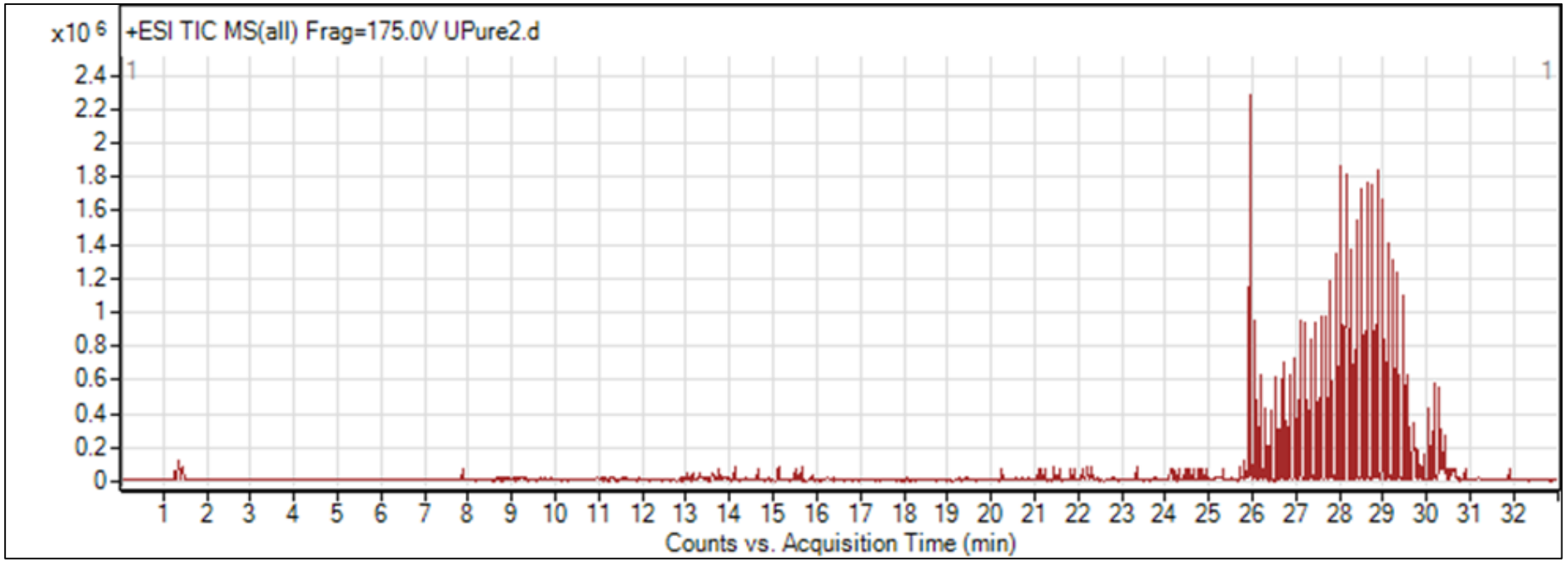
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APPENDICES

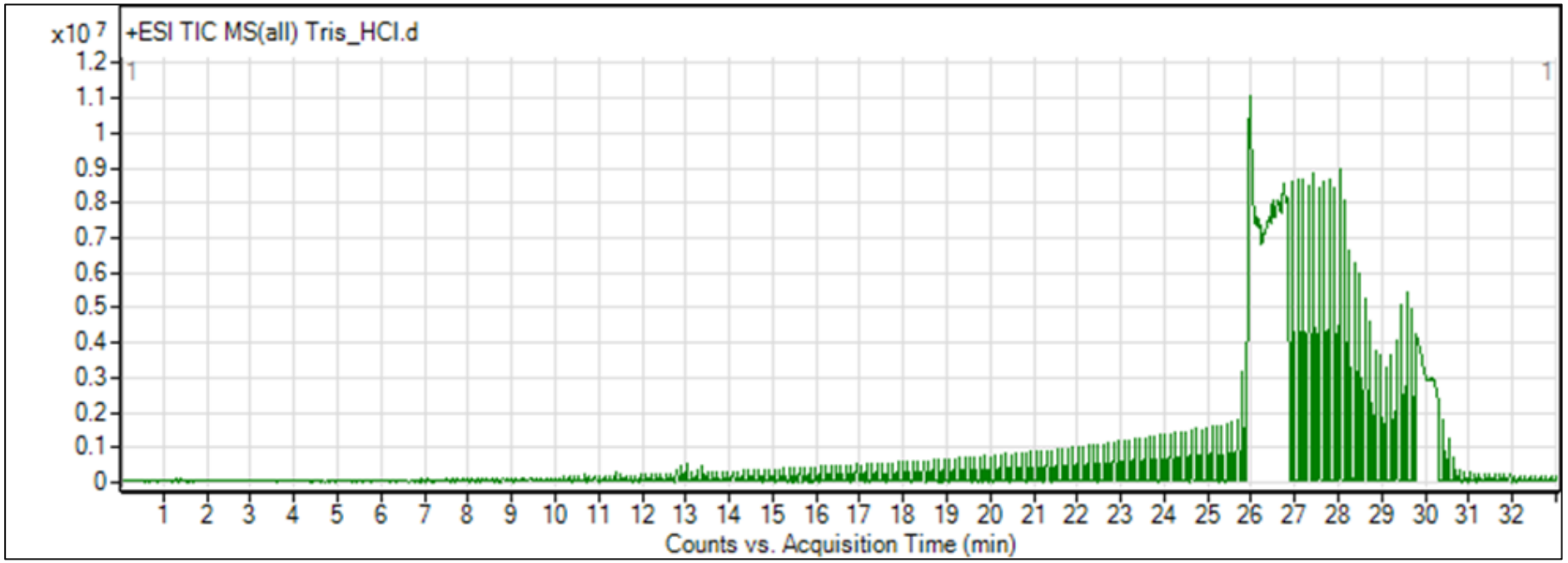
APPENDIX 1

IC of CBP in different extraction buffers

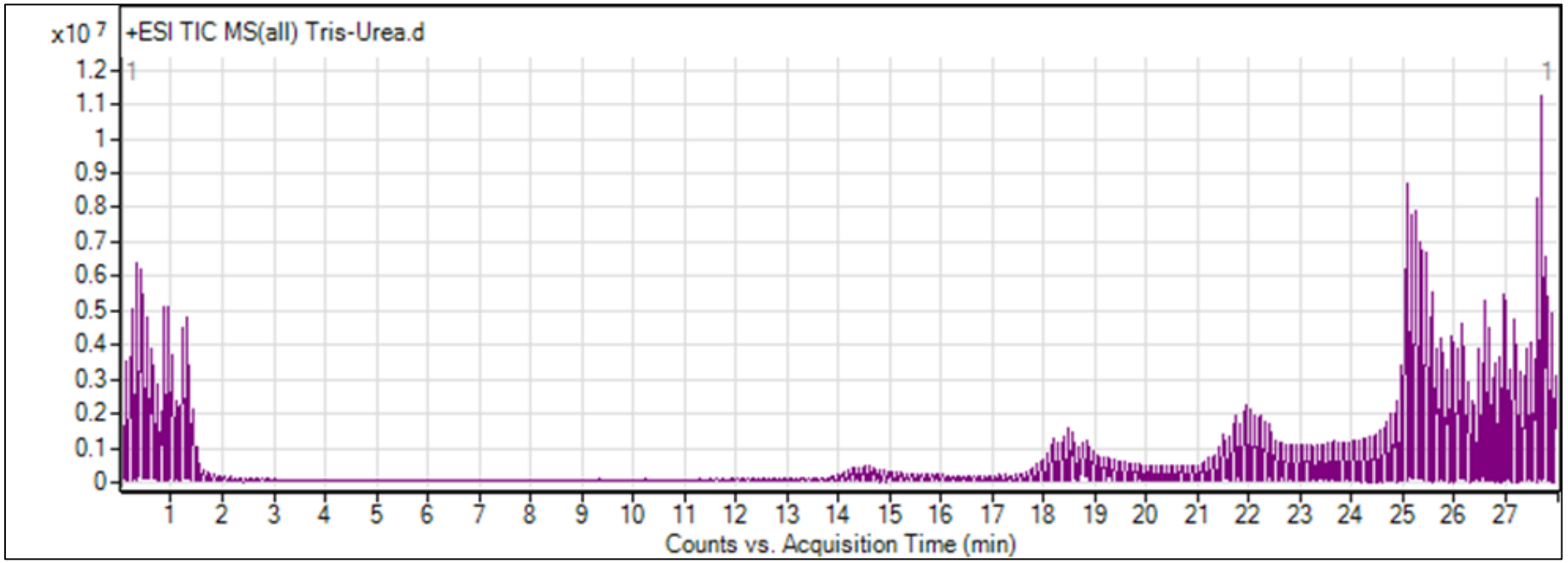
(a) ultrapure water, (b) 0.05 M Tris-HCl, (c) 0.05 M Tris-Urea, and (d) ultrapure water with acetone precipitation



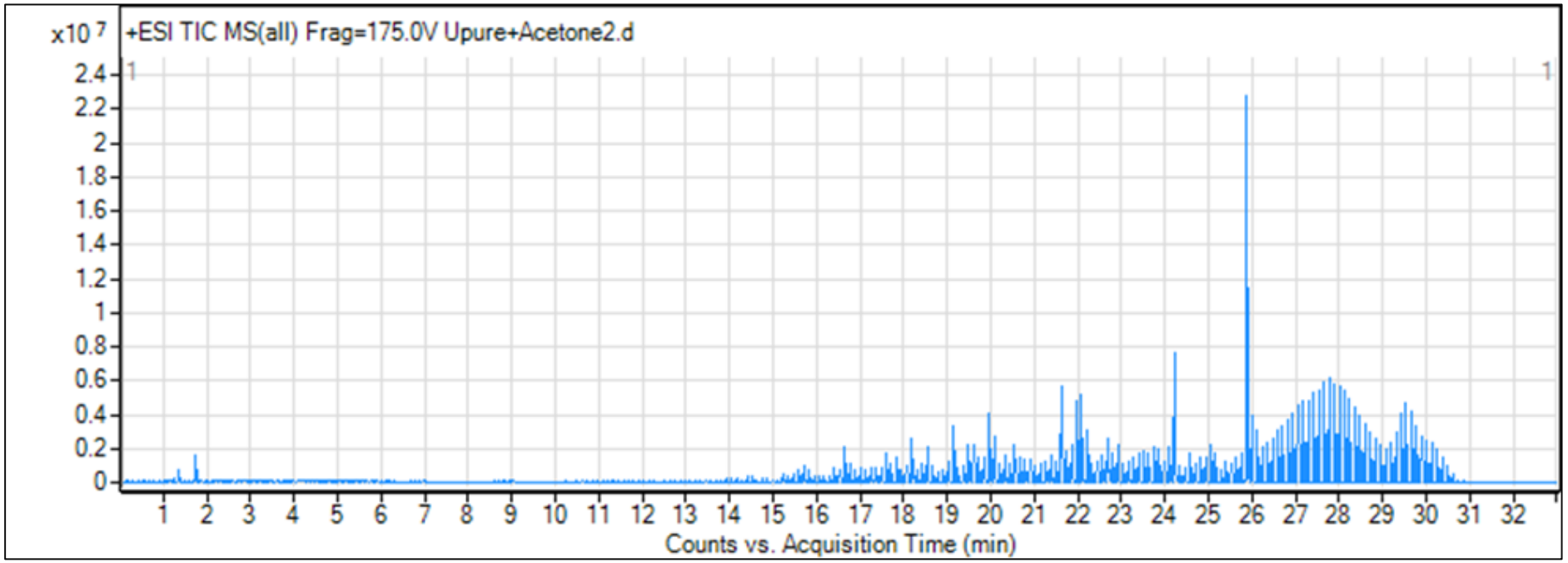
(a)


x10 7
+ESI TIC MS(all) Tris_HCl.d
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Counts vs. Acquisition Time (min)

(b)

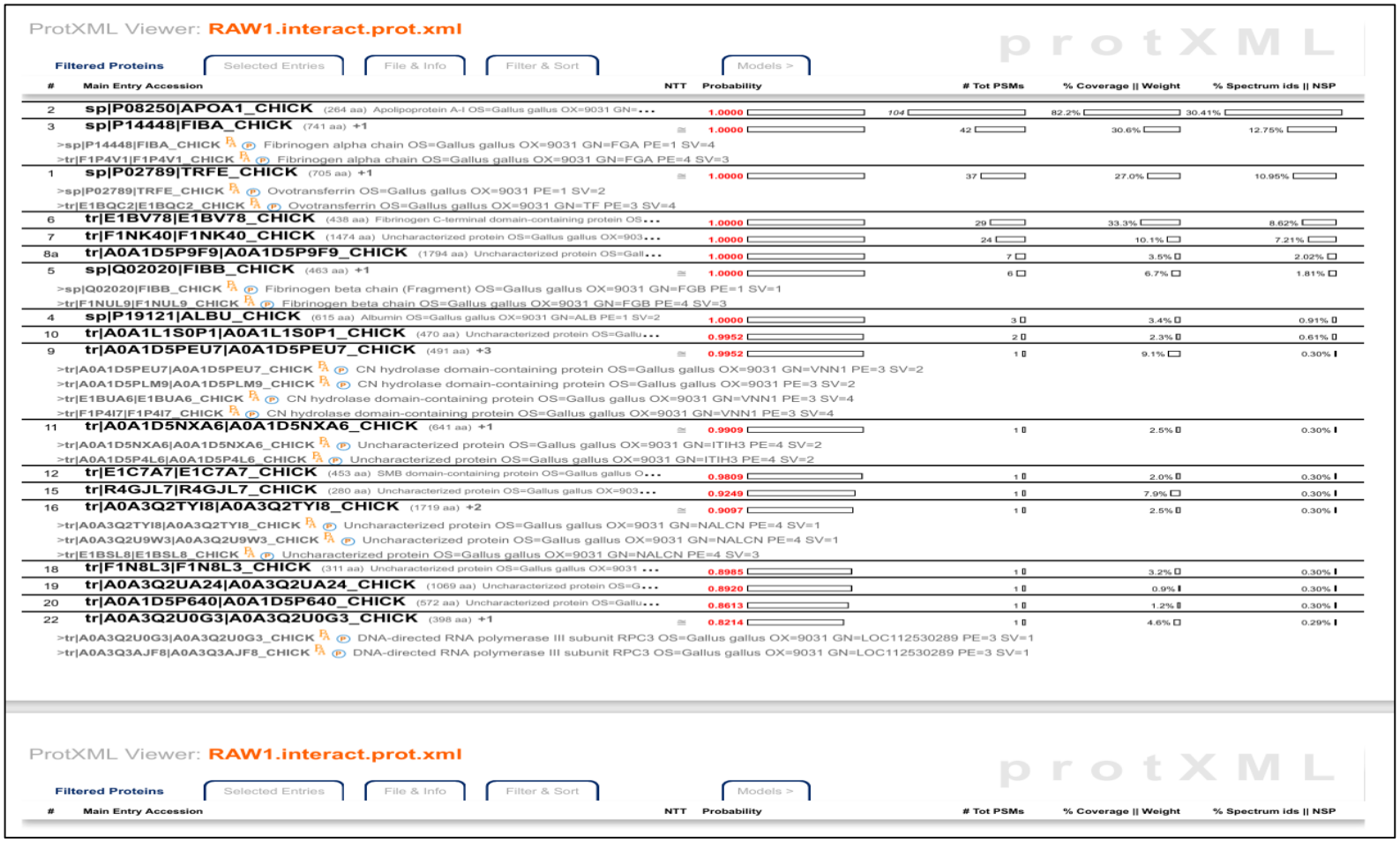

x10 7
+ESI TIC MS(all) Tris-Urea.d
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Counts vs. Acquisition Time (min)

(c)



(d)

Figure Appendix 1 : TIC of CBP in different extraction buffers (a) ultrapure water, (b) 0.05 M Tris-HCl, (c) 0.05 M Tris-Urea, and (d) ultrapure water with acetone precipitation



ProtXML Viewer: **RAW1.interact.prot.xml**

protXML

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#	Main Entry Accession	NTT	Probability	# Tot PSMs	% Coverage || Weight	% Spectrum ids || NSP
2	**sp|P08250|APOA1_CHICK** (264 aa) Apolipoprotein A-I OS=Gallus gallus OX=9031 GN=...		1.0000	104	82.2%	30.41%
3	**sp|P14448|FIBA_CHICK** (741 aa) +1	≅	1.0000	42	30.6%	12.75%
	>sp|P14448|FIBA_CHICK Fibrinogen alpha chain OS=Gallus gallus OX=9031 GN=FGA PE=1 SV=4					
	>tr|F1P4V1|F1P4V1_CHICK Fibrinogen alpha chain OS=Gallus gallus OX=9031 GN=FGA PE=4 SV=3					
1	**sp|P02789|TRFE_CHICK** (705 aa) +1	≅	1.0000	37	27.0%	10.95%
	>sp|P02789|TRFE_CHICK Ovotransferrin OS=Gallus gallus OX=9031 PE=1 SV=2					
	>tr|E1BQC2|E1BQC2_CHICK Ovotransferrin OS=Gallus gallus OX=9031 GN=TF PE=3 SV=4					
6	**tr|E1BV78|E1BV78_CHICK** (438 aa) Fibrinogen C-terminal domain-containing protein OS...		1.0000	29	33.3%	8.62%
7	**tr|F1NK40|F1NK40_CHICK** (1474 aa) Uncharacterized protein OS=Gallus gallus OX=903...		1.0000	24	10.1%	7.21%
8a	**tr|A0A1D5P9F9|A0A1D5P9F9_CHICK** (1794 aa) Uncharacterized protein OS=Gall...		1.0000	7	3.5%	2.02%
5	**sp|Q02020|FIBB_CHICK** (463 aa) +1	≅	1.0000	6	6.7%	1.81%
	>sp|Q02020|FIBB_CHICK Fibrinogen beta chain (Fragment) OS=Gallus gallus OX=9031 GN=FGB PE=1 SV=1					
	>tr|F1NUL9|F1NUL9_CHICK Fibrinogen beta chain OS=Gallus gallus OX=9031 GN=FGB PE=4 SV=3					
4	**sp|P19121|ALBU_CHICK** (615 aa) Albumin OS=Gallus gallus OX=9031 GN=ALB PE=1 SV=2		1.0000	3	3.4%	0.91%
10	**tr|A0A1L1S0P1|A0A1L1S0P1_CHICK** (470 aa) Uncharacterized protein OS=Gallu...		0.9952	2	2.3%	0.61%
9	**tr|A0A1D5PEU7|A0A1D5PEU7_CHICK** (491 aa) +3	≅	0.9952	1	9.1%	0.30%
	>tr|A0A1D5PEU7|A0A1D5PEU7_CHICK CN hydrolase domain-containing protein OS=Gallus gallus OX=9031 GN=VNN1 PE=3 SV=2					
	>tr|A0A1D5PLM9|A0A1D5PLM9_CHICK CN hydrolase domain-containing protein OS=Gallus gallus OX=9031 PE=3 SV=2					
	>tr|E1BUA6|E1BUA6_CHICK CN hydrolase domain-containing protein OS=Gallus gallus OX=9031 GN=VNN1 PE=3 SV=4					
	>tr|F1P4I7|F1P4I7_CHICK CN hydrolase domain-containing protein OS=Gallus gallus OX=9031 GN=VNN1 PE=3 SV=4					
11	**tr|A0A1D5NXA6|A0A1D5NXA6_CHICK** (641 aa) +1	≅	0.9909	1	2.5%	0.30%
	>tr|A0A1D5NXA6|A0A1D5NXA6_CHICK Uncharacterized protein OS=Gallus gallus OX=9031 GN=ITIH3 PE=4 SV=2					
	>tr|A0A1D5P4L6|A0A1D5P4L6_CHICK Uncharacterized protein OS=Gallus gallus OX=9031 GN=ITIH3 PE=4 SV=2					
12	**tr|E1C7A7|E1C7A7_CHICK** (453 aa) SMB domain-containing protein OS=Gallus gallus O...		0.9809	1	2.0%	0.30%
15	**tr|R4GJL7|R4GJL7_CHICK** (280 aa) Uncharacterized protein OS=Gallus gallus OX=903...		0.9249	1	7.9%	0.30%
16	**tr|A0A3Q2TYI8|A0A3Q2TYI8_CHICK** (1719 aa) +2	≅	0.9097	1	2.5%	0.30%
	>tr|A0A3Q2TYI8|A0A3Q2TYI8_CHICK Uncharacterized protein OS=Gallus gallus OX=9031 GN=NALCN PE=4 SV=1					
	>tr|A0A3Q2U9W3|A0A3Q2U9W3_CHICK Uncharacterized protein OS=Gallus gallus OX=9031 GN=NALCN PE=4 SV=1					
	>tr|E1BSL8|E1BSL8_CHICK Uncharacterized protein OS=Gallus gallus OX=9031 GN=NALCN PE=4 SV=3					
18	**tr|F1N8L3|F1N8L3_CHICK** (311 aa) Uncharacterized protein OS=Gallus gallus OX=9031 ...		0.8985	1	3.2%	0.30%
19	**tr|A0A3Q2UA24|A0A3Q2UA24_CHICK** (1069 aa) Uncharacterized protein OS=G...		0.8920	1	0.9%	0.30%
20	**tr|A0A1D5P640|A0A1D5P640_CHICK** (572 aa) Uncharacterized protein OS=Gallu...		0.8613	1	1.2%	0.30%
22	**tr|A0A3Q2U0G3|A0A3Q2U0G3_CHICK** (398 aa) +1	≅	0.8214	1	4.6%	0.29%
	>tr|A0A3Q2U0G3|A0A3Q2U0G3_CHICK DNA-directed RNA polymerase III subunit RPC3 OS=Gallus gallus OX=9031 GN=LOC112530289 PE=3 SV=1					
	>tr|A0A3Q3AJF8|A0A3Q3AJF8_CHICK DNA-directed RNA polymerase III subunit RPC3 OS=Gallus gallus OX=9031 GN=LOC112530289 PE=3 SV=1					

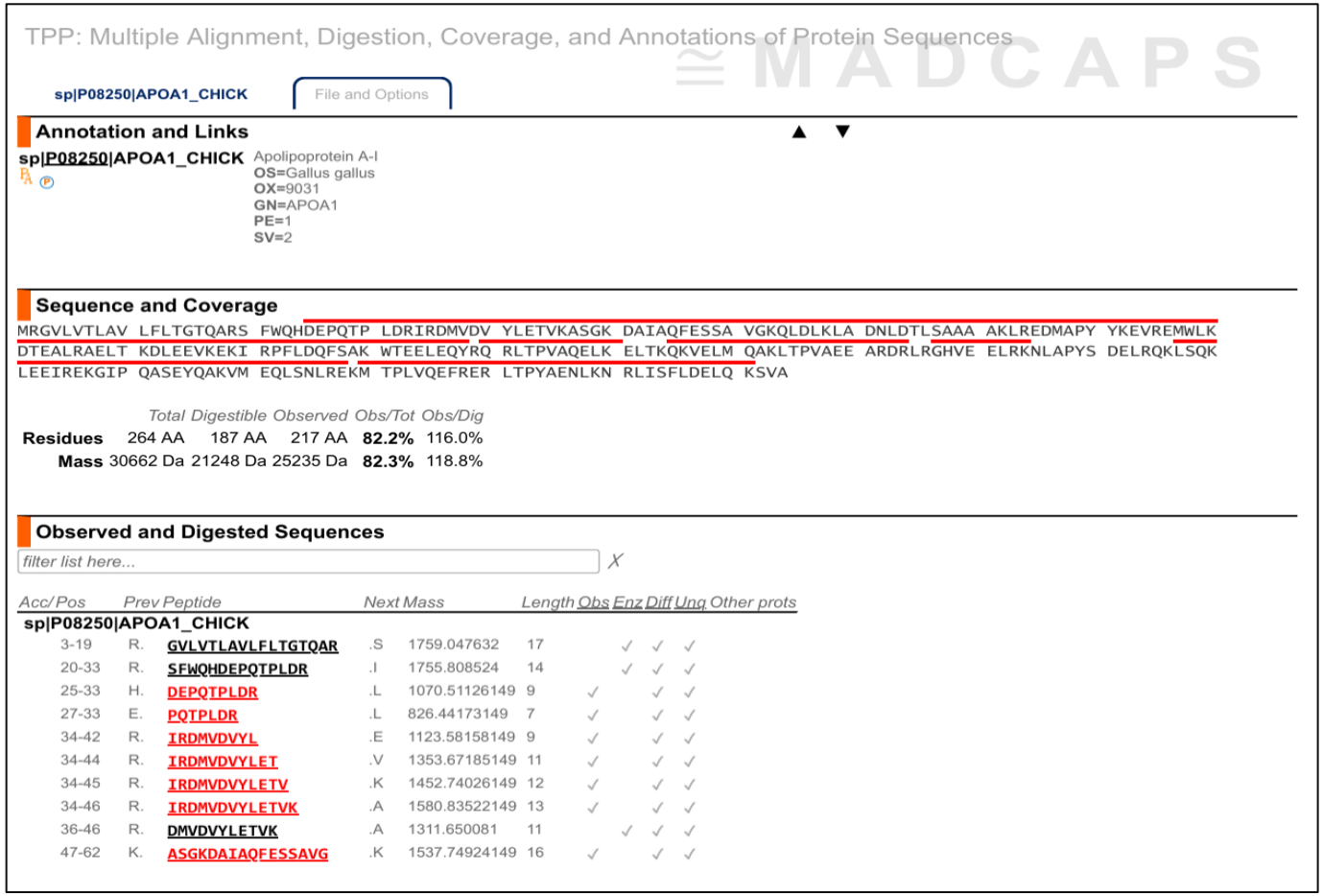
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Main Entry Accession NTT Probability # Tot PSMs % Coverage || Weight % Spectrum ids || NSP

Multiple alignment, Coverage and Annotations of Proteins sequence for ApoAI



TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences

≅MADCAPS

sp|P08250|APOA1_CHICK | File and Options

Annotation and Links

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OS=Gallus gallus
OX=9031
GN=APOA1
PE=1
SV=2

Sequence and Coverage

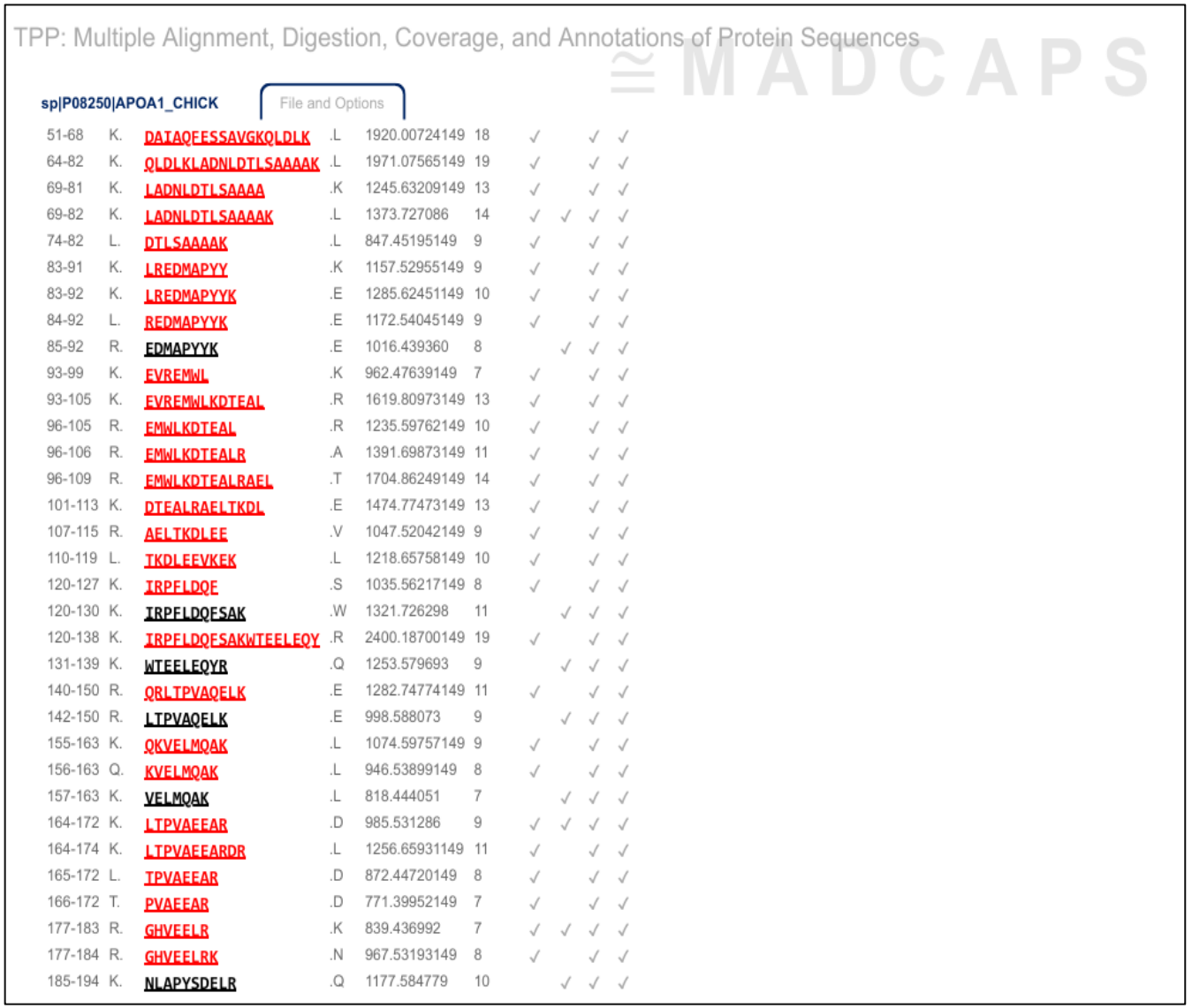
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DTEALRAELT KDLEEVKEKI RPFLDQFSAK WTEELEQYRQ RLTPVAQELK ELTKQKVELM QAKLTPVAEE ARDRLRGHVE ELRKNLAPYS DELRQKLSQK
LEEIREKGIP QASEYQAKVM EQLSNLREKM TPLVQEFRER LTPYAENLKN RLISFLDELQ KSVA

	Total	Digestible	Observed	Obs/Tot	Obs/Dig
Residues	264 AA	187 AA	217 AA	**82.2%**	116.0%
Mass	30662 Da	21248 Da	25235 Da	**82.3%**	118.8%

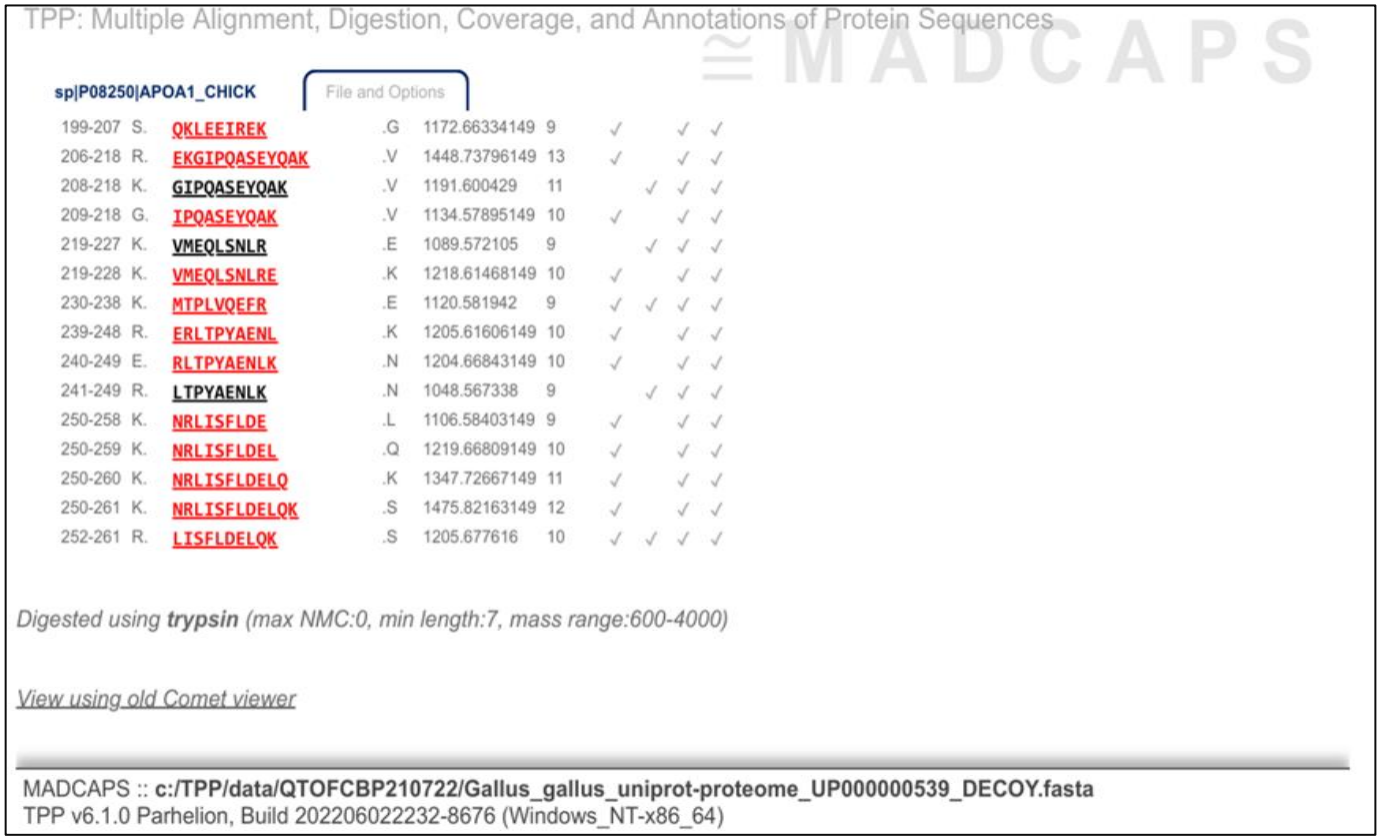
Observed and Digested Sequences

filter list here... ✗

Acc/Pos	Prev	Peptide	Next	Mass	Length	Obs	Enz	Diff	Unq	Other prots
sp|P08250|APOA1_CHICK										
3-19	R.	GVLVTLAVLFLTGTQAR	.S	1759.047632	17		✓	✓	✓	
20-33	R.	SFWQHDEPQTPLDR	.I	1755.808524	14		✓	✓	✓	
25-33	H.	DEPQTPLDR	.L	1070.51126149	9	✓		✓	✓	
27-33	E.	PQTPLDR	.L	826.44173149	7	✓		✓	✓	
34-42	R.	IRDMVDVYL	.E	1123.58158149	9	✓		✓	✓	
34-44	R.	IRDMVDVYLET	.V	1353.67185149	11	✓		✓	✓	
34-45	R.	IRDMVDVYLETV	.K	1452.74026149	12	✓		✓	✓	
34-46	R.	IRDMVDVYLETVK	.A	1580.83522149	13	✓		✓	✓	
36-46	R.	DMVDVYLETVK	.A	1311.650081	11		✓	✓	✓	
47-62	K.	ASGKDAIAQFESSAVG	.K	1537.74924149	16	✓		✓	✓	

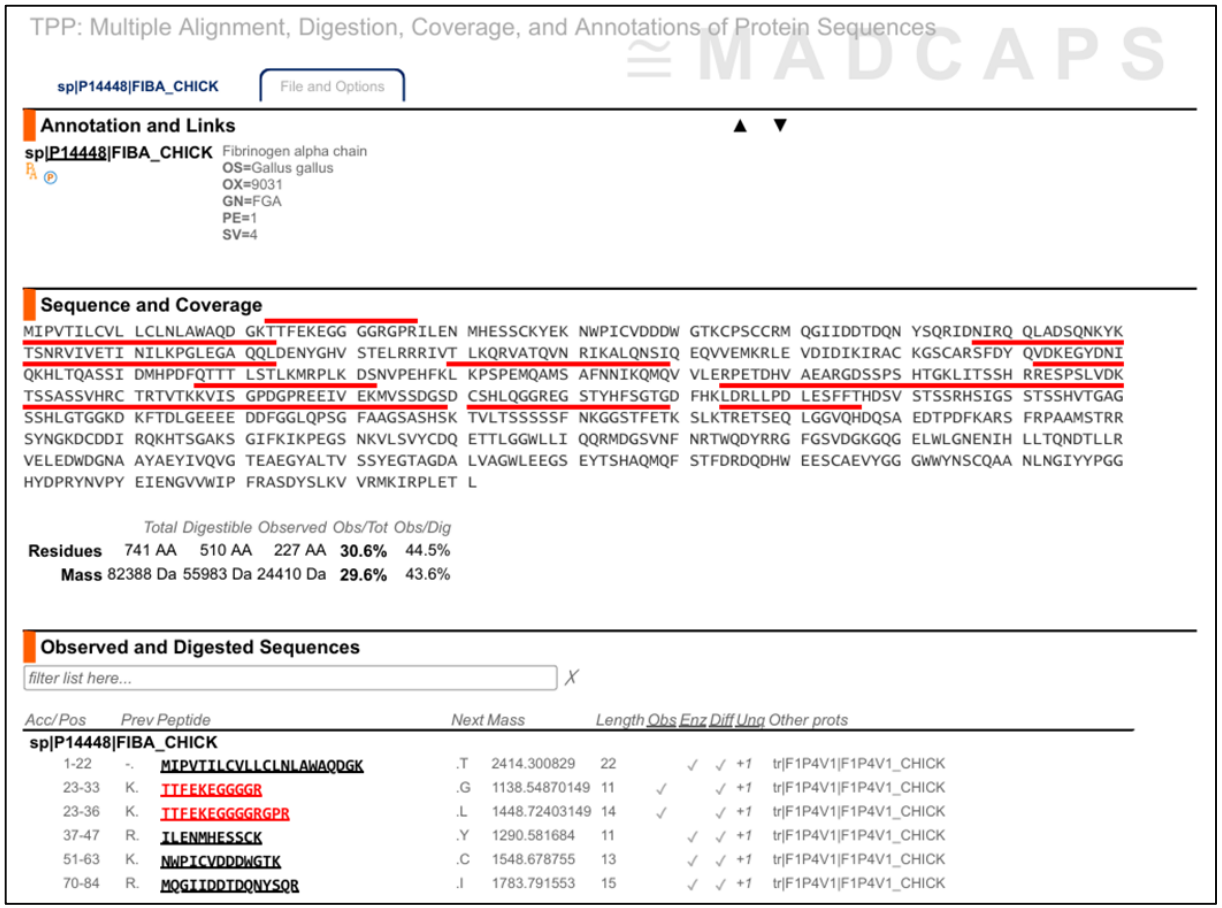


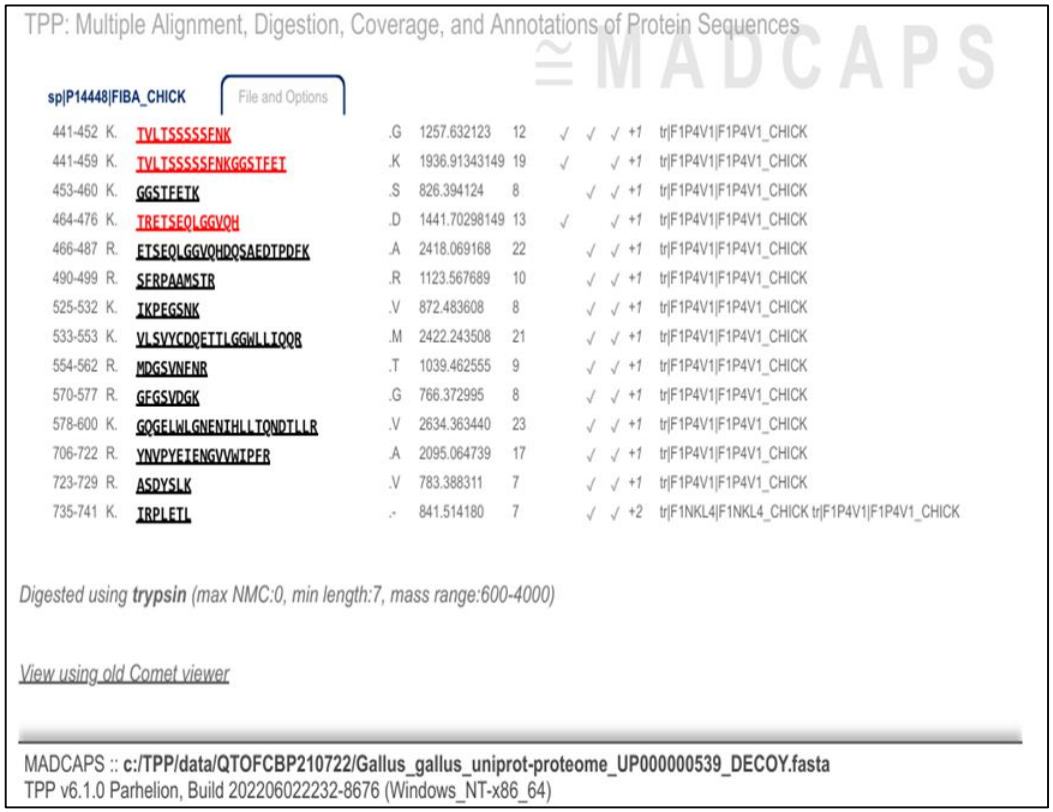
51-68	K.	DAIAQEESSAVGKQLDLK	.L	1920.00724149	18	✓		✓	✓
64-82	K.	QLDLKLADNLDTLSAAAAK	.L	1971.07565149	19	✓		✓	✓
69-81	K.	LADNLDTLSAAAA	.K	1245.63209149	13	✓		✓	✓
69-82	K.	LADNLDTLSAAAAK	.L	1373.727086	14	✓	✓	✓	✓
74-82	L.	DTLSAAAAK	.L	847.45195149	9	✓		✓	✓
83-91	K.	LREDMAPYY	.K	1157.52955149	9	✓		✓	✓
83-92	K.	LREDMAPYYK	.E	1285.62451149	10	✓		✓	✓
84-92	L.	REDMAPYYK	.E	1172.54045149	9	✓		✓	✓
85-92	R.	EDMAPYYK	.E	1016.439360	8		✓	✓	✓
93-99	K.	EVREMWL	.K	962.47639149	7	✓		✓	✓
93-105	K.	EVREMWLKDTEAL	.R	1619.80973149	13	✓		✓	✓
96-105	R.	EMWLKDTEAL	.R	1235.59762149	10	✓		✓	✓
96-106	R.	EMWLKDTEALR	.A	1391.69873149	11	✓		✓	✓
96-109	R.	EMWLKDTEALRAEL	.T	1704.86249149	14	✓		✓	✓
101-113	K.	DTEALRAELTKDL	.E	1474.77473149	13	✓		✓	✓
107-115	R.	AELTKDLEE	.V	1047.52042149	9	✓		✓	✓
110-119	L.	TKDLEEVKEK	.L	1218.65758149	10	✓		✓	✓
120-127	K.	IRPELDQE	.S	1035.56217149	8	✓		✓	✓
120-130	K.	IRPELDQESAK	.W	1321.726298	11		✓	✓	✓
120-138	K.	IRPELDQESAKWTEELEQY	.R	2400.18700149	19	✓		✓	✓
131-139	K.	WTEELEQYR	.Q	1253.579693	9		✓	✓	✓
140-150	R.	QRLTPVAQELK	.E	1282.74774149	11	✓		✓	✓
142-150	R.	LTPVAQELK	.E	998.588073	9		✓	✓	✓
155-163	K.	QKVELMQAK	.L	1074.59757149	9	✓		✓	✓
156-163	Q.	KVELMQAK	.L	946.53899149	8	✓		✓	✓
157-163	K.	VELMQAK	.L	818.444051	7		✓	✓	✓
164-172	K.	LTPVAEEAR	.D	985.531286	9	✓	✓	✓	✓
164-174	K.	LTPVAEEARDR	.L	1256.65931149	11	✓		✓	✓
165-172	L.	TPVAEEAR	.D	872.44720149	8	✓		✓	✓
166-172	T.	PVAEEAR	.D	771.39952149	7	✓		✓	✓
177-183	R.	GHVEELR	.K	839.436992	7	✓	✓	✓	✓
177-184	R.	GHVEELRK	.N	967.53193149	8	✓		✓	✓
185-194	K.	NLAPYSDELR	.Q	1177.584779	10		✓	✓	✓

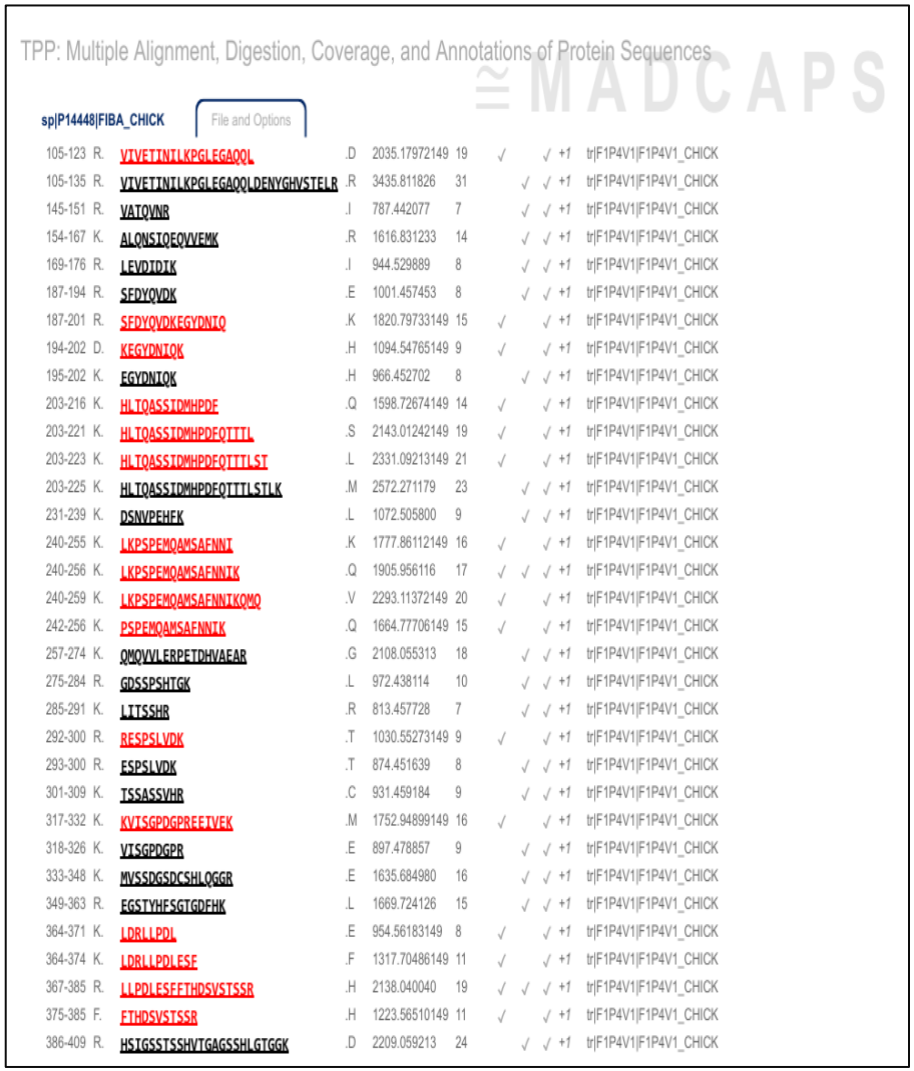

TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences
MADCAPS
sp|P08250|APOA1_CHICK
File and Options
199-207 S. QKLEEIREK .G 1172.66334149 9 ✓ ✓ ✓
206-218 R. EKGIPQASEYQAK .V 1448.73796149 13 ✓ ✓ ✓
208-218 K. GIPQASEYQAK .V 1191.600429 11 ✓ ✓ ✓
209-218 G. IPQASEYQAK .V 1134.57895149 10 ✓ ✓ ✓
219-227 K. VMEQLSNLR .E 1089.572105 9 ✓ ✓ ✓
219-228 K. VMEQLSNLRE .K 1218.61468149 10 ✓ ✓ ✓
230-238 K. MTPLVQEFR .E 1120.581942 9 ✓ ✓ ✓ ✓
239-248 R. ERLTPYAENL .K 1205.61606149 10 ✓ ✓ ✓
240-249 E. RLTPYAENLK .N 1204.66843149 10 ✓ ✓ ✓
241-249 R. LTPYAENLK .N 1048.567338 9 ✓ ✓ ✓
250-258 K. NRLISFLDE .L 1106.58403149 9 ✓ ✓ ✓
250-259 K. NRLISFLDEL .Q 1219.66809149 10 ✓ ✓ ✓
250-260 K. NRLISFLDELQ .K 1347.72667149 11 ✓ ✓ ✓
250-261 K. NRLISFLDELQK .S 1475.82163149 12 ✓ ✓ ✓
252-261 R. LISFLDELQK .S 1205.677616 10 ✓ ✓ ✓ ✓
Digested using trypsin (max NMC:0, min length:7, mass range:600-4000)
View using old Comet viewer
MADCAPS :: c:/TPP/data/QTOFCBP210722/Gallus_gallus_uniprot-proteome_UP000000539_DECOY.fasta
TPP v6.1.0 Parhelion, Build 202206022232-8676 (Windows_NT-x86_64)

APPENDIX 2

Multiple Alignment, Coverage and Annotations of Proteins Sequence for FGA

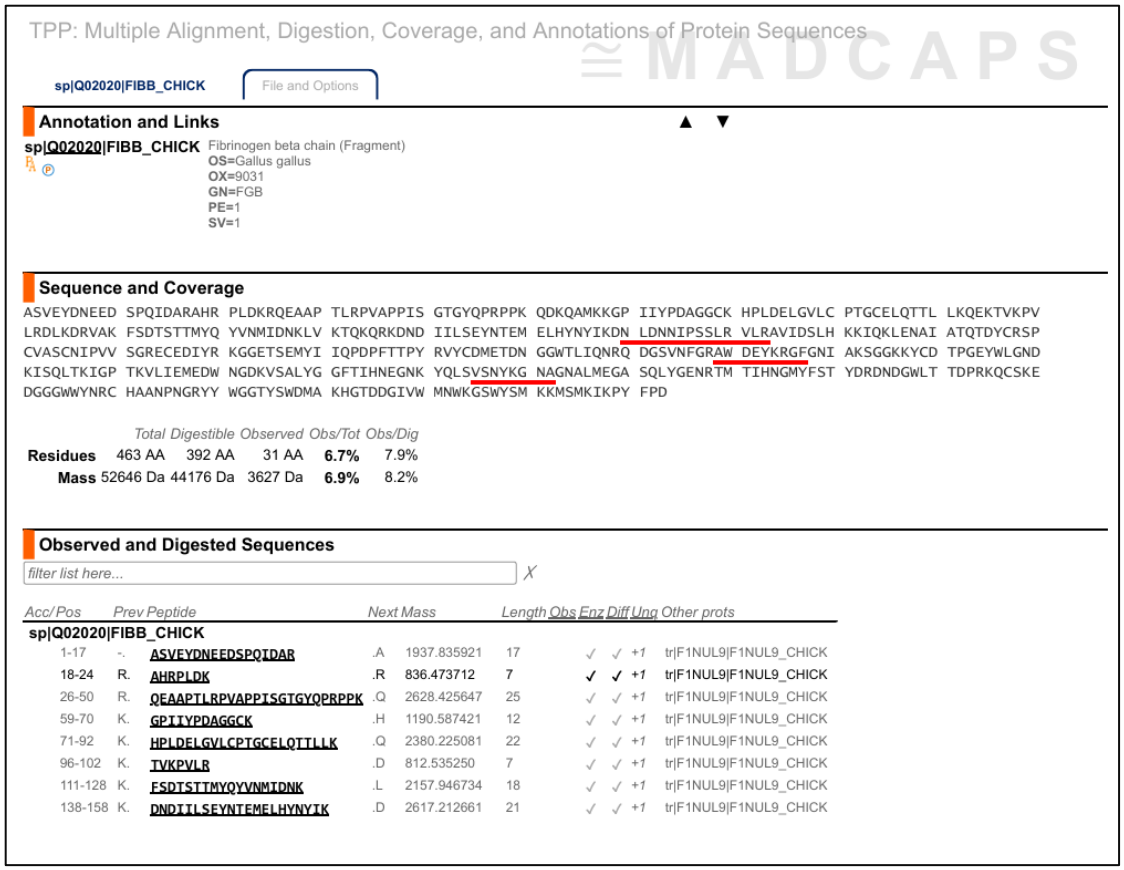



TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences
≅MADCAPS
sp|P14448|FIBA_CHICK
File and Options
441-452 K. TVLISSSSSFNK .G 1257.632123 12 √ √ √ +1 tr|F1P4V1|F1P4V1_CHICK
441-459 K. TVLISSSSSFNKGGSTFEI .K 1936.91343149 19 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
453-460 K. GGSTFEIK .S 826.394124 8 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
464-476 K. IREISEQLGGVQH .D 1441.70298149 13 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
466-487 R. EISEQLGGVQHDQSAEDIPDFK .A 2418.069168 22 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
490-499 R. SERPAAMSIR .R 1123.567689 10 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
525-532 K. IKPEGSNK .V 872.483608 8 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
533-553 K. VLSVYCDQETILGGWLLIQQR .M 2422.243508 21 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
554-562 R. MDGSVNFNR .T 1039.462555 9 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
570-577 R. GFGSVDGK .G 766.372995 8 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
578-600 K. GQGELWLGNENIHLLIQNDILLR .V 2634.363440 23 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
706-722 R. YNVPYEIENGVVWIPFR .A 2095.064739 17 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
723-729 R. ASDYSLK .V 783.388311 7 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
735-741 K. IRPLETL .- 841.514180 7 √ √ +2 tr|F1NKL4|F1NKL4_CHICK tr|F1P4V1|F1P4V1_CHICK
Digested using trypsin (max NMC:0, min length:7, mass range:600-4000)
View using old Comet viewer
MADCAPS :: c:/TPP/data/QTOFCBP210722/Gallus_gallus_uniprot-proteome_UP000000539_DECOY.fasta
TPP v6.1.0 Parhelion, Build 202206022232-8676 (Windows_NT-x86_64)


TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences
MADCAPS
sp|P14448|FIBA_CHICK
File and Options
105-123 R. VIVETINILKPGLEGAQQL .D 2035.17972149 19 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
105-135 R. VIVETINILKPGLEGAQQLDENYGHVSTELR .R 3435.811826 31 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
145-151 R. VATQVNR .I 787.442077 7 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
154-167 K. ALQNSIQEQVVEMK .R 1616.831233 14 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
169-176 R. LEVDIDIK .I 944.529889 8 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
187-194 R. SEDYQVDK .E 1001.457453 8 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
187-201 R. SEDYQVDKEGYDNIQ .K 1820.79733149 15 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
194-202 D. KEGYDNIQK .H 1094.54765149 9 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
195-202 K. EGYDNIQK .H 966.452702 8 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
203-216 K. HLTQASSIDMHPDF .Q 1598.72674149 14 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
203-221 K. HLTQASSIDMHPDFQTITL .S 2143.01242149 19 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
203-223 K. HLTQASSIDMHPDFQTITLST .L 2331.09213149 21 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
203-225 K. HLTQASSIDMHPDFQTITLSILK .M 2572.271179 23 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
231-239 K. DSNVPEHFK .L 1072.505800 9 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
240-255 K. LKPSPEMQAMSAFNNI .K 1777.86112149 16 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
240-256 K. LKPSPEMQAMSAFNNIK .Q 1905.956116 17 √ √ √ +1 tr|F1P4V1|F1P4V1_CHICK
240-259 K. LKPSPEMQAMSAFNNIKQMQ .V 2293.11372149 20 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
242-256 K. PSPEMQAMSAFNNIK .Q 1664.77706149 15 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
257-274 K. QMQVVLERPEIDHVAEAR .G 2108.055313 18 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
275-284 R. GDSSPSHTGK .L 972.438114 10 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
285-291 K. LITSSHR .R 813.457728 7 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
292-300 R. RESPSLVDK .T 1030.55273149 9 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
293-300 R. ESPSLVDK .T 874.451639 8 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
301-309 K. TSSASSVHR .C 931.459184 9 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
317-332 K. KVISGPDGPREEIVEK .M 1752.94899149 16 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
318-326 K. VISGPDGPR .E 897.478857 9 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
333-348 K. MVSSDGSDCSHLQGGR .E 1635.684980 16 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
349-363 R. EGSTYHFSGTGDFHK .L 1669.724126 15 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
364-371 K. LDRLLPDL .E 954.56183149 8 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
364-374 K. LDRLLPDLESF .F 1317.70486149 11 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
367-385 R. LLPDLESFETHDSVSTSSR .H 2138.040040 19 √ √ √ +1 tr|F1P4V1|F1P4V1_CHICK
375-385 F. ETHDSVSTSSR .H 1223.56510149 11 √ √ +1 tr|F1P4V1|F1P4V1_CHICK
386-409 R. HSTGSSTSSHVTGAGSSHLGTGGK .D 2209.059213 24 √ √ +1 tr|F1P4V1|F1P4V1_CHICK

APPENDIX 3

Multiple Alignment, Coverage and Annotations of Proteins Sequence for FGB



TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences

≅MADCAPS

sp|Q02020|FIBB_CHICK | File and Options

Annotation and Links ▲ ▼

sp|Q02020|FIBB_CHICK Fibrinogen beta chain (Fragment)
OS=Gallus gallus
OX=9031
GN=FGB
PE=1
SV=1

Sequence and Coverage

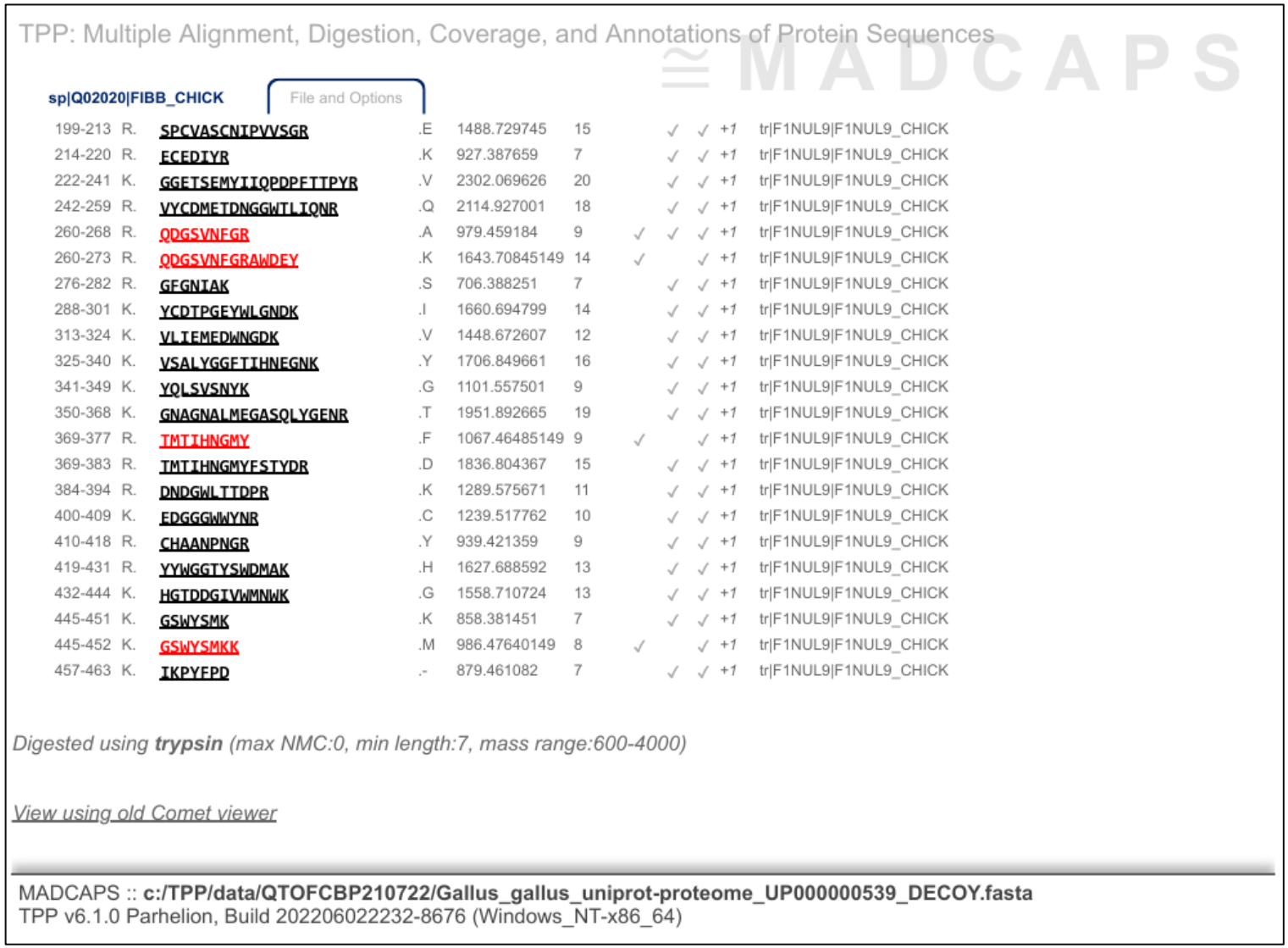
ASVEYDNEED SPQIDARAHR PLDKRQEAAP TLRPVAPPIS GTGYQPRPPK QDKQAMKKGP IIYPDAGGCK HPLDELGVLC PTGCELQTTL LKQEKTVKPV
LRDLKDRVAK FSDTSTTMYQ YVNMIDNKLV KTQKQRKDND IILSEYNTEM ELHYNYIKDN LDNNIPSSLR VLRAVIDSLH KKIQKLENAI ATQTDYCRSP
CVASCNIPVV SGRECEDIYR KGGETSEMYI IQPDPFTTPY RVYCDMETDN GGWTLIQNRQ DGSVNFGRAW DEYKRGFGNI AKSGGKKYCD TPGEYWLGND
KISQLTKIGP TKVLIEMEDW NGDKVSALYG GFTIHNEGNK YQLSVSNYKG NAGNALMEGA SQLYGENRTM TIHNGMYFST YDRDNDGWLT TDPRKQCSKE
DGGGWWYNRC HAANPNGRYY WGGTYSWDMA KHGTDDGIVW MNWKGSWYSM KKMSMKIKPY FPD

	Total	*Digestible*	*Observed*	*Obs/Tot*	*Obs/Dig*
Residues	463 AA	392 AA	31 AA	**6.7%**	7.9%
Mass	52646 Da	44176 Da	3627 Da	**6.9%**	8.2%

Observed and Digested Sequences

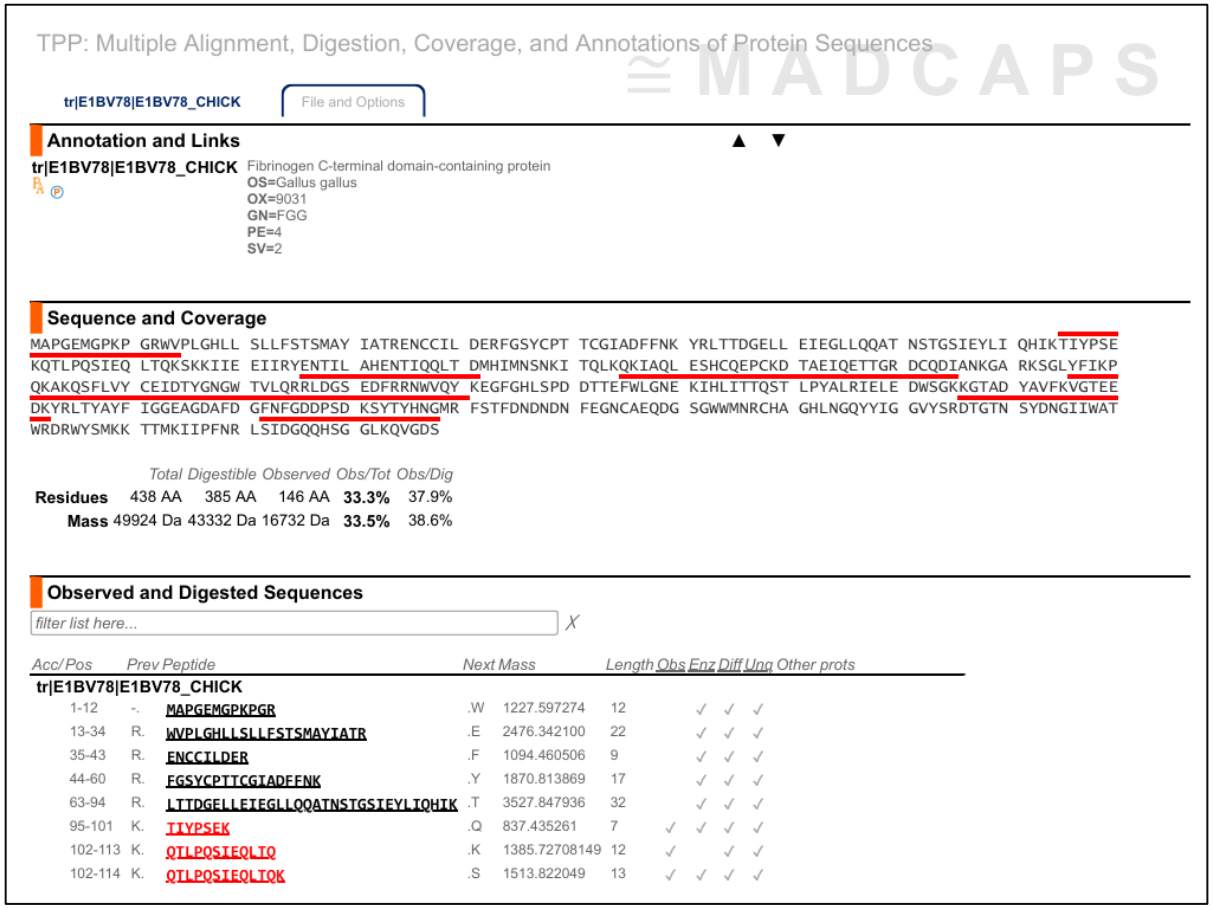
filter list here... ✗

Acc/Pos	*Prev*	*Peptide*	*Next*	*Mass*	*Length*	*Obs*	*Enz*	*Diff*	*Ung*	*Other prots*
sp|Q02020|FIBB_CHICK										
1-17	-.	ASVEYDNEEDSPQIDAR	.A	1937.835921	17	✓	✓	+1		tr|F1NUL9|F1NUL9_CHICK
18-24	R.	AHRPLDK	.R	836.473712	7	✓	✓	+1		tr|F1NUL9|F1NUL9_CHICK
26-50	R.	QEAAPTLRPVAPPISGTGYQPRPPK	.Q	2628.425647	25	✓	✓	+1		tr|F1NUL9|F1NUL9_CHICK
59-70	K.	GPIIYPDAGGCK	.H	1190.587421	12	✓	✓	+1		tr|F1NUL9|F1NUL9_CHICK
71-92	K.	HPLDELGVLCPTGCELQTTLLK	.Q	2380.225081	22	✓	✓	+1		tr|F1NUL9|F1NUL9_CHICK
96-102	K.	TVKPVLR	.D	812.535250	7	✓	✓	+1		tr|F1NUL9|F1NUL9_CHICK
111-128	K.	FSDTSTTMYQYVNMIDNK	.L	2157.946734	18	✓	✓	+1		tr|F1NUL9|F1NUL9_CHICK
138-158	K.	DNDIILSEYNTEMELHYNYIK	.D	2617.212661	21	✓	✓	+1		tr|F1NUL9|F1NUL9_CHICK


TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences
MADCAPS
sp|Q02020|FIBB_CHICK
File and Options
199-213 R. SPCVASCNIPVVSGR .E 1488.729745 15 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
214-220 R. ECEDIYR .K 927.387659 7 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
222-241 K. GGETSEMYIIQPDPETIPYR .V 2302.069626 20 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
242-259 R. VYCDMETDNGGWTLIQNR .Q 2114.927001 18 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
260-268 R. QDGSVNFGR .A 979.459184 9 ✓ ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
260-273 R. QDGSVNFGRAWDEY .K 1643.70845149 14 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
276-282 R. GFGNIAK .S 706.388251 7 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
288-301 K. YCDTPGEYWLGNDK .I 1660.694799 14 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
313-324 K. VLIEMEDWNGDK .V 1448.672607 12 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
325-340 K. VSALYGGFTIHNEGNK .Y 1706.849661 16 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
341-349 K. YQLSVSNYK .G 1101.557501 9 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
350-368 K. GNAGNALMEGASQLYGENR .T 1951.892665 19 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
369-377 R. TMTIHNGMY .F 1067.46485149 9 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
369-383 R. TMTIHNGMYFSTYDR .D 1836.804367 15 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
384-394 R. DNDGWLTTDPR .K 1289.575671 11 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
400-409 K. EDGGGWWYNR .C 1239.517762 10 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
410-418 R. CHAANPNGR .Y 939.421359 9 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
419-431 R. YYWGGTYSWDMAK .H 1627.688592 13 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
432-444 K. HGTDDGIVWMNWK .G 1558.710724 13 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
445-451 K. GSWYSMK .K 858.381451 7 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
445-452 K. GSWYSMKK .M 986.47640149 8 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
457-463 K. IKPYFPD .- 879.461082 7 ✓ ✓ +1 tr|F1NUL9|F1NUL9_CHICK
Digested using trypsin (max NMC:0, min length:7, mass range:600-4000)
View using old Comet viewer
MADCAPS :: c:/TPP/data/QTOFCBP210722/Gallus_gallus_uniprot-proteome_UP000000539_DECOY.fasta
TPP v6.1.0 Parhelion, Build 202206022232-8676 (Windows_NT-x86_64)

APPENDIX 4

Multiple Alignment, Coverage and Annotations of Proteins sequence for FGC



TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences

≅MADCAPS

tr|E1BV78|E1BV78_CHICK | File and Options

Annotation and Links

tr|E1BV78|E1BV78_CHICK Fibrinogen C-terminal domain-containing protein
OS=Gallus gallus
OX=9031
GN=FGG
PE=4
SV=2

Sequence and Coverage

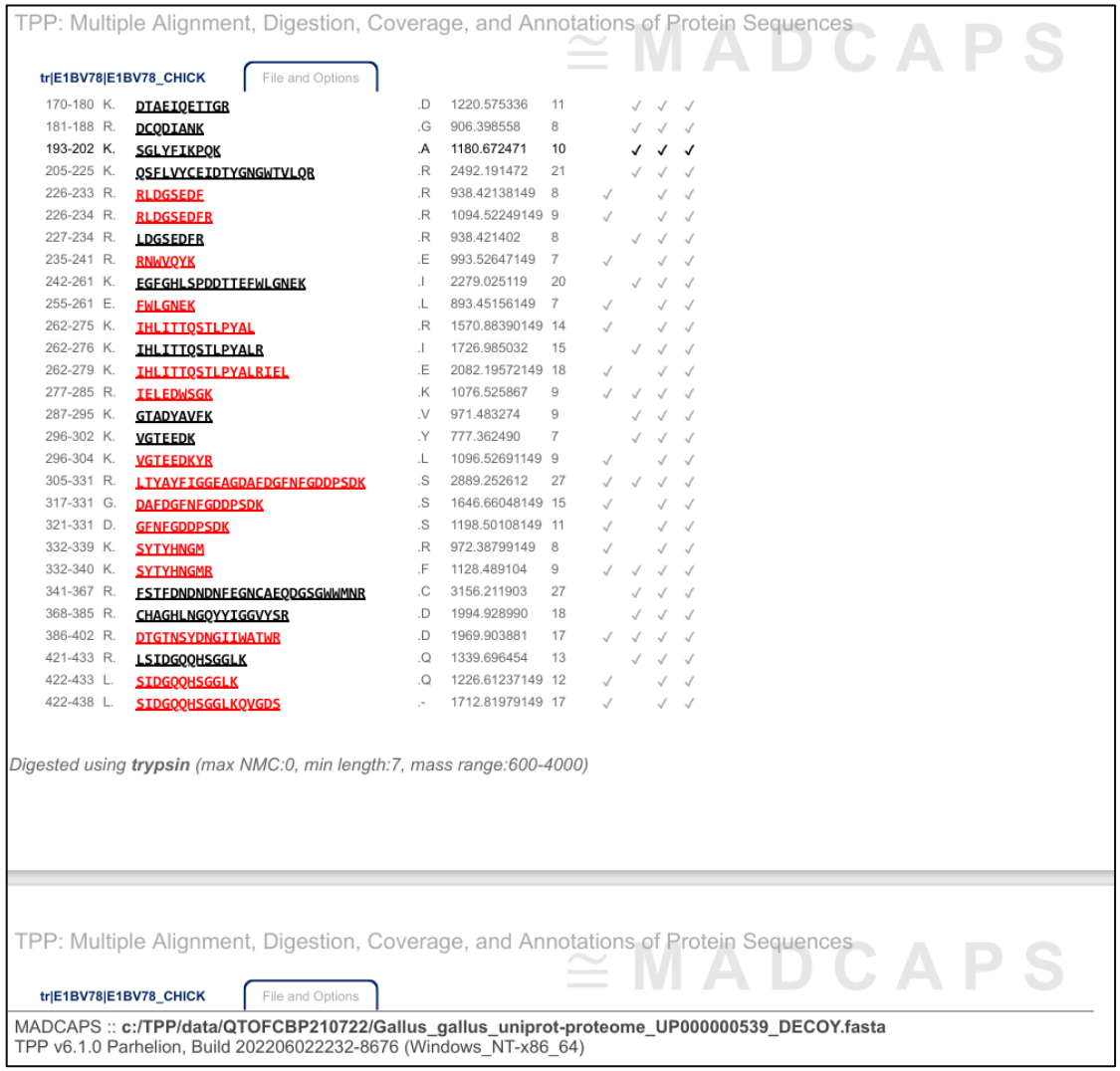
MAPGEMGPKP GRWVPLGHLL SLLFSTSMAY IATRENCCIL DERFGSYCPT TCGIADFFNK YRLTTDGELL EIEGLLQQAT NSTGSIEYLI QHIKTIYPSE
KQTLPQSIEQ LTQKSKKIIE EIIRYENTIL AHENTIQQLT DMHIMNSNKI TQLKQKIAQL ESHCQEPCKD TAEIQETTGR DCQDIANKGA RKSGLYFIKP
QKAKQSFLVY CEIDTYGNGW TVLQRRLDGS EDFRRNWVQY KEGFGHLSPD DTTEFWLGNE KIHLITTQST LPYALRIELE DWSGKKGTAD YAVFKVGTEE
DKYRLTYAYF IGGEAGDAFD GFNFGDDPSD KSYTYHNGMR FSTFDNDNDN FEGNCAEQDG SGWWMNRCHA GHLNGQYYIG GVYSRDTGTN SYDNGIIWAT
WRDRWYSMKK TTMKIIPFNR LSIDGQQHSG GLKQVGDS

	Total	Digestible	Observed	Obs/Tot	Obs/Dig
Residues	438 AA	385 AA	146 AA	**33.3%**	37.9%
Mass	49924 Da	43332 Da	16732 Da	**33.5%**	38.6%

Observed and Digested Sequences

filter list here...

Acc/Pos	Prev	Peptide	Next	Mass	Length	Obs	Enz	Diff	Ung	Other prots
tr|E1BV78|E1BV78_CHICK										
1-12	-.	MAPGEMGPKPGR	.W	1227.597274	12		✓	✓	✓	
13-34	R.	WVPLGHLLSLLFSTSMAYIATR	.E	2476.342100	22		✓	✓	✓	
35-43	R.	ENCCILDER	.F	1094.460506	9		✓	✓	✓	
44-60	R.	FGSYCPTTCGIADFFNK	.Y	1870.813869	17		✓	✓	✓	
63-94	R.	LTTDGELLEIEGLLQQATNSTGSIEYLIQHIK	.T	3527.847936	32		✓	✓	✓	
95-101	K.	TIYPSEK	.Q	837.435261	7	✓	✓	✓	✓	
102-113	K.	QTLPQSIEQLIQ	.K	1385.72708149	12	✓		✓	✓	
102-114	K.	QTLPQSIEQLIQK	.S	1513.822049	13	✓	✓	✓	✓	


TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences
MADCAPS
tr|E1BV78|E1BV78_CHICK
File and Options
170-180 K. DTAEIQETIGR .D 1220.575336 11 ✓ ✓ ✓
181-188 R. DCQDIANK .G 906.398558 8 ✓ ✓ ✓
193-202 K. SGLYFIKPQK .A 1180.672471 10 ✓ ✓ ✓
205-225 K. QSFLVYCEIDTYGNGWTVLQR .R 2492.191472 21 ✓ ✓ ✓
226-233 R. RLDGSEDF .R 938.42138149 8 ✓ ✓ ✓
226-234 R. RLDGSEDFR .R 1094.52249149 9 ✓ ✓ ✓
227-234 R. LDGSEDFR .R 938.421402 8 ✓ ✓ ✓
235-241 R. RNWVQYK .E 993.52647149 7 ✓ ✓ ✓
242-261 K. EGFGHLSPDDTTEFWLGNEK .I 2279.025119 20 ✓ ✓ ✓
255-261 E. FWLGNEK .L 893.45156149 7 ✓ ✓ ✓
262-275 K. IHLITTQSTLPYAL .R 1570.88390149 14 ✓ ✓ ✓
262-276 K. IHLITTQSTLPYALR .I 1726.985032 15 ✓ ✓ ✓
262-279 K. IHLITTQSTLPYALRIEL .E 2082.19572149 18 ✓ ✓ ✓
277-285 R. IELEDWSGK .K 1076.525867 9 ✓ ✓ ✓ ✓
287-295 K. GTADYAVFK .V 971.483274 9 ✓ ✓ ✓
296-302 K. VGTEEDK .Y 777.362490 7 ✓ ✓ ✓
296-304 K. VGTEEDKYR .L 1096.52691149 9 ✓ ✓ ✓
305-331 R. LTYAYFIGGEAGDAEDGENEGDDPSDK .S 2889.252612 27 ✓ ✓ ✓ ✓
317-331 G. DAEDGENEGDDPSDK .S 1646.66048149 15 ✓ ✓ ✓
321-331 D. GENEGDDPSDK .S 1198.50108149 11 ✓ ✓ ✓
332-339 K. SYTYHNGM .R 972.38799149 8 ✓ ✓ ✓
332-340 K. SYTYHNGMR .F 1128.489104 9 ✓ ✓ ✓ ✓
341-367 R. FSTFDNDNDNFEGNCAEQDGSGWWMNR .C 3156.211903 27 ✓ ✓ ✓
368-385 R. CHAGHLNGQYYIGGVYSR .D 1994.928990 18 ✓ ✓ ✓
386-402 R. DIGINSYDNGIIWATWR .D 1969.903881 17 ✓ ✓ ✓ ✓
421-433 R. LSIDGQQHSGGLK .Q 1339.696454 13 ✓ ✓ ✓
422-433 L. SIDGQQHSGGLK .Q 1226.61237149 12 ✓ ✓ ✓
422-438 L. SIDGQQHSGGLKQVGDS .- 1712.81979149 17 ✓ ✓ ✓
Digested using trypsin (max NMC:0, min length:7, mass range:600-4000)
TPP: Multiple Alignment, Digestion, Coverage, and Annotations of Protein Sequences
MADCAPS
tr|E1BV78|E1BV78_CHICK
File and Options
MADCAPS :: c:/TPP/data/QTOFCBP210722/Gallus_gallus_uniprot-proteome_UP000000539_DECOY.fasta
TPP v6.1.0 Parhelion, Build 202206022232-8676 (Windows_NT-x86_64)

APPENDIX 5

Percent Identity Matrix - created by Clustal2.1

```
#
#
#  Percent Identity  Matrix - created by Clustal2.1
#
#
```

1: ABY64539.1	100.00	15.38	12.50	16.92	15.38	13.04	13.85	18.46	23.08	23.08
2: AAI42073.1	15.38	100.00	63.22	32.67	36.17	21.34	26.01	28.00	31.58	30.24
3: XP_070102046.1	12.50	63.22	100.00	32.15	33.83	17.07	24.93	23.48	28.53	27.18
4: sp|P14448|FIBA_CHICK	16.92	32.67	32.15	100.00	83.99	26.67	25.08	41.22	32.06	43.34
5: XP_005021009.2	15.38	36.17	33.83	83.99	100.00	26.67	25.61	42.35	34.58	44.73
6: XP_042572819.1	13.04	21.34	17.07	26.67	26.67	100.00	24.24	22.29	21.95	21.95
7: XP_046693012.1	13.85	26.01	24.93	25.08	25.61	24.24	100.00	33.64	51.17	39.49
8: XP_056431720.1	18.46	28.00	23.48	41.22	42.35	22.29	33.64	100.00	52.27	57.44
9: XP_036842938.1	23.08	31.58	28.53	32.06	34.58	21.95	51.17	52.27	100.00	82.21
10: tr|A0A1S3SBW9|A0A1S3SBW9_SALSA	23.08	30.24	27.18	43.34	44.73	21.95	39.49	57.44	82.21	100.00

APPENDIX 6

Percent Identity Matrix - created by Clustal2.1

```
#
#
#  Percent Identity  Matrix - created by Clustal2.1
#
#
```

1: XP_005021009.2	100.00	31.06	30.42	29.87	29.87	29.79	30.11	29.25	32.75	29.94	30.15	30.00
2: XP_056431764.1	31.06	100.00	68.70	68.31	68.52	65.62	62.99	54.64	52.83	53.42	54.04	53.94
3: tr|A0A8C7QHC1|A0A8C7QHC1_ONCMY	30.42	68.70	100.00	93.47	94.11	70.32	68.29	55.60	57.29	53.38	53.38	53.59
4: tr|A0A1S3NAJ7|A0A1S3NAJ7_SALSA	29.87	68.31	93.47	100.00	98.97	69.64	67.36	55.29	58.02	54.47	54.47	55.00
5: tr|A0A674ALX8|A0A674ALX8_SALTR	29.87	68.52	94.11	98.97	100.00	70.09	67.56	55.08	57.78	54.26	54.26	54.79
6: tr|A0A9J8AZ34|A0A9J8AZ34_CYPCA	29.79	65.62	70.32	69.64	70.09	100.00	72.87	52.68	58.65	53.81	54.26	55.06
7: XP_046693367.1	30.11	62.99	68.29	67.36	67.56	72.87	100.00	55.31	58.56	54.07	54.07	55.23
8: sp|Q02020|FIBB_CHICK	29.25	54.64	55.60	55.29	55.08	52.68	55.31	100.00	68.91	67.10	65.58	65.73
9: XP_070102050.1	32.75	52.83	57.29	58.02	57.78	58.65	58.56	68.91	100.00	79.71	78.04	79.19
10: NP_001231042.1	29.94	53.42	53.38	54.47	54.26	53.81	54.07	67.10	79.71	100.00	84.04	84.62
11: DAA20936.1	30.15	54.04	53.38	54.47	54.26	54.26	54.07	65.58	78.04	84.04	100.00	91.30
12: XP_005691215.1	30.00	53.94	53.59	55.00	54.79	55.06	55.23	65.73	79.19	84.62	91.30	100.00

AUTHOR'S PROFILE



Nurhazirah Azmi received her Bachelor of Science in Biology from the Faculty of Science, Universiti Putra Malaysia (UPM) in 2010. She completed her Master of Science in Halal Product Science at the Halal Product Research Institute (HPRI), UPM in 2014. Her MSc thesis, titled *"Nutritional and Bioactivity Properties of Selected Earthworm Species for Potential Cosmeceutical Application"*, focused on protein extraction from earthworms, with emphasis on their antioxidant and anti-ageing potential. Following her postgraduate studies, she began her academic career as a lecturer in Biology at Universiti Teknologi MARA (UiTM) Perak, Tapah Campus. She later advanced her research by pursuing a PhD in Food Science with a specialization in halal authentication, integrating proteomics and electrochemical immunosensor approaches to detect blood plasma proteins in food products.

LIST OF PUBLICATION

Azmi, N., Abidin, S. a. S. Z., Zakaria, N. A., Shukor, M. S. A., Karsani, S. A., Fatt, L. K., & Yuswan, M. H. (2025). Evaluation of Extraction Buffers for Protein Identification in Fish Paste with Chicken Blood Plasma (CBP) Spikes: A Preliminary Mass Spectrometry Study. Indonesian Journal of Chemistry, 25(2), 497. https://doi.org/10.22146/ijc.100755

Azmi, N., Nordin, N. A., Abidin, S. A. S. Z., Nizar, N. N. A., Mutalib, S. R. A., & Ismail-Fitry, M. R. (2025). Valorization of tomato peel waste: antioxidant, physicochemical properties and acceptability of dried tomato peel in beef patties. *Food Research*, *9*(2), 372–380.

Azmi, N., Zainal Abidin, S. A. S., Low, K. F., Mahmud, A. H., Gati, N. I., & Karsani, S. A. (2024). Development of an electrochemical immunosensor using gold nanoparticles (AuNPs) for the detection of Apolipoprotein AI (ApoAI) in chicken blood plasma. *ESTEEM Academic Journal*, *20*, 1-11

Ain, N., Sarah, S. A., **Azmi, N**., Bujang, A., & Ab Mutalib, S. R. (2023). Response surface methodology (RSM) identifies the lowest amount of chicken plasma protein (CPP) in surimi-based products with optimum protein solubility, cohesiveness, and whiteness. *CyTA-Journal of Food*, *21*(1), 646-655.

Ain, N., Ab Mutalib, S. R., **Azmi, N**., Bujang, A., & Sarah, S. A. (2023). Determining the Minimum Contamination Percentage of Chicken Plasma Protein in Surimi-Based Products using Response Surface Methodology. *AIJR Abstracts*, 158-162.

LIST OF PRESENTATIONS

Azmi, N., Zainal Abidin, S. A. S., Low, K. F., & Karsani, S. A. Utilizing Gold Nanoparticles in Electrochemical Immunosensors for the Detection of Apolipoprotein AI in Chicken Blood Plasma for Surimi-based products. In: International Halal Symposium Halal, 20 – 22 August 2024,
Kota Kinabalu, Sabah. (Universiti Malaysia Sabah) – ***Best Oral Presenter***

Nor Izzati Gati, Abdul Hadi Mahmud, Mawar Hasyikin Abu Seman, **Nurhazirah Azmi**, Abdul Mutalib Mad Jani, Low Kim Fatt, Hanani Yazid. On the spot Halal test on raw & rocessed meats. In : In The 6^{6h} International Innovation, Design and Articulation (i-IDeA), 9 – 11 May 2023, Arau, Perlis. (Universiti Teknologi MARA Perlis) – ***Silver award***