Applied Food Research 5 (2025) 101428

Contents lists available at ScienceDirect

Applied Food Research

journal homepage: www.elsevier.com/locate/afres

Differentiation of fish species based on *O*-acetylated *N*-glycan fragments using LC-IM-MS to combat seafood adulteration

Ian Walsh [a,1], Thimo Ruethers [b,c,d,1], Sim Lyn Chiin [a], Gavin Teo [a], Shi Jie Tay [a], Corrine Wan [a], Kuin Tian Pang [a], Sean Chia [a], Andreas L. Lopata [b,c,d,*], Beiying Qiu [a,*]

[a] *Bioprocessing Technology Institute (BTI), Agency for Science, Technology and Research (A*STAR), 20 Biopolis Way, #06-01 Centros, Singapore 138668, Republic of Singapore*
[b] *Tropical Futures Institute, James Cook University, 149 Sims Drive, Singapore 387380, Republic of Singapore*
[c] *Molecular Allergy Research Laboratory, Centre for Sustainable Tropical Fisheries and Aquaculture, College of Science and Engineering, James Cook University, 1 James Cook Drive, Queensland 4811, Australia*
[d] *Biomolecular Sequence To Function Division (BSFD), Bioinformatics Institute (BII), Agency for Science, Technology and Research (A*STAR), 30 Biopolis St, Singapore 138671, Republic of Singapore*

ARTICLE INFO

Keywords:
Food identification and authentication
Liquid chromatography ion mobility-mass spectrometry
Food fraud
N-glycan fingerprinting
O-acetylated sialic acid
Glycomic biomarkers
Food safety

ABSTRACT

Food fraud poses a serious safety risk and affects the economy, with seafood being particularly vulnerable due to high species diversity and complex global supply chains. Accurate fish species identification is crucial for sustainable fishery management, food safety and protecting consumers with species-specific fish allergies who are at risk of life-threatening anaphylaxis. While some methods such as DNA-based PCR are well-established, they have limitations for processed foods and are costly, complex and time-consuming. Glycan markers are highly stable and have recently emerged as a tool for food authentication due to their unique species-specific characteristics. This study introduces *N*-glycan profiling as a novel technique for fish species authentication and addresses the need for reliable methods applicable to processed seafood products. By employing liquid chromatography ion mobility-mass spectrometry analysis, we examined *N*-glycan profiles of raw and heated fish muscle tissues from three fish species, which represent widely consumed seabass and snapper as well as their potential counterfeit substitute, tilapia from markets and restaurants. *N*-glycan structures containing different degrees of *O*-acetylated sialic acids (*O*-Ac-Sias) were identified as species-specific markers and clustering based on their percentage abundance enabled species classification. This study provides the foundation for the development of a rapid, species-specific authentication tool, which could be employed throughout the seafood supply chain, from harvest to retail, improving traceability and reducing mislabeling in markets and restaurants.

1. Introduction

Seafood, including fish and shellfish, is one of the most widely traded and consumed foods globally. High demand (>24 kg per capita), intricate supply chains and species complexity contribute to an estimated 25 % of catches being attributed to Illegal, Unreported, and Unregulated (IUU) fishing, which threatens ecosystems, industries and sustainability efforts (Agnew et al., 2009; Cawthorn et al., 2018; Romero, 2023). The absence of clear regulations and effective enforcement against mislabeling poses a challenge to both markets and consumers (Kroetz et al., 2020). Notably, seabasses (e.g., barramundi) and snappers are the most commonly mislabeled fish globally (Warner et al., 2019). The ramifications of seafood fraud extend beyond potential health risks for consumers, particularly those with seafood allergies, to the erosion of consumer confidence in the integrity of the food supply chain and severe environmental impact (Fox et al., 2018). As allergy risks are species-specific to individuals, correct labeling is crucial for affected consumers to safely manage this life-threatening condition (Davis et al., 2020; Ruethers et al., 2018). Fish is included in the European mandatory labeling legislation along with 13 other allergens due to its prevalence in

* Correspondence authors.
E-mail addresses: ian_walsh@a-star.edu.sg (I. Walsh), thimo.ruethers@jcu.edu.au (T. Ruethers), sim_lyn_chiin@a-star.edu.sg (S.L. Chiin), gavteo@gmail.com (G. Teo), tay_shi_jie@a-star.edu.sg (S.J. Tay), corrine_wan@a-star.edu.sg (C. Wan), zach_pang@a-star.edu.sg (K.T. Pang), sean_chia@a-star.edu.sg (S. Chia), lopata@jcu.edu.au (A.L. Lopata), qiu_beiying@a-star.edu.sg (B. Qiu).
[1] Equal contributions.

https://doi.org/10.1016/j.afres.2025.101428
Received 9 July 2025; Received in revised form 3 October 2025; Accepted 15 October 2025
Available online 16 October 2025

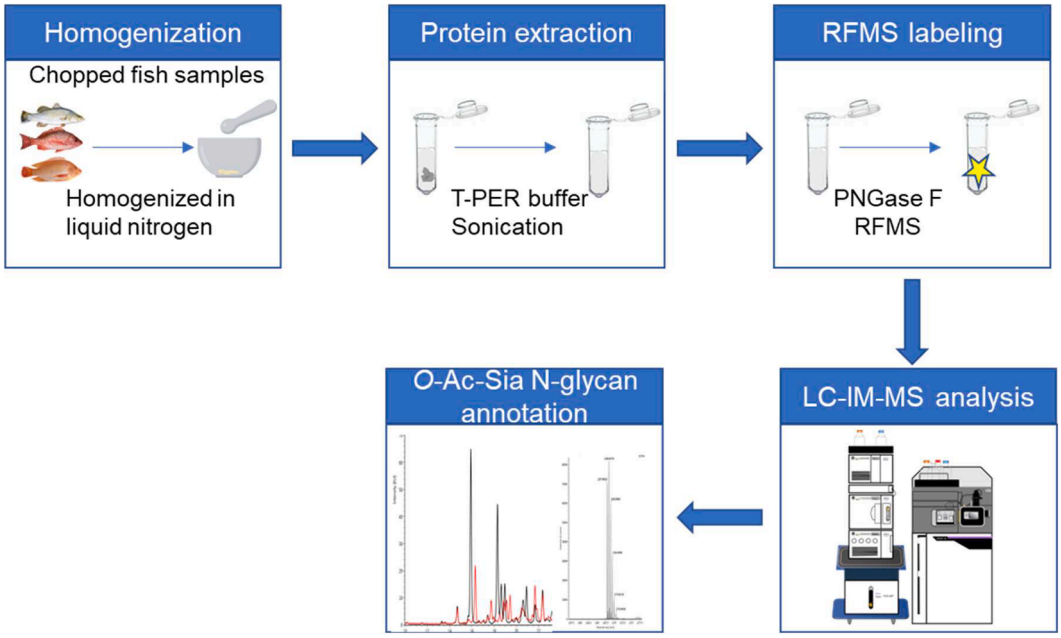

Fig. 1. Glycomics-based workflow for fish authentication of raw fish muscle tissue. Proteins were first extracted from raw tissue followed by the release of *N*-linked glycans. *N*-linked glycans were labeled by RFMS and characterized via LC-IM-MS.

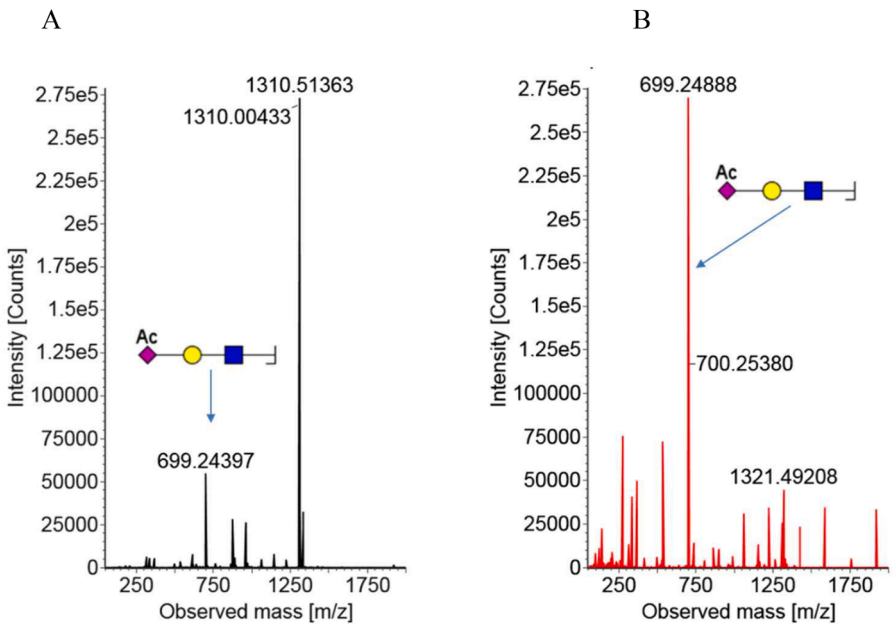

Fig. 2. Diagnostic ion, GlcNAc(1)Gal(1)Neu5Ac(1)OAc(1), was observed in both low energy (A) and high energy (B) mass spectrometry channel. ■: GlcNAc; ●: galactose; ◆: Neu_5NAc (sialic acid); Ac: *O*-acetylation.

causing allergies (Rona et al., 2007; Taylor & Baumert, 2015). Although optimizing regulations, improving enforcement, and implementing stricter traceability systems are crucial measures for preventing fish mislabeling, more effective methods to differentiate fish species are urgently needed for combating fraud.

It is imperative to develop reliable and rapid high-throughput methods for seafood authentication to counteract seafood mislabeling and adulteration throughout the supply chain. Traditional methods, such as identifying species based on morphological and morphometric features, are often inadequate, particularly for processed seafood where distinguishing anatomical features are removed or altered during processing. More state-of-the-art methods involve DNA barcoding, a technique leveraging genetically variable DNA sequences to discern between species (ISO17174, 2024; Kotsanopoulos et al., 2021). This molecular approach relies on a unique mitochondrial COI DNA sequence of approximately 650 base pairs (bp) that is amplified for each biological species by using a universal set of polymerase chain reaction (PCR) primers. Following Sanger sequencing, this DNA sequence is matched against a DNA barcode database to determine the biological identity of the sample (Bemis et al., 2023). Authentication is then confirmed by comparing the identified species from the barcoded DNA sequence with the label on the sample. However, several limitations constrain the widespread application of DNA barcoding in commercial seafood testing. The technique is inherently time-consuming, typically requiring

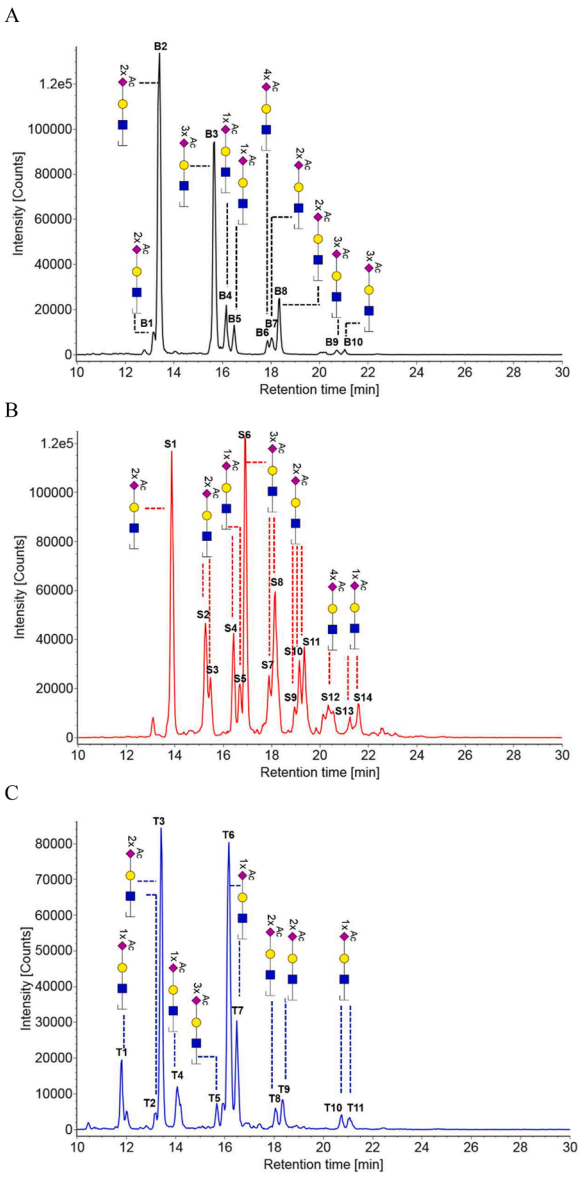

Fig. 3. Representative extracted ion chromatogram (EIC) of the diagnostic ion: GlcNAc(1)Gal(1)Neu5Ac(1)OAc(1). A: raw barramundi; B: raw red snapper; C: raw red tilapia.

2–3 days from sample preparation to final identification, and the effectiveness of fish species identification through barcoding depends critically on the completeness and accuracy of DNA reference libraries (Delrieu-Trottin et al., 2019; Silva & Hellberg, 2021). While public sequence databases such as the Barcode of Life Database (http://www.boldsystems.org/index.php) and GenBank (https://www.ncbi.nlm.nih.gov) are widely used resources for DNA barcoding, misidentified sequences have been reported, creating potential for false identifications (Mulcahy et al., 2022; Phillips et al., 2022). Moreover, this method is only well-established for raw and unprocessed fish, as DNA degradation during thermal processing, canning, or fermentation can compromise amplification success and sequence quality. Additionally, the cost per sample and requirement for specialized laboratory infrastructure limit its scalability for routine industry use. Alternative molecular approaches, including lipidomic, metabolomic and proteomic methods for fish authentication have shown promising results. However, their implementation in routine testing protocols is hindered by significant cost and time constraints, often requiring even more specialized equipment and expertise than DNA barcoding (Braconi et al., 2021). Consequently, there remains an urgent need for rapid, cost-effective, and field-deployable authentication methods that can complement or potentially replace current approaches while maintaining high accuracy across diverse seafood products and processing states.

Among emerging omics approaches for species authentication, glycomics represents a promising method with analytical advantages (Wang et al., 2025). Glycomics focuses particularly on sugar chains covalently linked to asparagine residues in peptide chains known as *N*-glycans. Glycans exhibit diverse structural combinations that play multiple biological roles in species-specific glycoproteins (Schjoldager et al., 2020). *N*-glycans are composed of various monosaccharides that serve as essential molecular building blocks and exhibit complex branched structures attached to membranes and secreted glycoproteins and glycolipids (Marth, 2008). Protein *N*-glycosylation is controlled by diverse glyco-enzymes, including glycosidases and glycotransferases, within organisms, creating species-specific enzymatic fingerprints. The activity of these enzymes depends on multiple factors, such as environment, tissues, organs, and, notably, species, resulting in reproducible glycosylation patterns that can serve as taxonomic markers. Therefore, *N*-glycans have emerged as promising novel markers for species identification (Solorzano et al., 2009) and may even be able to detect environmental cues from farming or wild-reared specimens, offering potential advantages over DNA-based methods in terms of information content. Previously, *N*-glycans have been identified as biomarkers for meat and milk authentication and showed potential for high-throughput applications (Chia et al., 2022; Liu et al., 2023). In seafood, unique *N*-glycan structures containing *O*-Ac-Sias have been reported in various

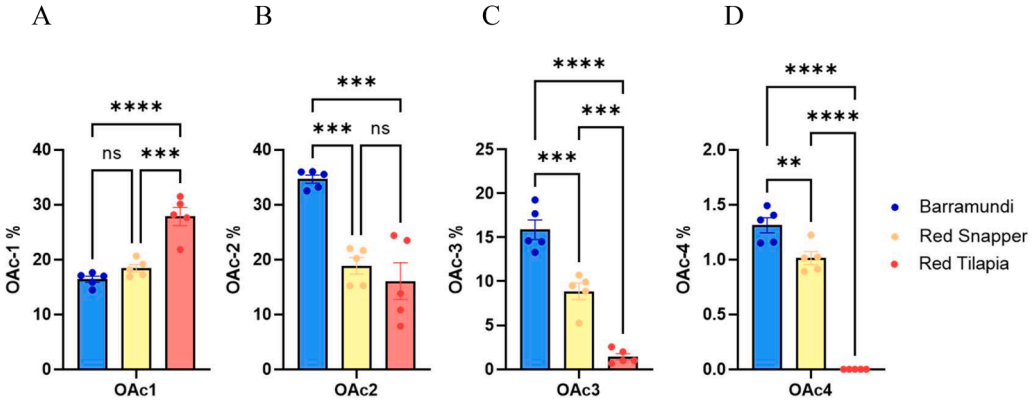

Fig. 4. The relative abundance of *N*-glycan containing various numbers of *O*-acetylated sialic acid in raw fish samples. A. *N*-glycan with one *O*-Ac-Sia (OAc1). B. *N*-glycan with two *O*-Ac-Sias (OAc2). C. *N*-glycan with three *O*-Ac-Sias (OAc3). D. *N*-glycan with four *O*-Ac-Sias (OAc4). One-way ANOVA followed by Tukey's post hoc test, multiple comparisons were performed among barramundi, red snapper, and red tilapia, $n = 5$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

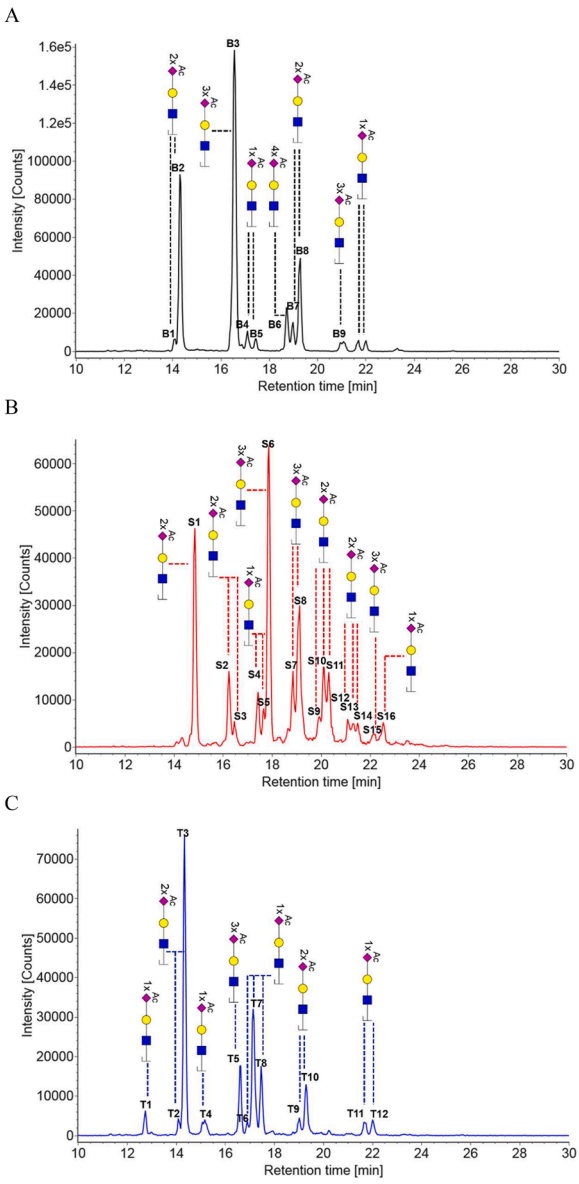

Fig. 5. Extracted ion chromatogram (EIC) of a diagnostic ion: GlcNAc(1)Gal(1)Neu5Ac(1)OAc(1). A: heated barramundi; B: heated red snapper; C: heated red tilapia.

organs including serum, muscle, intestine and skin Aamelfot et al. (2014); Liu et al. (2008); Wu et al. (2017); Ylönen et al. (2001); Zhao et al. (2024). In the serum of Atlantic salmon (*Salmo salar*), mono--*O*-acetylated sialic acids accounted for up to 83 % of total sialic acids, with patterns that shifted under long-term handling stress which indicates environmentally driven modulation (Liu et al., 2008). While meats from common livestock such as beef, pork, and chicken have not been reported to contain significant levels of *O*-acetylated sialic acids (Chia et al., 2022), their presence in seafood suggests that *O*-Ac Sias could serve as unique biomarkers for seafood authentication.

In this study, we aimed to explore the utility of *O*-Ac Sia abundances for fish species authentication. An analytical process utilizing *N*-glycan structures containing *O*-Ac Sias was developed to identify and differentiate widely consumed seabass, snapper and their potential counterfeit cheaper substitute, tilapia. Raw and heated fish muscle tissue (meat) was analyzed, which represent fish fillets from markets and restaurants for which authentication by morphology is impossible. The *N*-glycan profiles of all samples were analyzed by a Waters SYNAPT XS Liquid chromatography–ion mobility mass spectrometer (LC-IM-MS) of which unique *N*-glycan structures containing *O*-Ac-Sias were identified. Additionally, LC-IM-MS analyses revealed differential abundances of *O*-Ac-Sias, which could be used as markers to distinguish fish species.

2. Methods

The workflow of sample preparation and analyses is illustrated in Fig. 1.

2.1. Fish sample collection

Five whole, deceased specimens of each species, barramundi (Asian seabass, *Lates calcarifer*), red snapper (*Lutjanus malabaricus*) and red tilapia (*Oreochromis* sp.), were procured from aquaculture farms in Singapore. Standardized fish muscle tissue samples were collected from the center of each fillet and stored at −80 °C as described previously (Ruethers et al., 2020).

2.2. Protein extractions from fish muscle

Raw fish samples were generated by first homogenizing the tissue using a pestle and mortar under liquid nitrogen. Proteins were then extracted in 800 µL of T-PER tissue protein extraction reagent (ThermoFisher, UK), supplemented with freshly added protease and phosphatase inhibitor (1:100, ThermoFisher, UK). The protein lysates underwent six cycles of sonication, each consisting of 10 s of sonication followed by a 10-second interval. Subsequently, the protein lysates were centrifuged at 13,000 rcf for 15 min, and the supernatant was subjected to buffer exchange into 50 mM HEPES (pH 8.0). To mimic cooked fish, raw tissue samples were first heated at 95–100 °C in T-PER reagent for 20 min and the proteins were extracted as described above (Ruethers et al., 2021). The protein concentration of all extracts was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, MA, USA), with bovine serum albumin as the standard. Fish protein was dried using a centrifuge vacuum concentrator (Labconco, MO, USA).

2.3. N-glycan release and labeling

Dried fish protein (15 µg) was reconstituted in 22.8 µL of LC-MS-grade water (Merck, NJ, USA) and 6 µL of a 5 % RapiGest solution (Waters Corporation, MA, USA). Protein denaturation was achieved through incubation at 95 °C for 5 min. Subsequently, 600 U of recombinant PNGase F (Waters Corporation, MA, USA) was added, to facilitate the enzymatic release of *N*-glycans into the solution during a 10-minute incubation at 50 °C. The liberated glycans were then labeled using 12 µL of RapiFluor-MS's (RFMS) solution (Waters Corporation, MA, USA) at room temperature for 10 min. RFMS-labeled glycans were subjected to purification using a GlycoWorks HILIC µElution plate (Waters Corporation, MA, USA). The isolated *N*-glycans were dried for subsequent analysis. For reconstitution, the dried samples were mixed in 9 µL of LC-MS-grade water, 10 µL of dimethylformamide, and 21 µL of acetonitrile before transferring into a glass vial. The injection volume for each run was set at 10 µL.

2.4. LC-IM-MS analysis of RFMS-Labeled N-Glycan

The analysis of released *N*-glycans was performed as described previously (Chia et al., 2022; Pang et al., 2021). Initially, 10 µL of the reconstituted *N*-glycans was injected into an ACQUITY H—Class UPLC system paired with a SYNAPT XS mass spectrometer (both from Waters Corporation, Milford, MA, USA). Samples underwent separation using an ACQUITY UPLC Glycan BEH amide column (Waters Corporation, MA, USA) at 60 °C with a flow rate of 400 µL/min, using a 40-minute gradient from 25 % to 49 % of 50 mM ammonium formate (mobile phase A). Mobile phase B consisted of 100 % acetonitrile. RFMS-labeled glycans were excited at 265 nm and their emission was recorded at 425

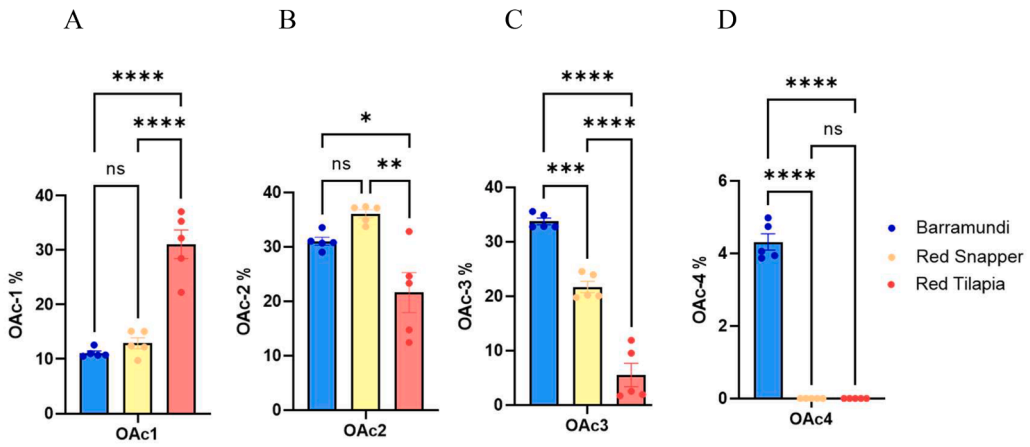

Fig. 6. The relative abundance of *N*-glycan containing various numbers of *O*-acetylated sialic acid in heated fish samples. A. *N*-glycan with one *O*-Ac-Sia (OAc1). B. N-glycan with two *O*-Ac-Sias (OAc2). C. N-glycan with three *O*-Ac-Sias (OAc3). D. *N*-glycan with four *O*-Ac-Sias (OAc4). One-way ANOVA followed by Tukey's post hoc test, multiple comparisons were performed among Barramundi, Red Snapper, and Red Tilapia, $n = 5$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$.

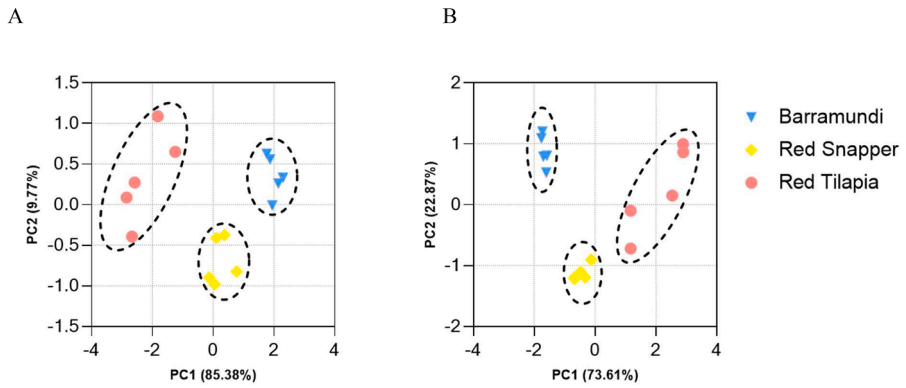

Fig. 7. Principal component (PC) analysis was performed on relative abundances of *N*-glycans containing *O*-Ac-Sias of raw fish samples (A) and heated fish samples (B) from barramundi, red snapper and red tilapia. Distinct separation was observed between different species. Blue inverted triangle: barramundi; yellow diamond: red snapper; pink circle: red tilapia.

nm using an ACQUITY UPLC FLR detector. The mass spectrometer conducted MS profile scans from *m/z* 400 to 2000 in positive mode at a rate of 1 Hz. The instrument settings included an electrospray ionization capillary voltage of 1.8 kV, cone voltage of 30 V, desolvation gas flow of 850 L/h, and ion source and desolvation temperatures maintained at 120 °C and 350 °C, respectively. Leucine enkephalin served as the LockSpray compound for real-time mass accuracy, and a RapiFluor-MS dextran calibration ladder was used to calibrate the retention times of the sample peaks. LC-MS retention times were subsequently normalized to glucose units (GU) using the dextran calibration curve. Data was acquired with MassLynx (Version 4.2, Waters Corporation, MA, USA).

2.5. Determination of the relative abundance of O-Ac-Sia-containing N-glycans in different fish species

Released *N*-glycans were analyzed using the UNIFI Scientific Information System (Version 1.8, Waters Corporation, MA, USA). Fluorescence peaks were manually integrated within the same system, and their relative quantity was determined by area-under-curve measures, which were normalized to the total area. *N*-glycan composition was determined from the neutral mass and where possible matching the *N*-glycan database in the UNIFI software. Fragmented *O*-Ac-Sia-containing diagnostic ions were determined using the SYNAPT XS high-voltage MS^e capabilities and low energy in-source. *O*-Ac-Sia-containing *N*-glycans were annotated through extracted ion chromatogram by *O*-Ac-Sia diagnostic ion.

2.6. Distribution of collision cross section (CCS) value of diagnostic ion across different fish species by script

The diagnostic ion, GlcNAc(1)Gal(1)Neu5Ac(1)OAc(1), with an *m/z* value of 699.24 was extracted across each sample replicate. Drift time and collision cross section (CCS) values were determined by IM-MS. CCS of *O*-Ac-Sia diagnostic ion observed between 200 and 300 $Å^2$ were binned into 50 CCS windows. That is, 200–202 $Å^2$: bin 1, 202–204 $Å^2$: bin 2, .., 298–300 $Å^2$:bin 50. Each observed CCS intensity (normalized between 0–100 %) was averaged in each bin for each species.

2.7. Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Statistical analysis was performed using a two-tailed, unpaired student's *t*-test or analysis of variance (ANOVA) followed by a Tukey post-hoc analysis as appropriate, using Prism 9 (GraphPad Software Inc., La Jolla, CA, USA) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). The relative abundance of structures containing *O*-Ac-Sia was analyzed using principal component analysis (PCA).

3. Results

3.1. Distinct glycan signatures in different fish species

N-glycan distribution in raw fish fillets from three fish species was

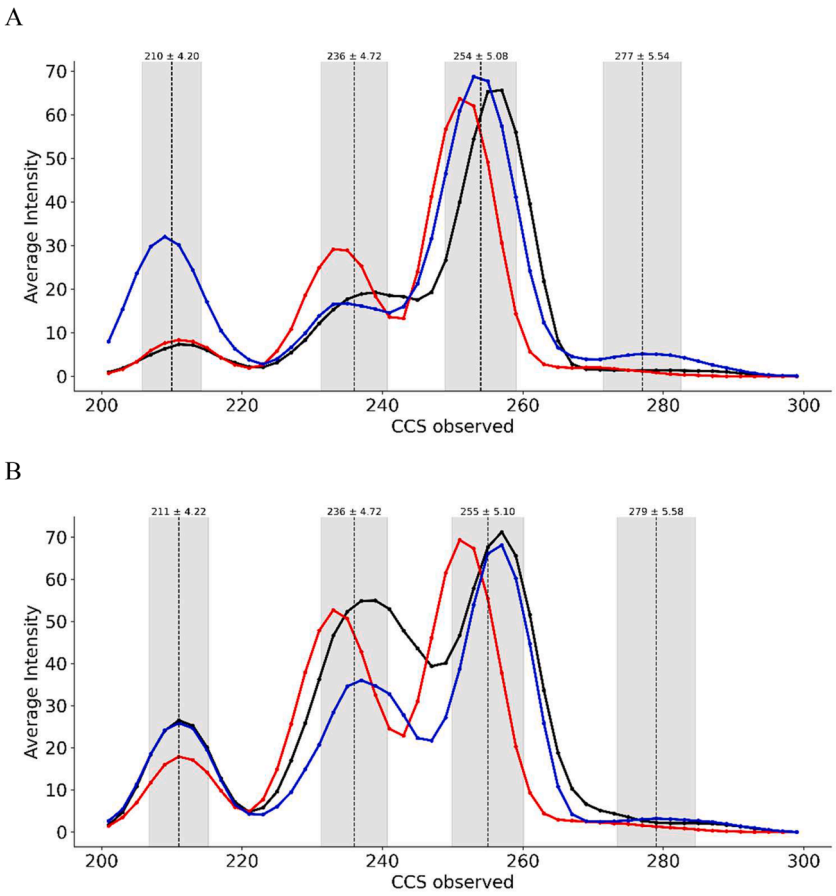

Fig. 8. Isomeric GlcNAc(1)Gal(1)Neu5Ac(1)OAc(1) CCS distribution of three fish species. Each dot represents one CCS bin of width (e.g. most intensities fell into the bin 256–258 in barramundi and snapper). A. raw fish samples. B. heated fish samples. Black line: barramundi; Red line: red snapper; Blue line: red tilapia.

investigated after *N*-glycan release by PNGase F and labeling with RFMS, followed by LC-IM-MS *N*-glycan analysis (Fig. 1). *N*-glycan structures containing *O*-Ac-Sias were observed and can be characterized through a diagnostic ion fragment with a *m/z* of 699.24 in both low and high energy MS channels (Fig. 2A-B). As our protocol consists of PNGase F release of N-glycans, it is known that this fragment consists of *O*-acetylated sialic acid (*O*-Ac-Sia) bonded to galactose and then to *N*-Acetylglucosamine monosaccharides (GlcNAc(1)Gal(1)Neu5Ac(1)OAc(1)) (Varki et al., 2015). Extracted ion chromatograms (EICs) of each fish species, obtained using the diagnostic ion, and the observed *m/z* of the parent N-glycan enabled annotation of the number of *O*-Ac-Sias (Fig. S1 and Fig. 3A-C). For example, *m/z* values of 1310.00 correspond to the $[M + 2H]^{+2}$ ions of the intact N-glycan GlcNAc(4)Hex(5)Neu5Ac(2)OAc (2) and therefore must have only 2 *O*-Ac-Sia's (Fig S1). *O*-Ac-Sias were then grouped according to their degree and subsequently quantified using their parent *N*-glycan abundance based on RFMS fluorescence intensity in LC (Fig. S1). Significant differences in the relative abundance were observed among species (Fig. 4A-D). For instance, four *O*-Ac-Sias were observed in both barramundi and red snapper, but it was absent in red tilapia. The statistically different *O*-Ac Sia abundances shown in Fig. 4A-D demonstrate the feasibility of using *O*-Ac-Sia glycan profiles for fish authentication. The EICs of samples from each species showed excellent reproducible across biological replicates, supporting the potential of specific *O*-Ac-Sia structures as biomarkers for fish authentication (Fig. S2A-S2C). Furthermore, given *O*-Ac-Sias known abundance in fish (Aamelfot et al., 2014; Liu et al., 2008; Wu et al., 2017; Ylönen et al., 2001; Zhao et al., 2024) extracting their EIC avoids the very complex full *N*-glycan characterization of each fish sample.

It is also crucial for customers to know which fish they consume after cooking, e.g., in restaurant settings. Therefore, the same workflow with minor modification was used to determine the *N*-glycan profiles of heated fish, representing cooked products (Fig. S3). Small pieces of raw fish muscle tissue were heated for 20 min at 95–100 °C before protein extraction. EICs were plotted based on same diagnostic ion indicating the unique distribution of *O*-Ac-Sia-containing *N*-glycan structures (Fig. 5A-C). As with the raw samples, the profiles were reproducible in all biological replicates (Fig. S4A-S4C). Although the percentages of *O*-Ac-Sia changed between raw and heated samples, possibly due to protein degradation (Fig. S5A-S5C), the *O*-Ac-Sia levels are reproducible between biological replicates and significant differences between species were observed (Fig. 6A-D and Fig. S4A-S4C). All three fish species exhibited a high abundance of *O*-Ac-Sia-containing *N*-glycans. *O*-Ac-Sia structures accounted for 40–60 % of the total *N*-glycan structures in raw fish meat and 60–70 % in heated fish meat (Fig. S6A and S6B).

3.2. Species classification

PCA was conducted to examine data variability and determine if the raw fish samples could be differentiated from one another (Fig 7A). The PCA clusters were determined based on the percentage abundance of *O*-Ac-Sias in different species of fish (i.e. the features used in the PCA were the percentages in Fig. 4). Barramundi, red snapper and red tilapia were clearly differentiated in the PCA plot, with no crossover or overlap among samples of different species and relatively concentrated sample distributions within the same species. In the heated samples, PCA based on the relative abundance of *O*-Ac-Sias (Fig. 6) revealed distinct clustering patterns, indicating that the three species can still be effectively differentiated even after cooking (Fig. 7B). In summary, despite species-specific changes in *O*-Ac-Sia after heating compared to that in raw fish samples, the quantification was still reproducible within the same

species after heating. Therefore *O*-Ac-Sia quantification can also potentially be used to classify species from cooked seafood.

3.3. Different isomeric O-Ac-Sia structures' distribution across fish species

In both raw and heated fish samples, multiple *N*-glycan isomers were observed (results not shown). To understand this isomerism better, we investigated the CCS attributes derived from ion mobility of the *O*-Ac-Sia diagnostic ion GlcNAc(1)Gal(1)Neu5Ac(1)OAc(1) which was also reported in Vos's study (Vos et al., 2023). Observed CCS values of the 699.24 fragments were binned and used to analyze and visualize the distribution of the diagnostic ion across three different fish species. This allowed us to comparatively assess structural isoform presence and average CCS intensity among the samples. (Fig. 8A and B). The results revealed the presence of four isomeric forms of this specific fragment, with notable variation in their relative intensities across the samples. The four regions were identified from the mean observed CCS and a 2 % error margin (Song et al., 2022). In raw flesh Type 1 (mean CCS 210 $Å^2$), type 2 (mean CCS 236 $Å^2$) and type 3 (mean 254 $Å^2$) isoforms of GlcNAc (1)Gal(1)Neu5Ac(1)OAc(1) were observed in all species. Interestingly, Tilapia has a unique fourth type of GlcNAc(1)Gal(1)Neu5Ac(1)OAc(1) with a mean CCS of 277 $Å^2$ in the raw samples. The same four CCS peaks were observed in heated tissue (Fig 8B) albeit with slightly different mean CCS (type 1: 211 $Å^2$, type 2: 236 $Å^2$, type 3: 255 $Å^2$, and type 4: 279 $Å^2$).

4. Discussion

Food fraud is one of the most persistent issues that affect consumer confidence and is a potential public health concern globally. Adulteration and mislabeling of fish have become increasingly common due to economic interests. Currently, several techniques can be used to authenticate fish species based on DNA, proteins, lipid and other components, but they all involve complicated procedures, resulting in lengthy analysis methods (Chien et al., 2022; Naaum et al., 2021; Shen et al., 2022). DNA-based methods can produce false positive or false negative results due to issues with cross-reactivity or sensitivity and are highly dependent on reference libraries and processing state. To overcome these shortcomings, more precise, reliable, cost-effective, high--throughput analytical methods are urgently needed.

N-glycosylation is the most extensive post-translational modification of proteins in nature (Reily et al., 2019). They are highly specific and unique to each species, and they are more stable under heat and pressure compared to DNA, proteins, and fatty acids (Shi et al., 2019). Although *N*-glycans are composed of similar monosaccharides, the glycomes of different species differ greatly from each other because of the complex glycosylation pathways in the Golgi, resulting in multiple combinations of monosaccharides and linkages. Consequently, *N*-glycans are widely used as biomarkers for predicting hallmarks of human health and diseases (Kuzmanov et al., 2013). Food-glycomics has been implemented in recent years as a new strategy for controlling food quality and safety (Tang et al., 2022). Milk from different sources can be differentiated through structural differences in *N*-glycans (Liu et al., 2023; Wang et al., 2017) and successful meat authentication has been achieved by comparing *N*-glycan profiles among different meats, demonstrating the utility of *N*-glycan profiling in the food industry (Chia et al., 2022; Shi et al., 2019). These reports have highlighted the potential for seafood/fish authentication through *N*-glycans.

In this study, a simple and efficient workflow was developed to authenticate fish species through *N*-glycan signatures. Consistent *O*-Ac-Sia-containing *N*-glycan profiles in biological replicates and unique abundance distribution of *O*-Ac-Sia-containing *N*-glycans among different species were observed and well characterized with established fluorescent labeling techniques, mass spectrometry, and ion-mobility. All *N*-glycans containing *O*-Ac-Sias were extracted by using its diagnostic ion and grouped according to the degree of *O*-Ac-Sias. This approach greatly simplifies the analysis for separating fish species by the percentage of *N*-glycans containing different numbers of *O*-Ac-Sias. Concentrating solely on O-Ac-Sia significantly reduces analysis time, which is indispensable for platform development. In contrast, intact *O*-acetylated *N*-glycans are difficult to separate and identify unambiguously (Vreeker & Wuhrer, 2017), hampering the development of glycomic species detection assays, particularly when including many more fish species.

Sialic acid can be modified by acetyl esters at the 4-, 7-, 8-, and/or 9-position which results in various structures (Visser et al., 2021). Additionally, sialic acid can have different linkages to its neighboring galactose, thus multiple isomers of *N*-glycans containing *O*-Ac-Sias are possible. We extracted the *O*-Ac-Sia diagnostic ion distribution across different fish species and each species *O*-Ac-Sia profile was found to be substantially different in each species (Fig 3). This perhaps suggests that specific glycoenzymes, especially enzymes for *O*-Ac modification and sialylation, are involved in *N*-glycan synthesis among different fish species. Given that *O*-Ac modifications are associated with health status and various diseases in humans (Cavdarli et al., 2019; Visser et al., 2021), it will be interesting to further investigate their potential nutritional value and even their effects on human allergies.

IM-MS has emerged as a powerful analytical separation technique and its applicability has expanded beyond traditional uses and is now widely employed for the characterization of biomolecules, including proteins, glycans, and lipids (Ben Faleh et al., 2022). Here, IM-MS provided deeper insights, enabling us to determine four *O*-Ac-Sia isoforms present in the three species (Fig. 8A-B). Notably, the four detected isoforms remained intact upon heating (Fig. 8B), suggesting that despite undergoing some abundance changes between raw and heated fish samples (Fig. S5), the *O*-Ac-Sias retain their structure and capacity to differentiate between the fish species (Fig. 7). This criterion is important because fish mislabeling occurs throughout the entire supply chain, including markets and restaurants. A limitation of the IM-MS analysis was the inability to determine the exact structure of the four *O*-Ac-Sias. Utilizing standards of *O*-Ac-Sia isomers into the analysis would facilitate more accurate structural annotation in the future.

For the first time, we report an approach using LC-IM-MS to differentiate fish species based on *N*-glycans *O*-acetylated fragment. The consistency of the *O*-Ac-Sia-containing *N*-glycan structures found in raw or heated fish muscle tissue, allows this method to be used at various stages of the supply chain, from harvesting to consumer sales either from markets or restaurants. This study highlights the application of glycomics as a robust alternative to DNA barcoding for food authentication. While DNA templates are often degraded during cooking or processing, *N*-glycan markers remain stable and species-specific, enabling reliable identification even in heat-treated samples. The refined workflow employs food glycomics as an alternative method to authenticate fish species. The study is a proof-of-concept and it can be further developed for the identification of other seafood species by identifying and quantitating *O*-Ac-Sias. To this end, we hope to expand our platform to a wide range of species, different cooking methods, and compare them with PCR and other established identification methods. Additionally, we can investigate the changes on *N*-glycan *O*-Ac modifications in wild and farmed seafood – potentially even discriminating among these food products. Future work will focus on these areas, and we will aim to develop a high throughput and robust workflow with lower costs.

5. Conclusions

LC-IM-MS analysis was performed on raw and heated muscle tissues from three fish species. *O*-Ac-Sia signatures were identified for each fish species in both raw and heated samples. Clustering effectively separated different fish species based on the percentage of *N*-glycans containing varying numbers of *O*-Ac-Sias. Overall, four *O*-Ac-Sia isoforms and the degree of *O*-Ac-Sia on *N*-glycans are biomarker candidates for fish authentication, potentially reducing ambiguous and inaccurate labeling

of fish in the markets and restaurants. This study is the first to demonstrate that glycomics can be applied for fish species authentication using *N*-glycan profiling via LC-IM-MS. We show that species-specific *O*-acetylated sialic acid markers remain stable and distinct even after heat treatment, overcoming the limitations of DNA barcoding in processed foods. Our work establishes glycomics as a robust and complementary approach for food authentication beyond conventional genetic methods.

Funding

This work was supported by the Singapore Ministry of Health's National Medical Research Council under its Open Fund-Young Individual Research Grant (OF-YIRG) (MOH-001132–00) (awarded to S.C.), the Agency for Science, Technology, and Research (A*STAR) under the A*STAR Start Up Fund (awarded to S.C.) and the Agency for Science, Technology, and Research (A*STAR) under Career Development Fund (awarded to K.T.P.). Further support was received from the National Research Foundation, Singapore and A*STAR under the Singapore Food Story R&D Programme (W22W3D0003) and the Good Food Institute under its Research Grant Program (Fish tissue proteomics as baseline evaluation for safer cultivated seafood with known allergen profile).

Ethical statement

No ethical approval was required for this study as fish samples were purchased from aquaculture farms in Singapore and were already deceased at the time of purchase.

CRediT authorship contribution statement

Ian Walsh: Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization. **Thimo Ruethers:** Writing – review & editing, Validation, Investigation, Conceptualization. **Sim Lyn Chiin:** Validation, Investigation, Formal analysis. **Gavin Teo:** Validation, Investigation, Formal analysis. **Shi Jie Tay:** Writing – review & editing, Methodology, Formal analysis. **Corrine Wan:** Writing – review & editing, Methodology, Formal analysis. **Kuin Tian Pang:** Writing – review & editing, Validation, Funding acquisition. **Sean Chia:** Writing – review & editing, Validation, Funding acquisition. **Andreas L. Lopata:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Beiying Qiu:** Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

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

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.afres.2025.101428.

Data availability

Data will be made available on request.

References

Aamelfot, M., Dale, O. B., Weli, S. C., Koppang, E. O., & Falk, K. (2014). The in situ distribution of glycoprotein-bound 4-O-Acetylated sialic acids in vertebrates. *Glycoconjugate Journal, 31*, 327–335. https://doi.org/10.1007/s10719-014-9529-7

Agnew, D. J., Pearce, J., Pramod, G., Peatman, T., Watson, R., Beddington, J. R., & Pitcher, T. J. (2009). Estimating the worldwide extent of illegal fishing. *PloS one, 4* (2), e4570. https://doi.org/10.1371/journal.pone.0004570

Bemis, K. E., Girard, M. G., Santos, M. D., Carpenter, K. E., Deeds, J. R., Pitassy, D. E., Flores, N. A. L., Hunter, E. S., Driskell, A. C., & Macdonald, K. S., III (2023). Biodiversity of Philippine marine fishes: A DNA barcode reference library based on voucher specimens. *Scientific Data, 10*(1), 411. https://doi.org/10.1038/s41597-023-02306-9

Ben Faleh, A., Warnke, S., Bansal, P., Pellegrinelli, R. P., Dyukova, I., & Rizzo, T. R. (2022). Identification of mobility-resolved N-glycan isomers. *Analytical Chemistry, 94* (28), 10101–10108. https://doi.org/10.1021/acs.analchem.2c01181

Braconi, D., Millucci, L., Parisi, M. L., Spiga, O., & Santucci, A. (2021). Omics-based technologies for food authentication and traceability. *Food authentication and traceability* (pp. 215–245). Elsevier. https://doi.org/10.1016/B978-0-12-821104-5.00003-9

Cavdarli, S., Dewald, J. H., Yamakawa, N., Guérardel, Y., Terme, M., Le Doussal, J.-M., Delannoy, P., & Groux-Degroote, S. (2019). Identification of 9-O-acetyl-N-acetylneuraminic acid (Neu5, 9Ac 2) as main O-acetylated sialic acid species of GD2 in breast cancer cells. *Glycoconjugate Journal, 36*, 79–90. https://doi.org/10.1007/s10719-018-09856-w

Cawthorn, D. M., Baillie, C., & Mariani, S. (2018). Generic names and mislabeling conceal high species diversity in global fisheries markets. *Conservation Letters, 11*(5), Article e12573. https://doi.org/10.1111/conl.12573

Chia, S., Teo, G., Tay, S. J., Loo, L. S. W., Wan, C., Sim, L. C., Yu, H., Walsh, I., & Pang, K. T. (2022). An integrative glycomic approach for quantitative meat species profiling. *Foods, 11*(13). https://doi.org/10.3390/foods11131952

Chien, H.-J., Huang, Y.-H., Zheng, Y.-F., Wang, W.-C., Kuo, C.-Y., Wei, G.-J., & Lai, C.-C. (2022). Proteomics for species authentication of cod and corresponding fishery products. *Food Chemistry, 374*, Article 131631. https://doi.org/10.1016/j.foodchem.2021.131631

Davis, C. M., Gupta, R. S., Aktas, O. N., Diaz, V., Kamath, S. D., & Lopata, A. L. (2020). Clinical management of seafood allergy. *The Journal of Allergy and Clinical Immunology: In Practice, 8*(1), 37–44. https://doi.org/10.1016/j.jaip.2019.10.019

Delrieu-Trottin, E., Williams, J., Pitassy, D., Driskell, A., Hubert, N., Viviani, J., Cribb, T., Espiau, B., Galzin, R., & Kulbicki, M. (2019). A DNA barcode reference library of french polynesian shore fishes. *Scientific Data, 6*(1), 114. https://doi.org/10.1038/s41597-019-0123-5

Fox, M., Mitchell, M., Dean, M., Elliott, C., & Campbell, K. (2018). The seafood supply chain from a fraudulent perspective. *Food Security, 10*, 939–963. https://doi.org/10.1007/s12571-018-0826-z

ISO17174. (2024). https://www.iso.org/obp/ui/en/#iso:STd:ISo:17174:ED-1:V1:EN.

Kotsanopoulos, K. V., Exadactylos, A., Gkafas, G. A., Martsikalis, P. V., Parlapani, F. F., Boziaris, I. S., & Arvanitoyannis, I. S. (2021). The use of molecular markers in the verification of fish and seafood authenticity and the detection of adulteration. *Comprehensive Reviews in Food Science and Food Safety, 20*(2), 1584–1654. https://doi.org/10.1111/1541-4337.12719

Kroetz, K., Luque, G. M., Gephart, J. A., Jardine, S. L., Lee, P., Chicojay Moore, K., Cole, C., Steinkruger, A., & Donlan, C. J. (2020). Consequences of seafood mislabeling for marine populations and fisheries management. *Proceedings of the National Academy of Sciences, 117*(48), 30318–30323. https://doi.org/10.1073/pnas.2003741117

Kuzmanov, U., Kosanam, H., & Diamandis, E. P. (2013). The sweet and sour of serological glycoprotein tumor biomarker quantification. *BMC Medicine, 11*, 1–14. https://doi.org/10.1186/1741-7015-11-31

Liu, X., Afonso, L., Altman, E., Johnson, S., Brown, L., & Li, J. (2008). O-acetylation of sialic acids in N-glycans of Atlantic salmon (Salmo salar) serum is altered by handling stress. *Proteomics, 8*(14), 2849–2857. https://doi.org/10.1002/pmic.200701093

Liu, Y., Hu, X., Voglmeir, J., & Liu, L. (2023). N-glycan profiles as a tool in qualitative and quantitative analysis of goat milk adulteration. *Food Chemistry, 423*, Article 136116. https://doi.org/10.1016/j.foodchem.2023.136116

Marth, J. D. (2008). A unified vision of the building blocks of life. *Nature cell Biology, 10* (9), 1015. https://doi.org/10.1038/ncb0908-1015. -1015.

Mulcahy, D. G., Ibáñez, R., Jaramillo, C. A., Crawford, A. J., Ray, J. M., Gotte, S. W., Jacobs, J. F., Wynn, A. H., Gonzalez-Porter, G. P., & McDiarmid, R. W. (2022). DNA barcoding of the National Museum of Natural History reptile tissue holdings raises concerns about the use of natural history collections and the responsibilities of scientists in the molecular age. *PloS one, 17*(3), Article e0264930. https://doi.org/10.1371/journal.pone.0264930

Naaum, A. M., Cusa, M., Singh, M., Bleicher, Z., Elliott, C., Goodhead, I. B., Hanner, R. H., Helyar, S. J., Mariani, S., & Rice, J. E. (2021). Validation of FASTFISH-ID: A new commercial platform for rapid fish species authentication via universal closed-tube barcoding. *Food Research International, 141*, Article 110035. https://doi.org/10.1016/j.foodres.2020.110035

Pang, K. T., Tay, S. J., Wan, C., Walsh, I., Choo, M. S. F., Yang, Y. S., Choo, A., Ho, Y. S., & Nguyen-Khuong, T. (2021). Semi-automated glycoproteomic data analysis of LC-MS data using GlycopeptideGraphMS in process development of monoclonal antibody biologics. *Frontiers in Chemistry, 9*, Article 661406. https://doi.org/10.3389/fchem.2021.661406

Phillips, M. J., Westerman, M., & Cascini, M. (2022). The value of updating GenBank accessions for supermatrix phylogeny: The case of the New Guinean marsupial carnivore genus Myoictis. *Molecular Phylogenetics and Evolution, 166*, Article 107328. https://doi.org/10.1016/j.ympev.2021.107328

Reily, C., Stewart, T. J., Renfrow, M. B., & Novak, J. (2019). Glycosylation in health and disease. *Nature Reviews. Nephrology, 15*(6), 346–366. https://doi.org/10.1038/s41581-019-0129-4

Romero, L. (2023). *Per capita seafood consumption in singapore from 2011 to 2020*. https://www.statista.com/statistics/1038132/per-capita-seafood-consumption-singapore/.

Rona, R. J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., Sigurdardottir, S. T., Lindner, T., Goldhahn, K., & Dahlstrom, J. (2007). The prevalence of food allergy: A meta-analysis. *Journal of Allergy and Clinical Immunology, 120*(3), 638–646. https://doi.org/10.1016/j.jaci.2007.05.026

Ruethers, T., Taki, A. C., Johnston, E. B., Nugraha, R., Le, T. T., Kalic, T., McLean, T. R., Kamath, S. D., & Lopata, A. L. (2018). Seafood allergy: A comprehensive review of fish and shellfish allergens. *Molecular Immunology, 100*, 28–57. https://doi.org/10.1016/j.molimm.2018.04.008

Ruethers, T., Taki, A. C., Karnaneedi, S., Nie, S., Kalic, T., Dai, D., Daduang, S., Leeming, M., Williamson, N. A., & Breiteneder, H. (2021). Expanding the allergen repertoire of salmon and catfish. *Allergy, 76*(5), 1443–1453. https://doi.org/10.1111/all.14574

Ruethers, T., Taki, A. C., Khangurha, J., Roberts, J., Buddhadasa, S., Clarke, D., Hedges, C. E., Campbell, D. E., Kamath, S. D., Lopata, A. L., & Koeberl, M. (2020). Commercial fish ELISA kits have a limited capacity to detect different fish species and their products. *Journal of the Science of Food and Agriculture, 100*(12), 4353–4363. https://doi.org/10.1002/jsfa.10451

Schjoldager, K. T., Narimatsu, Y., Joshi, H. J., & Clausen, H. (2020). Global view of human protein glycosylation pathways and functions. *Nature Reviews Molecular Cell Biology, 21*(12), 729–749. https://doi.org/10.1038/s41580-020-00294-x

Shen, Q., Song, G., Zhao, Q., Wang, P., Yang, H., Xue, J., Wang, H., Cui, Y., & Wang, H. (2022). Detection of lipidomics characterization of tuna meat during different wet-aging stages using iKnife rapid evaporative ionization mass spectrometry. *Food Research International, 156*, Article 111307. https://doi.org/10.1016/j.foodres.2022.111307

Shi, Z., Yin, B., Li, Y., Zhou, G., Li, C., Xu, X., Luo, X., Zhang, X., Qi, J., & Voglmeir, J. (2019). N-glycan profile as a tool in qualitative and quantitative analysis of meat adulteration. *Journal Of Agricultural And Food Chemistry, 67*(37), 10543–10551. https://doi.org/10.1021/acs.jafc.9b03756

Silva, A. J., & Hellberg, R. S. (2021). DNA-based techniques for seafood species authentication. In *Advances in food and nutrition research, 95* pp. 207–255). Elsevier. https://doi.org/10.1016/bs.afnr.2020.09.001

Solorzano, Y., Viana, M. T., López, L. M., Correa, J. G., True, C. C., & Rosas, C. (2009). Response of newly hatched Octopus bimaculoides fed enriched Artemia salina: Growth performance, ontogeny of the digestive enzyme and tissue amino acid content. *Aquaculture (Amsterdam, Netherlands), 289*(1–2), 84–90. https://doi.org/10.1016/j.aquaculture.2008.12.036

Song, X.-C., Canellas, E., Dreolin, N., Goshawk, J., & Nerin, C. (2022). A collision cross section database for extractables and leachables from food contact materials. *Journal Of Agricultural And Food Chemistry, 70*(14), 4457–4466. https://doi.org/10.1021/acs.jafc.2c00724

Tang, W., Liu, D., & Nie, S.-P. (2022). Food glycomics in food science: Recent advances and future perspectives. *Current Opinion in Food Science, 46*, Article 100850. https://doi.org/10.1016/j.cofs.2022.100850

Taylor, S. L., & Baumert, J. L. (2015). Worldwide food allergy labeling and detection of allergens in processed foods. *Food Allergy: Molecular Basis And Clinical Practice, 101*, 227–234. https://doi.org/10.1159/000373910

Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Darvill, A. G., Kinoshita, T., Packer, N. H., & Prestegard, J. H. (2015). *Essentials of glycobiology* (3rd ed.). Cold Spring Harbor Laboratory Press.

Visser, E. A., Moons, S. J., Timmermans, S. B., de Jong, H., Boltje, T. J., & Büll, C. (2021). Sialic acid O-acetylation: From biosynthesis to roles in health and disease. *Journal of Biological Chemistry, 297*(2). https://doi.org/10.1016/j.jbc.2021.100906

Vos, G. M., Hooijschuur, K. C., Li, Z., Fjeldsted, J., Klein, C., de Vries, R. P., Toraño, J. S., & Boons, G.-J. (2023). Sialic acid O-acetylation patterns and glycosidic linkage type determination by ion mobility-mass spectrometry. *Nature Communications, 14*(1), 6795. https://doi.org/10.1038/s41467-023-42575-x

Vreeker, G. C., & Wuhrer, M. (2017). Reversed-phase separation methods for glycan analysis. *Analytical And Bioanalytical Chemistry, 409*, 359–378. https://doi.org/10.1007/s00216-016-0073-0

Wang, W. L., Du, Y. M., Wang, W., Conway, L. P., Cai, Z. P., Voglmeir, J., & Liu, L. (2017). Comparison of the bifidogenic activity of human and bovine milk N-glycome. *Journal of Functional Foods, 33*, 40–51. https://doi.org/10.1016/j.jff.2017.03.017

Wang, Z., Zhang, J., & Li, L. (2025). Recent advances in labeling-based quantitative glycomics: From high-throughput quantification to structural elucidation. *Proteomics, 25*(1–2), Article e202400057. https://doi.org/10.1002/pmic.202400057

Warner, K., Roberts, W., Mustain, P., Lowell, B., & Swain, M. (2019). Casting a wider net: More action needed to stop seafood fraud in the United States. *Oceana*.

Wu, Z., Li, H., Zhang, Q., Liu, X., Zheng, Q., & Li, J. (2017). Characterization of O-acetylation in sialoglycans by MALDI-MS using a combination of methylamidation and permethylation. *Scientific Reports, 7*(1), Article 46206. https://doi.org/10.1038/srep46206

Ylönen, A., Kalkkinen, N., Saarinen, J., Bøgwald, J., & Helin, J. (2001). Glycosylation analysis of two cysteine proteinase inhibitors from Atlantic salmon skin: Di-O-acetylated sialic acids are the major sialic acid species on N-glycans. *Glycobiology, 11*(7), 523–531. https://doi.org/10.1093/glycob/11.7.523

Zhao, C., Wang, X., Wu, J., Hu, Y., Zhang, Q., & Zheng, Q. (2024). Analysis of O-acetylated sialic acids by 3-nitrophenylhydrazine derivatization combined with LC-MS/MS. *Analytical Methods, 16*(16), 2472–2477. https://doi.org/10.1039/d4ay00330f