FISH METABOLOMES: APPLICATION OF ^{1}H NMR SPECTROSCOPY IN QUALITY AND ORIGIN DETERMINATION

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Abstract: The global fish industry faces persistent challenges related to product quality, safety, and authenticity, driven by complex supply chains, increasing demand, and the perishable nature of aquatic products. Traditional analytical methods often fall short in providing rapid, comprehensive, and non-destructive insights into the complex biochemical changes occurring in fish. Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy has emerged as a powerful and versatile tool for metabolomics, offering a holistic view of the low-molecular-weight compounds (metabolites) present in biological samples. The present study applied ^{1}H High-Resolution Nuclear Magnetic Resonance for chemical fingerprint identification in mullets from Araruama Lagoon, with the objective of obtaining a geographical identification brand for fish from the Lakes Region of Rio de Janeiro State, Brazil. Lyophilized mullet muscle samples were used to prepare aqueous extracts. Subsequently, the samples were carefully transferred to 5 mm NMR tubes and placed in the NMR spectrometer (600 MHz for ^{1}H frequency) for analysis. As partial results, 22 metabolites related to degradation biomarkers, essential metabolites, energy expenditure, and muscle structure were identified. The ^{1}H NMR tool was efficient in determining metabolites that can be considered biomarkers in analyses for fish characterization.

Keywords: mullet, metabolites, biomarkers, ^{1}H NMR analyses, muscle.

METABOLOMAS DE PEIXE: DETERMINAÇÃO DA QUALIDADE E ORIGEM PELA APLICAÇÃO DA ESPECTROSCOPIA DE 1H-RMN

Resumo: A indústria global pesqueira enfrenta desafios persistentes relacionados à qualidade, segurança e autenticidade dos produtos, impulsionados por cadeias de suprimentos complexas, demanda crescente e natureza perecível dos produtos aquícolas. Os métodos analíticos tradicionais muitas vezes não conseguem fornecer percepções rápidas, abrangentes e não destrutivas sobre as complexas mudanças bioquímicas que ocorrem no peixe. A espectroscopia de Ressonância Magnética Nuclear de Prótons (RMN de ^{1}H) surgiu como uma ferramenta poderosa e versátil para metabolômica, oferecendo uma visão holística dos compostos de baixa massa molecular (metabólitos) presentes em amostras biológicas. No presente estudo a RMN de ^{1}H foi utilizada para identificação de impressões digitais químicas em tainhas da Lagoa de Araruama, com o objetivo de obter um registro de identificação geográfica para peixes da Região dos Lagos do Estado do Rio de Janeiro, Brasil. Amostras liofilizadas de músculo de tainha foram usadas para preparar os extratos aquosos. Posteriormente, as amostras foram cuidadosamente transferidas para tubos de RMN de 5 mm e colocadas no espectrômetro de RMN (600 MHz na frequência de ^{1}H) para análise. Como resultados parciais, sugere-se a identificação de 22 metabólitos relacionados a biomarcadores de degradação, metabólitos

essenciais, gasto energético e estrutura muscular. A ferramenta de RMN de ^{1}H foi eficiente na determinação de metabólitos que podem ser considerados biomarcadores em análises para caracterização do peixe.

Palavras-chave: tainha, metabolitos, biomarcadores, ^{1}H RMN análises, músculo.

1. Introduction (Title in Arial, 12, bold)

Fish, a vital component of global food security and a rich source of essential nutrients, is highly susceptible to rapid deterioration post-harvest. Factors such as enzymatic activity, microbial growth, and lipid oxidation lead to significant losses in quality, nutritional value, and economic viability (Karanth et al., 2023). Ensuring the authenticity and safety of seafood products, from species identification to geographical origin, is paramount for consumer trust and regulatory compliance (Yang et al., 2024).

Conventional analytical techniques, while valuable, often provide limited information, are time-consuming, or require destructive sample preparation (Edwards et al., 2021). Nuclear Magnetic Resonance (NMR) spectroscopy, particularly ^{1}H NMR, has revolutionized the field of metabolomics, offering a non-destructive, highly sensitive, and comprehensive approach to analyze the molecular composition of complex biological matrices. Unlike targeted analyses that focus on specific compounds, NMR metabolomics provides a "fingerprint" of hundreds to thousands of metabolites simultaneously, reflecting the physiological state, biochemical processes, and environmental influences on an organism (Wishart et al., 2022).

This capability makes ^{1}H NMR an ideal tool for understanding the dynamic changes in fish metabolites related to freshness, spoilage, processing, and origin. The technique relies on the interaction of atomic nuclei (specifically protons, 1H) with radiofrequency when the samples are placed in an magnetic field. The method generates unique spectral signals that correspond to different chemical environments of the molecules within the sample. The intensity of these signals is directly proportional to the concentration of the respective metabolites, enabling both qualitative identification and quantitative determination (Picone, 2024). Its ability to provide a holistic metabolic profile makes it particularly well-suited for addressing the multifaceted challenges in fish quality and traceability.

Mullet (*Mugil liza*) is an important fishery resource in Brazil, both for artisanal and industrial fishing. In the state of Rio de Janeiro, specifically in the Araruama Lagoon, mullet is one of the main sources of income for local communities. The Araruama Lagoon is one of the most salinized environments in the world (> 55 ppm), directly influencing the quality of fish in the region. Thus, our project is determining biomarkers for mullet from the Araruama Lagoon, applying NMR techniques to correlate its results with conventional analyses for its nutritional and elemental characterization.

2. Materials and Methods

2.1 Samples

Fish were collected at different points in Lagoa de Araruama, between January and July 2024. They were transported to Embrapa in isothermal boxes with potable ice. Mullets were characterized by biometrics with measurements of weight and length, visual identification of sex, and gonad maturity. Fillets were removed, homogenized, and lyophilized for chemical characterization analyses.

2.2. Metabolomics analysis ^{1}H NMR

For the extraction of polar metabolites, approximately 20 mg of lyophilized mullet muscle

was homogenized for 30 s by using a commercial tip ultrasound with 0.685 mL of a cold methanol-water solution, followed by a second homogenization with cold chloroform. Samples were kept on ice throughout the procedure. Then, the homogenates were centrifuge for 10 min at 12,700 rpm at 4◦C to remove precipitated protein and fat. Supernatants were carefully collected, transferred to Eppendorf tubes, and dried in a centrifugal concentrator (Speed-Vac, Thermo Savant, Holbrook, NY, USA) overnight, at room temperature. The dried extract was resuspended in 500 μL of phosphate buffer (0.10 mol/L, pH = 7.4), prepared in D_2O (99.9%; Sigma-Aldrich, San Luis, CA, USA), containing 0.5 mmol/L of 2,2-dimethyl-2-silapentane-5-sulfonate-d_6 (DSS-d_6) as an internal standard. The mixture was vortexed briefly and centrifuged at 14,000 × g for 5 minutes. Subsequently, the supernatant was carefully transferred to 5 mm NMR tubes and placed in the NMR spectrometer for analysis.

A 14.1 T Bruker spectrometer (600 MHz for hydrogen frequency) fitted with a 5-mm BBA probe was used to record NMR spectra at 298 K. The following acquisition parameters were assumed: number of scans (128), relaxation delay (4 s), spectral width (30 ppm), acquisition time (3.635 s), four dummy scans, and a 90◦ pulse time (9.75 μs) in order to acquire the 1 D ^{1}H-NMR spectra using a standard Bruker pulse sequence with a water pre-saturation signal adopting a continuous wave. For every sample, the procedure was carried out in fully automatic mode via the ICON-NMR interface utilizing Bruker routines (load, automatic tuning, locking, phase, shimming, acquisition, process). Information from the database, the Chenomx NMR suite (free-trial version), and the values from the literature were used to assign the one-dimension spectra.

2.3. Regulatory aspects

The project was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) with the number AEC8FA4.

3. Results and Discussion

Representative ^{1}H NMR spectra of mullet muscles are displayed in Fig. 1. Based on the literature (Bodin et al., 2022), the peaks were assigned to particular metabolites. As seen in fish muscle generally, the ^{1}H NMR spectra of mullet muscle samples included a variety of assignable amino acids, organic acids, glucose, and lipids (Pinheiro et al., 2003). Based on their 1D and 2D spectra, 22 metabolites were found; the chemicals ascribed are listed in Table 1.

For geographical origin, principal aim of our work, the following metabolites such as lactate, hypoxantine, inosine-5-monophosphate (IMP), carnosine, anserine and creatine, can be considered biomarkers, as observed in studies with tuna species (Bodin et al., 2022).

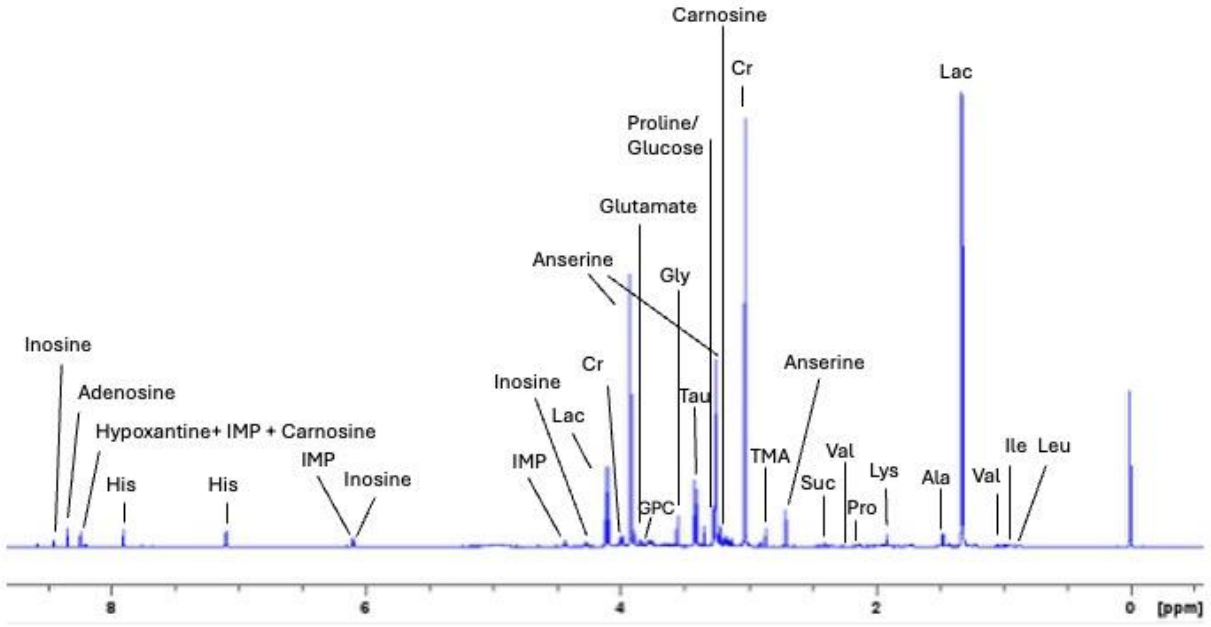

Figure 1. Typical 600 MHz ^{1}H NMR spectra from white muscle of wild mullet. His: histidine; IMP: Inosine-5-monophosphate; Lac: lactate; GPC: Glycerophosphorylcholine; Gly: glycine; Tau: taurine; Cr: creatine; TMA: Trimethylamine; Suc: succinate; Val: valine; Pro: proline; Lys: lysine; Ala: alanine; Ile: isoleucine; Leu: leucine.

Table 1. Representative ^{1}H NMR assignments for white muscles of wild mullet at 600 MHz.

Metabolite	Group	Type	Classification
Inosine	I	Nucleoside	Degradation biomarkers
Adenosine	I	Nucleoside	Degradation biomarkers
Hypoxantine	I	Purine	Degradation biomarkers
Inosine-5-monophosphate	I	Enzyme	Degradation biomarkers
Lactate	II	Organic acid	Essential metabolites
Glycerophosphorylcholine	II	Essential nutrient	Essential metabolites
Glucose	II	Monosaccharide	Essential metabolites
Succinate	III	Organic acid	Energy expenditure
Anserine	III	Dipeptide	Energy expenditure
Carnosine	III	Dipeptide	Energy expenditure
Trimethylamine	III	Amine	Energy expenditure
Creatine	III	Nitrogen organic acid	Energy expenditure
Glutamate	III	Organic acid	Energy expenditure
Histidine	IV	Amino acid	Muscle structure
Glycine	IV	Amino acid	Muscle structure
Taurine	IV	Amino acid	Muscle structure
Proline	IV	Amino acid	Muscle structure
Valine	IV	Amino acid	Muscle structure
Lysine	IV	Amino acid	Muscle structure
Alanine	IV	Amino acid	Muscle structure
Isoleucine	IV	Amino acid	Muscle structure
Leucine	IV	Amino acid	Muscle structure

Bodin et al. (2022).

4. Conclusions

In summary, aqueous extracts from mullet muscles demonstrated extensive metabolic information (amino acids, dipeptides, organic acids, and other vital nutrients) using high-resolution ^{1}H NMR spectroscopy.

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