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Article

Application of Magnetic Resonance Tools for Qualification and Traceability of Mullet

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Abstract

The global seafood 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 intricate biochemical changes occurring in seafood. ¹H Nuclear Magnetic Resonance (¹H NMR) spectroscopy has emerged as a powerful and versatile tool for metabolomics, offering a holistic view of the low-molecular-mass compounds (metabolites) present in biological samples. The present study applied ¹H NMR for chemical fingerprint identification in mullets (*Mugil liza*) from Brazil. Dorsal muscle samples were taken from samples during summer, autumn, and winter. The procedure involved freeze-drying the muscle tissue, thereafter extracting polar metabolites using designated solvents (methanol, water, and chloroform), and analyzing them using a 600 MHz spectrometer. As results, 23 metabolites related to degradation biomarkers, essential metabolites, energy expenditure, and muscle structure were identified. The statistical analysis demonstrated a distinct separation between the geographical origins (RJ vs. SC), mostly influenced by variations in the concentrations of lactate, histidine, threonine, phenylalanine, and ornithine. Factors like fish size and seasonal variations did not markedly affect the overall metabolic profile, so underscoring the reliability of these chemicals as stable origin indicators. The Principal Components Analysis identified two distinct groups of metabolites, establishing a profile for each geographical origin. The developed protocol can be applied in the processes for geographical identification. Thus, the ¹H NMR tool was efficient in determining metabolites that can be considered biomarkers in analyses for seafood traceability.

Keywords: authenticity; metabolites; biomarkers; fingerprinting; Proton Nuclear Magnetic Resonance

Key Contribution: the work emphasizes metabolic fingerprinting of biological samples, transitioning from targeted analysis of markers to a comprehensive view of low-molecular-mass compounds. ¹H NMR is a non-destructive way to monitor a sample's metabolic state, effectively distinguishing the geographical origin and quality of species. By analyzing specific metabolites, including flavor-related amino acids and biomarkers of muscle integrity, distinct chemical profiles can be developed, serving as barcodes for seafood products.

1. Introduction

The global seafood industry is currently operating under complex supply chains, constant environmental changes, and customers who demand transparency, safety, and authenticity. The inherent perishability of aquatic products, coupled with complex processing and worldwide trading, has significantly increased the likelihood of mislabeling, quality degradation, and geographical and

species fraud [1]. In 2022, the aquaculture and fisheries business was valued at approximately \$264.8 billion, underscoring the necessity for the development of validated authentication solutions. Conventional analytical techniques are applicable for certain situations; however, they frequently lack the requisite speed, repeatability, or multidimensional depth necessary to get a comprehensive biochemical profile of seafood products [2]. The application of modern molecular techniques, particularly ^1H Nuclear Magnetic Resonance (^1H NMR) spectroscopy in conjunction with metabolomics, has emerged as a transformative method for the qualification and tracing of seafood. This novel approach pertains to the metabolic fingerprinting of biological specimens. ^1H NMR is an effective method for determining the origin and quality of a species, due to its rapid, non-destructive, and highly reliable assessment of a sample's metabolic state [3]. The technique relies on the interaction of atomic nuclei (specifically, ^1H) with radiofrequency when the samples are placed in a 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 [4]. Its ability to provide a holistic metabolic profile makes it particularly well-suited for addressing the multifaceted challenges in seafood quality and traceability.

Several studies have demonstrated the efficacy of ^1H NMR, exemplified by the accurate differentiation of five fish species, including flounder (*Glyptocephalus cynoglossus*) and European sole (*Lepidopsetta* spp.), with 100% precision [5]. Investigations on the Amazonian fish pirarucu (*Arapaima gigas*) have revealed biomarkers, including acetate, lactate, and creatinine, to assess molecular alterations during the salting and drying processes [6]. A pertinent study examined the traceability of tropical tunas, successfully distinguishing samples by species, size, geographical origin (including the Mozambique Channel), and onboard storage conditions [7]. Additionally, ^1H NMR has been employed to examine the liberation of metabolites and protein breakdown during the *in vitro* digestion of sea bass (*Dicentrarchus labrax*) fillets [8].

The mullets (*Mugil* spp.) are tropical fish species residing in coastal and estuarine habitats, found along the southwestern coast of South America. In Brazil, two species are particularly abundant: *Mugil liza*, prevalent in the Southeast region, and *Mugil curema*, found in the Northeast region. *M. liza* have economic significance for both industrial and artisanal fishing, ranking among the most intensively caught fishery resources in Brazil.

Similar to all marine fish, mullets possess significant nutritional value, particularly for macronutrients (proteins and lipids) and micronutrients (inorganic elements, amino acids, and fatty acids). These nutrients and their metabolites reflect the environmental history, nutritional condition, and physiological adaption of the fish. They may be regarded as biomarkers, signifying a distinct identity for fish from specific locales.

Thus, this study established the profile for *M. liza* from Araruama lagoon by analyzing their concentrations of specific metabolites, such as flavor-enhancing amino acids, osmolytes, and muscle health biomarkers. These profiles could function as mullets chemical barcodes.

2. Materials and Methods

2.1. Study Area

The research was performed using mullet specimens from the State of Rio de Janeiro, southeastern Brazil. The Araruama Lagoon (-22.788, -42.275), situated in the Lakes Region, is acknowledged as the biggest permanently hypersaline lagoon globally, including roughly 225 km² of water surface and exhibiting an average salinity of 52 ‰ (range from 12 to 60) [9,10] (Figure 1).

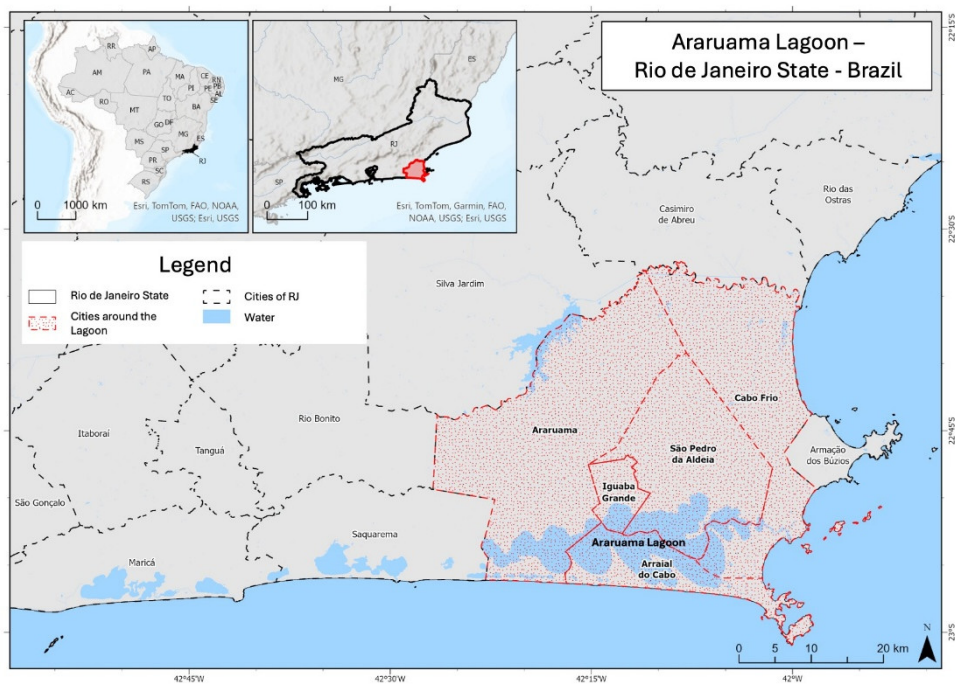


Figure 1. Location of Araruama Lagoon.

2.2. Samples

Fish were collected at four different points in Araruama Lagoon, in the summer, autumn, and winter, between January and July 2024. A total of 16-24 fishes were collected per season of the year. They were transported in styrofoam boxes with potable ice (-18 °C). Table 1 shows the mass (g), dimensions and males/females percentage of the fish collected in summer, autumn and winter. A sample of about 10 g on a wet mass (WM) basis was collected from the dorsal muscle [7]. Muscles were homogenized, lyophilized, and preserved at -80 °C until chemical characterization analyses. Six mullets (1,200 ± 132 g) from Santa Catarina State were purchased at the fish market during the winter for comparative analysis. To mitigate the specific impact of each fish on the analytical results, pools of 10 fillets each were obtained per season of the year.

Table 1. Biometric parameters for the mullets of Araruama Lagoon, RJ.

Season	Total length (cm)	Standard length (cm)	Total mass (g)	Height (cm)	Males/Females (%)
Summer ¹	44.48 ± 10.66	35.63 ± 4.94	834.92 ± 362.33	11.62 ± 1.93	37.50/62.50
Autumn ²	54.27 ± 3.85	44.81 ± 3.56	1,539.45 ± 367.06	12.06 ± 0.37	43.75/56.25
Winter ³	47.40 ± 11.89	40.78 ± 9.59	977.94 ± 704.19	10.62 ± 2.30	68.42/31.58

¹n=24, one female with developed gonads; ²n=16, two females with developed gonads; ³n=19, all samples were adults reproductive phase.

Water samples, collected at the same points of Araruama Lagoon, were obtained with a Van Dorn bottle to assess the physicochemical properties (temperature, pH, salinity, dissolved oxygen, and conductivity) (Table 2).

Table 2. Water physicochemical properties, Araruama Lagoon, RJ.

Season	Dissolved oxygen (mg/L)	pH	Temperature (°C)	Conductivity (mS)	Salinity (‰)
Summer ¹	6.73 ± 0.12	7.99 ± 0.10	29.78 ± 0.67	73.08 ± 4.30	50.50 ± 1.00

Autumn ²	8.78 ± 2.76	7.93 ± 0.11	28.48 ± 0.31	81.20 ± 5.86	54.43 ± 2.19
Winter ³	7.43 ± 1.32	7.84 ± 0.21	26.23 ± 0.54	78.78 ± 3.35	53.31 ± 2.25

Mean ± SD. n=4 samples/season.

2.3. Metabolomics Analysis ¹H NMR

For the extraction of polar metabolites, approximately 20 mg of lyophilized mullet muscle was homogenized for 30 sec 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 lipid supernatants. Aqueous extracts were carefully collected, transferred to Eppendorf tubes, and dried in a centrifugal concentrator (Speed-Vac, Thermo Savant, Holbrook, NY, USA) overnight (13 hours), at room temperature (25 °C). The dried extract was resuspended in 500 µL of phosphate buffer (0.10 M, pH = 7.4), prepared in D₂O (99.9%; Sigma-Aldrich, San Luis, CA, USA), containing 0.5 mM of 2,2-dimethyl-2-silapentane-5-sulfonate-d₆ (DSS-d₆) 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 × 178 mm thin-walled NMR tubes (VWR International), and placed in the NMR spectrometer for analysis.

A 14.1 T Bruker spectrometer (600 MHz for ¹H frequency) fitted with a 5-mm BBA probe was used to record NMR spectra at 298 K. The ¹H-NMR spectra were acquired with a standard Bruker pulse sequence with a water pre-saturation (ZG) with the following parameters: number of scans (128), recycle delay (4 s), spectral width (30 ppm), acquisition time (3.635 s), dummy scans (4), and a 90° pulse time (9.75 s). 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).

The ¹H NMR spectra were analyzed utilizing the Chenomx NMR Suite Professional 7.7 software (Chenomx Inc., Edmonton, Canada). Phasing and baseline correction were carried out and pH was calibrated via imidazole resonances. The spectra were calibrated to the DSS methyl peak at 0.00 ppm. The identical peak was utilized as a chemical shape indication, serving as an internal reference for quantification [11].

2.4. Metabolite Identification and Quantification

Metabolites in the ¹H NMR spectra were identified using the integrated 1D spectrum library of Magnet metabolomic software [12]. A total of 23 metabolites were quantified in muscle extracts utilizing the profiler module (Figure 2). Quantification was conducted by comparing the identified metabolite peaks area to the area beneath the DSS methyl peak, which is associated with a known concentration of 0.5 mM in each sample. Then, metabolite concentrations were exported to Excel for data processing.

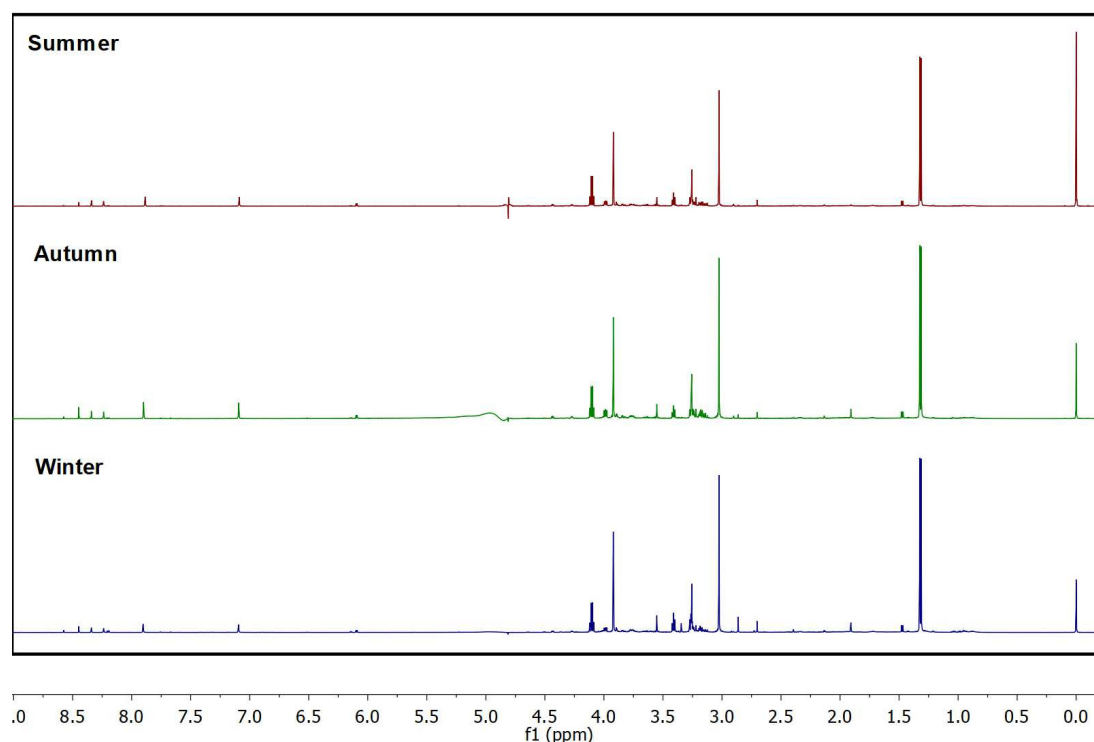


Figure 2. Typical 600 MHz ^1H NMR spectra from muscle of wild mullet collected in Summer, Autumn and winter.

2.5. Statistical Analysis

Means and standard deviations were determined using Excel. PCA, PLS, normality tests (Shapiro-Wilk), and ANOVA (Kruskal-Wallis) were conducted utilizing PAST (Paleontological Statistics Software Package, version 5) [13]. The estimation of missing values was carried out utilizing the k-nearest neighbors (KNN) approach, relying on analogous samples [11]. A one-way ANOVA was conducted with Tukey's HSD as a post-hoc analysis, and significance was defined at $P < 0.05$.

3. Results

3.1. Identification of Mullet Muscle Metabolite Profiles

The ^1H NMR spectra of mullet muscle samples revealed multiple recognizable amino acids, organic acids, glucose, and lipids, aligning with previous research on fish muscle [14]. Twenty-three metabolites were identified by their 1D and 2D spectra, with the corresponding compounds listed in Table 3. The analysis of muscle spectra permitted the identification of four categories of signals (according to 7) corresponding to metabolites: (i) Group I – degradation biomarkers: the NMR spectra from 8.5 to 4.2 ppm displays signals belonging to adenosine triphosphate (ATP) degraded compounds as hypoxanthine and inosine-5-monophosphate; (ii) Group II – essential metabolites: the NMR spectra from 4.4 to 3.5 ppm, display the signals of choline derivate metabolites as glycerophosphorycholine (GPC), and also characterized by glucose presence; (iii) Group III – energy expenditure: the highest intensity of signals was observed for lactate in the high-field (2.7 - 1.4 ppm), and creatine in the mild-field (4.2 – 3.0 ppm) NMR spectra regions; (iv) Group IV – muscle structure and its degradation: the signals from 2.8 to 0.8 ppm, from 3.5 to 3.4 ppm, and from 7.0 - 7.9 ppm correspond to aliphatic and aromatic groups, with 14 amino acids determined (Table 3).

Table 3. Representative ¹H NMR assignments for mullet muscles at 600 MHz.

Metabolite	Concentration range*	Group	Type
Hypoxanthine	543.2 – 6.5	I	Purine
Inosine-5-monophosphate	97.8 – 0.2	I	Enzyme
Glycerophosphorylcholine	171.1 – 4.9	II	Essential nutrient
Glucose	4,563.3 – 215.8	II	Monosaccharide
Lactate	24,418.9 – 522.7	III	Organic acid
Succinate	155.3 – 12.6	III	Organic acid
Carnosine	372.0 – 14.1	III	Dipeptide
Creatine	10,918.0 – 381.1	III	Nitrogen organic acid
Glutamate	512.1 – 29.8	III/IV	Organic acid
Histidine	4,422.5 – 338.8	IV	Amino acid
Glycine	2,188.3 – 43.1	IV	Amino acid
Taurine	5,303.4 – 14.4	IV	Amino acid
Proline	1,476.4 – 216.7	IV	Amino acid
Valine	483.7 – 21.2	IV	Amino acid
Lysine	6,522.5 – 3.8	IV	Amino acid
Alanine	518.3 – 11.0	IV	Amino acid
Isoleucine	707.8 – 23.0	IV	Amino acid
Leucine	1,505.4 – 35.8	IV	Amino acid
Methionine	834.3 – 28.2	IV	Amino acid
Threonine	470.2 – 3.1	IV	Amino acid
Phenylalanine	849.1 – 12.9	IV	Amino acid
Serine	868.6 – 16.3	IV	Amino acid
Ornithine	862.5 – 17.7	IV	Non-proteinogenic amino acid

*(mM/g muscle).

3.2. Effect of Season on Mullet Muscle Metabolite Profiles

The metabolite profile of fish captured across different seasons was analyzed to determine the impact of the fishing season on fish quality in terms of metabolites. There was a significant difference between the samples only for succinate, a metabolite for energy expenditure (Table 4). This result indicates that the fish from the Lagoon exhibit a chemical composition pattern associated with environmental characteristics (diet, salinity, inorganic element content in the water, etc.), irrespective of seasonal variations that result from environmental changes, especially in temperature (Table 2).

Table 4. Metabolite profile of mullet muscle across different seasons, from Araruama Lagoon, RJ.

Metabolite*	Summer	Autumn	Winter
Hypoxanthine	298.81 ± 120.0 ^a	319.49 ± 153.65 ^a	248.10 ± 73.35 ^a
Inosine-5-monophosphate	7.93 ± 7.13 ^a	4.50 ± 4.91 ^a	11.93 ± 6.62 ^a
Glycerophosphorylcholine	96.92 ± 26.49 ^a	108.40 ± 35.52 ^a	96.41 ± 41.15 ^a
Glucose	908.75 ± 396.77 ^a	888.74 ± 432.70 ^a	677.16 ± 160.31 ^a
Lactate	15,539.75 ± 3,631.36 ^a	17,739.46 ± 5,781.62 ^a	15,760.72 ± 1,918.71 ^a
Succinate	60.66 ± 41.18 ^a	65.16 ± 33.13 ^a	34.76 ± 21.13 ^b
Carnosine	210.37 ± 78.33 ^a	230.50 ± 84.53 ^a	248.14 ± 83.21 ^a
Creatine	7,310.01 ± 1,405.24 ^a	7,423.95 ± 3,357.65 ^a	6,172.06 ± 1,567.88 ^a

Glutamate	206.20 ± 133.84 ^a	150.80 ± 131.80 ^a	146.81 ± 50.29 ^a
Histidine	3,080.28 ± 643.25 ^a	2,755.87 ± 943.66 ^a	3,368.12 ± 637.02 ^a
Glycine	1,058.33 ± 409.53 ^a	1,209.52 ± 721.59 ^a	740.02 ± 413.83 ^a
Taurine	39.16 ± 17.21 ^a	40.40 ± 19.87 ^a	34.69 ± 16.83 ^a
Proline	657.98 ± 216.23 ^a	650.01 ± 331.77 ^a	786.22 ± 385.54 ^a
Valine	97.43 ± 37.62 ^a	105.37 ± 59.55 ^a	80.51 ± 34.79 ^a
Lysine	300.62 ± 160.11 ^a	442.71 ± 349.81 ^a	290.69 ± 160.17 ^a
Alanine	772.48 ± 258.15 ^a	869.11 ± 344.94 ^a	767.01 ± 282.91 ^a
Isoleucine	53.85 ± 24.50 ^a	54.32 ± 32.53 ^a	102.94 ± 110.44 ^a
Leucine	133.17 ± 63.83 ^a	131.96 ± 57.65 ^a	239.73 ± 216.45 ^a
Methionine	131.63 ± 111.34 ^a	146.47 ± 90.11 ^a	92.35 ± 40.99 ^a
Threonine	243.19 ± 124.22 ^a	285.18 ± 111.34 ^a	322.95 ± 66.23 ^a
Phenylalanine	111.54 ± 112.74 ^a	38.04 ± 25.04 ^a	361.37 ± 358.70 ^a
Serine	398.12 ± 225.72 ^a	315.46 ± 193.02 ^a	356.23 ± 262.78 ^a
Ornithine	607.13 ± 226.42 ^a	583.96 ± 263.07 ^a	458.03 ± 381.40 ^a

*(mM/g muscle). Mean ± SD. n=12 samples/season. Different letters in the columns indicate a statistically significant variance (p<0.05) between the treatment means (summer, autumn, and winter).

3.3. Effect of Size Category on Mullet Muscle Metabolite Profiles

To evaluate the impact of fish size, samples were categorized into three groups: (I) mass up to 700 g; (II) mass from 701 to 1,400 g; and (III) mass over 1,401 g. No significant differences were identified among the various mass classes assessed (P>0.05), as observed for the collection periods. For easier visualization of the results, a PCA analysis was performed to assess the clustering of the different groups (Figure 3, a). The figure indicates an absence of group segregation for the metabolites determined based on sample mass. The dendrogram illustrates a correlation among the samples; however, it reveals no discernible pattern, indicating similarities among the samples irrespective of the fish mass (Figure 3, b).

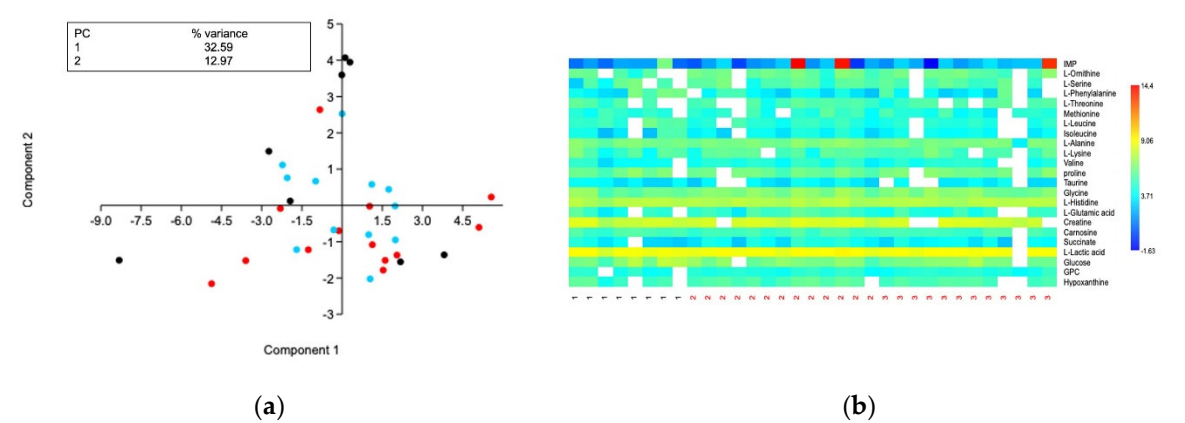


Figure 3. Metabolite profile of mullet muscle across different sizes, from Araruama Lagoon, RJ. Group I (black points): ≤ 700g; group II (red points): 701g - 1,400g; group III (blue points): ≥ 1,401g. (a) PCA analysis between groups; (b) Dendrogram with correlations of groups.

3.4. Effect of Geographical Origin on Mullet Muscle Metabolite Profiles

When comparing mullets with different origins (Araruama Lagoon, RJ, and Santa Catarina State), collected simultaneously in winter, a segregation of groups was observed, indicating differences in the metabolite profiles between the samples (Figure 4). The statistical analysis validated the distinctions between the metabolites: lactate, histidine, threonine, phenylalanine and ornithine (Table 5).

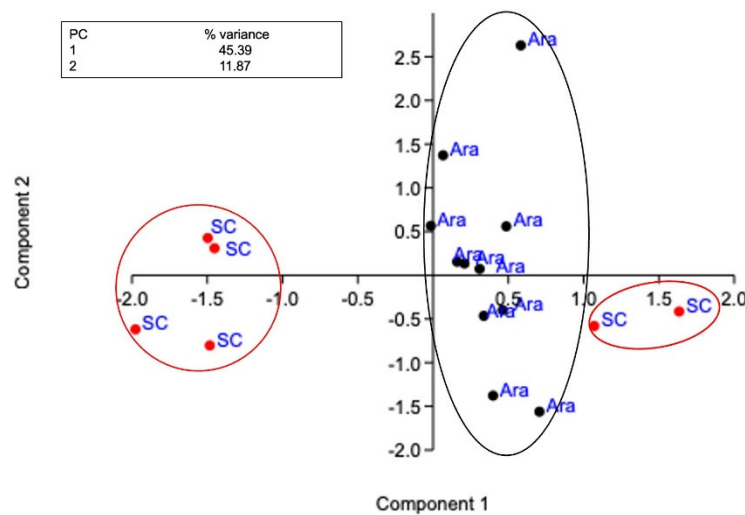


Figure 4. PCA analysis of metabolite profile of mullet muscle from Araruama Lagoon, Rio de Janeiro State (black points) and Santa Catarina States (red points).

Table 5. Metabolite profile of mullet muscle from Araruama Lagoon, RJ, and Santa Catarina State.

Metabolite*	Ara	SC	P value
Hypoxanthine	248.10 ± 73.35 a	345.73 ± 254.75 a	0.8864
Inosine-5-monophosphate	11.93 ± 6.62 a	9.52 ± 6.75 a	0.4791
Glycerophosphorylcholine	96.41 ± 41.15 a	73.60 ± 54.69 a	0.1931
Glucose	677.16 ± 160.31 a	474.63 ± 248.18 a	0.1003
Lactate	15,760.72 ± 1,918.71 a	7,420.12 ± 5,183.60 b	0.0066
Succinate	34.76 ± 21.13 a	33.52 ± 21.86 a	0.8137
Carnosine	248.14 ± 83.21 a	191.85 ± 127.67 a	0.1931
Creatine	6,172.06 ± 1,567.88 a	3,969.27 ± 2,451.98 a	0.0593
Glutamate	146.81 ± 50.29 a	188.75 ± 166.96 a	0.5677
Histidine	3,368.12 ± 637.02 a	2,037.26 ± 1,281.17 b	0.0187
Glycine	740.02 ± 413.83 a	487.03 ± 347.10 a	0.1289
Taurine	34.69 ± 16.83 a	33.26 ± 27.95 a	0.6631
Proline	786.22 ± 385.54 a	315.98 ± 227.24 a	0.0424
Valine	80.51 ± 34.79 a	98.45 ± 71.88 a	0.6704
Lysine	150.93 ± 100.13 a	123.70 ± 106.38 a	0.7540
Alanine	767.01 ± 282.91 a	577.45 ± 400.51 a	0.1585
Isoleucine	102.94 ± 110.44 a	59.07 ± 44.02 a	0.5186
Leucine	239.73 ± 216.45 a	113.18 ± 82.92 a	0.0707
Methionine	92.35 ± 40.99 a	76.20 ± 44.00 a	0.5582
Threonine	322.95 ± 66.23 a	158.82 ± 81.34 b	0.0283
Phenylalanine	361.37 ± 358.70 a	39.38 ± 29.38 b	0.0019
Serine	356.23 ± 262.78 a	279.70 ± 219.49 a	0.4822
Ornithine	458.03 ± 381.40 a	162.33 ± 111.72 b	0.0152

*(mM/g muscle). Mean ± SD. n=10 samples for Araruama (ARA) and n=6 samples for Santa Catarina State (SC). Different letters in the columns indicate a statistically significant variance (p<0.05) between the treatment means (Ara x SC).

4. Discussion

Fish from Araruama Lagoon showed elevated levels of lactate and creatine (Table 3), related with increased muscular activity, supporting the hypothesis of a metabolic shift following escape

from fishing vessels [15] or for reproduction at ocean. Lactate accumulates in white muscle during burst swimming (anaerobic) and also during post mortem alterations [16]. That accumulation causes intracellular acidosis, leading to muscle fatigue, while creatine helps buffer this and accelerates muscle recovery [17].

The mullet exhibits a typical behavior for marine-reproducing species, wherein its juveniles inhabit estuaries and lagoons for feeding and growth, subsequently returning to the ocean once reaching reproductive maturity to ensure species perpetuation [18,19]. In the Araruama lagoon, juvenile recruitment occurs throughout the closed season, ranging from August to November annually. During summer and autumn, fishing targets adult specimens in the early stages of gonadal maturation [20], as indicated by the samples in this study (Table 1). During winter, the mature adults left the Lagoon for the sea via a channel [21], where fishing cages or hooks are situated, resulting in the catch of some of these fish. Consequently, there exists a persistent pattern of foraging for sustenance, incessant swimming, and returning to the ocean during winter. This trend can be attributed to the increased concentrations of lactate and creatine in the analyzed samples.

Mullets also exhibit elevated concentrations of taurine and lysine (Table 3). Taurine has a crucial role in osmoregulation, membrane integrity, and the metabolism of energy, amino acids, proteins, and lipids, in addition to promoting development and facilitating antioxidative processes [22–25]. Lysine is an essential amino acid that significantly contributing to protein synthesis, muscle development, and the maintenance of a positive nitrogen balance [26]. It is crucial for collagen synthesis, immunological response, and the production of carnitine, which facilitates energy metabolism. The presence of these two amino acids indicates the good nutritional status of the fish in the Lagoon. Research indicates that wild fish exhibit elevated taurine levels compared to cultivated and escaped fish from marine aquaculture farms [15]. Recently, studies determined that taurine may serve as a potential biomarker for identifying the origin of fish and can also indicate the health of fish cultivated in marine aquaculture facilities in contrast to wild fish [23,27].

The reduced concentrations of IMP, GCP, and succinate indicate the favorable nutritional condition of the fish. Elevated levels of succinate and inosine monophosphate (IMP) are frequently associated with metabolic stress. IMP and GCP are metabolites resulting from the degradation of ATP during intense muscle exercise or post-mortem decomposition (loss of freshness). Spoilage changes nucleotides (e.g., $\text{ATP} \rightarrow \text{IMP} \rightarrow \text{inosine}$) providing these metabolites significant markers of freshness [28,29]. Succinic acid increases during hypoxia or metabolic stress; it functions as a signaling molecule to activate the immune response [30].

Concerning seasonal variations, only the fish harvested in winter exhibited reduced succinate levels relative to those gathered in summer and autumn. Succinate is recognized for its role as a growth enhancer and is utilized in feed to enhance protein efficiency in juveniles [31,32]. As the fish progresses beyond the high-growth phase, the physiological demand for elevated succinate levels for swift growth decreases. It is an essential step in the tricarboxylic acid (TCA) cycle, which is involved in energy metabolism. The elevated activity levels and swift growth in juveniles necessitate accelerated and more vigorous energy processing in contrast to the slower metabolic state observed in many adult fish [33]. Table 1 illustrates that during winter, we gathered a greater number of adults in the reproductive phase, but in summer and fall, we collected more juveniles, which accounts for the variation in succinate levels.

Previous studies indicate that the results for mullets (*Mugil liza*) in Brazil can be directly associated with the application of metabolites for geographical differentiation, a method already substantiated for species such as tunas (*Thunnus albacares*, *Katsuwonus pelamis*, and *T. obesus*) [7], salmon (*Salmo salar*) [34,35], pikeperch (*Sander lucioperca*), perch (*Perca fluviatilis*), and bream (*Abramis brama*) [36]. The study substantiates three primary dimensions: the effectiveness of NMR spectroscopy, the detection of certain biomarkers, and the impact of behavior and environment on the metabolic profile.

Similar to research conducted on tropical tunas and fish from Northeast Europe, the ^1H -NMR technique demonstrated exceptional efficacy in discerning a chemical “fingerprint” of mullets,

facilitating a distinct differentiation between the origins of Lagoa de Araruama (RJ) and Santa Catarina through Principal Component Analysis (PCA), which established the correlation among the metabolite profiles of samples from various locations. The regional differentiation of mullets was confirmed by notable variations in particular metabolites (lactate, histidine, threonine, and ornithine), several of which also serve as critical differentiators in other species. Lactate and creatine exhibited higher levels in the mullets from Araruama Lagoon, attributed to vigorous muscle activity (explosive swimming and reproductive migration). In the study involving tunas, lactate and creatine/phosphocreatine were recognized as essential metabolites for differentiating fish collected in various regions (e.g., the Mozambique Channel versus the Central Indian Ocean), indicating variations in hunting behavior and habitat [7].

In terms of amino acids, the concentrations of histidine, threonine, and ornithine were essential for geographical differentiation in mullets. In tunas and seabass, free amino acids and dipeptides, including anserine and carnosine, were significant factors in distinguishing between geographical origins and natural diets [7,15]. Histidine is an essential amino acid associated with muscle formation and its decomposition, serving as a precursor to important dipeptides, including carnosine and anserine [37]. These chemicals are present in elevated concentrations in pelagic and migratory species, such as tunas and mullets, since they serve as chemical buffers that mitigate the acidity resulting from lactate accumulation during anaerobic activity [38] associated with long-distance migration and reproduction [39].

Environmental and adaptation factors directly influence the metabolic profile of fish [40,41]. Salinity and osmoregulation may affect the biomarker profile [42]. The Araruama Lagoon is hypersaline (50-55‰), affecting the osmolyte composition of the mullets. The coast of Santa Catarina exhibits typical saltwater salinity levels of 30-35‰. The study developed with *Sparus aurata*, *Dicentrarchus labrax* and *Argyrosomus regius* substantiates this occurrence, emphasizing trimethylamine (TMAO) and taurine as significant biomarkers of environmental adaptation and exposure to varying salinities in natural habitats [15].

An additional significant influence is the nutritional condition of the fish. Elevated concentrations of taurine and lysine in mullets signify a favorable nutritional condition in the lagoon. The utilization of taurine as a biomarker of origin is substantiated by sources to differentiate not only geographical provenance but also life history (wild versus domesticated) [15]. Threonine is an indispensable amino acid that contributes to the chemical “fingerprint” characteristic of fish. This amino acid serves as a structural element of muscle proteins [43,44], and its concentrations indicate the nutritional background and dietary availability [45] in various environments. A distinction in origin for Brazilian mullets was identified using the quantification of threonine, corroborating its application as a biomarker for origin designation. Ornithine, unlike other amino acids, is a non-proteinogenic amino acid, indicating that it is not utilized in protein synthesis; instead, it plays a role in intermediary metabolic pathways, including nitrogen metabolism and the urea cycle [46]. In the mullets of Araruama Lagoon, ornithine was recognized as a key metabolite facilitating the segmentation of groups based on geographic origin by PCA analysis. This illustrates particular physiological adaptations to the environment, including the lagoon’s hypersalinity, which influences the equilibrium of free amino acids in the tissue [47].

Thus, mullets exhibit a globally recognized pattern wherein the metabolic profile (lactate, creatine, and amino acids) functions as a chemical “barcode” that synthesizes the local diet, the physical exertion demanded by the environment, and the physiological adaptations to the physicochemical conditions of the water.

5. Conclusion

¹H NMR spectroscopy is a potent technique for mapping the metabolism of mullet (*Mugil liza*), identifying metabolites that function as species-specific chemical fingerprints. The metabolic profile varies significantly based on geographical origin. This registration enhances traceability, combats fraud, and ensures food safety and market value.

6. Patents

The findings will constitute the report for the registration of the Geographical Indication under the Denomination of Origin category for the mullet from the Araruama Lagoon at the Brazilian National Institute of Intellectual Property (INPI).

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Abbreviations

The following abbreviations are used in this manuscript:

ATP	Adenosine Triphosphate
IMP	Inosine-5-monophosphate
GCP	Glycerophosphorylcholine
PCA	Principal Component Analysis
TMAO	trimethylamine

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