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ARTIGO *REVISTA BRASILEIRA DE ENGENHARIA DE PESCA*

BIOCHEMICAL PROSPECTION OF GLYCOSAMINOGLYCANS IN FISH WASTES AIMING AT CIRCULAR ECONOMY

PROSPECÇÃO BIOQUÍMICA DE GLICOSAMINOGLICANOS EM RESÍDUOS DE PEIXE VISANDO ECONOMIA CIRCULAR

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Abstract Wastes of *Prochilodus brevis* and *Oreochromis niloticus* fishes could be reservoirs in glycosaminoglycans (GAGs) for circular economy. The aim of this study was to biomass prospect for biochemical analysis of GAGs, from cultured fish wastes, on yield and physical/chemical/structural features. Dehydrated wastes showed both skins (0.51/0.65%, w w^{-1}, respectively) with higher volume than *O. niloticus* eyeball (0.29%), intestine (0.08%) and gonad (0.03%) from the total fish mass. Papain digestion had an inverse rate ($p < 0.05$) in yield (%) of GAGs (0.35 ± 0.03/0.18 ± 0.02-skin; 0.16 ± 0.03-eyeball; 0.36 ± 0.10-intestine; 0.40 ± 0.08-gonad). Combined electrophoresis and infrared techniques revealed a difference in charge density among the samples in mixture or not of GAGs, suggesting dermatan (skin), chondroitin/hyaluronic acid (eyeball), heparan/hyaluronic acid (intestine) and dermatan/hyaluronic acid (gonad) the classes found from ~8 to >100 kDa. The scenario presumed a dominant yield of GAGs in abdominal cavity while a more charged composition for those located in external anatomy. Therefore, the study contributed to partial description of freshwater fish waste GAGs of bioeconomic interest.

Key Words: aquaculture, residual discard, polymers, bioeconomy.

Resumo Resíduos de peixes *Prochilodus brevis* e *Oreochromis niloticus* poderiam ser, para economia circular, reservatórios em glicosaminoglicanos (GAGs). Este estudo prospectou biomassa, de resíduos de peixes cultivados, para análise bioquímica de GAGs sobre rendimento e características físicas/químicas/estruturais. Resíduos desidratados mostraram, com volume maior, ambas as peles (0,51/0,65%, m m^{-1}, respectively) que olho (0,29%), intestino (0,08%) e gônada (0,03%) de *O. niloticus*, da massa total dos peixes. A digestão com papaína apresentou uma taxa inversa ($p < 0,05$) no rendimento (%) de GAGs (0,35 ± 0,03/0,18 ± 0,02-pele; 0,16 ± 0,03-olho; 0,36 ± 0,10-intestino; 0,40 ± 0,08-gônada). As técnicas combinadas de eletroforese e infravermelho revelaram, entre as amostras em mistura ou não, uma diferença na densidade de cargas, sugerindo dermatam (pele), condroitim/ácido hialurônico (olho), heparam/ácido hialurônico (intestino) e dermatam/ácido hialurônico (gônada) as classes encontradas de ~8 a >100 kDa. O cenário presumiu, na cavidade abdominal, um rendimento dominante de GAGs, enquanto para aqueles localizados na anatomia externa, uma composição mais carregada. Portanto, o estudo contribuiu para descrição parcial de GAGs em resíduos de peixes dulcícolas de interesse bioeconômico.

Palavras-chave: aquicultura, descarte residual, polímeros, bioeconomia.

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Introduction

In the last decades, human practices have created interactions between humanity and the environment incompatibles with the sustainability of the planet (Machado et al., 2020). Within the context of circular economy, the production of fish is preponderantly derived through aquaculture due to its technological efficiency, farmed species variety and market opportunities (Cooney et al., 2023). Because the fish (industrial and artisanal) production systems generate a large amount of filleting operation-discarded solid wastes (*e.g.*, fins, heads, gills, spines, scales visceras and skins) harming the environment (Moreira et al., 2001; Oetterer et al., 2014), the change in human behaviour must be directed towards the transition of new mentalities of ecological protection and combating waste through biological prospecting in valorization, avoiding the loss of valuable materials with biotechnological potential and promoting more sustainable models, consequently, providing market opportunities (Cooney et al., 2023) by reducing environmental and economic damage (Machado et al., 2020).

Based on this concept (Figure 1), the animal-derived glycosaminoglycan (GAG) industry has emerged for more than 50 years (Badri et al., 2018), but with growing interest in those derived from fish processing waste it has renewed (Oetterer et al., 2014) *vs*. to those obtained from terrestrial sources that have infection risks (Volpi, 2011; Badri et al., 2018). The presence of high amounts of organic compounds in fish waste arises as multiple social-economic-environmental advantages (Cooney et al., 2023). Based on full use of fish (Ogawa & Maia, 1999; Oetterer et al., 2014), the biochemical knowledge about the GAGs-related composition of the solid waste discarded in the fish production would support the rational use of this valuable biological material (Badri et al., 2018).

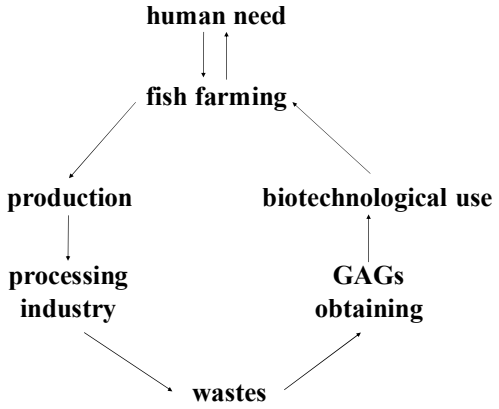

Figure 1. Model of circular economy for fish GAGs industry based on Cooney et al. (2023).

GAGs are the major components occuping and hydratating the extracellular matrix, playing functionalities related to since ionic balance to biomechanical properties (Badri et al., 2018), as well as microbial defence (Moreira et al., 2001; Ali et al., 2023). These regulatory macromolecules also form GAG-protein interactions contributing to the structural biology of the matrix of complex multicellular organisms (Gandhi & Mancera, 2008). GAGs chains are classified as linear and highly charged polymers (10-100 kDa) varying in sulfation patterns, different linkages and specific saccharide sequences (Gandhi & Mancera, 2008; Badri et al., 2018), being heparin (HEP, highly charged), chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronic acid (HA, nonsulfated), keratan sulfate and heparan sulfate (HS) the classes most found in animals (Gandhi & Mancera, 2008). Global demand for GAGs have been elevated (HEP, bovine/porcine lung and intestine; HA, microbial fermentation; and CS, bovine/porcine trachea and shark cartilage) due to their pharmaceutical consumption over time, such as anticoagulant/antithrombotic, cosmetic and anti-inflammatory (Volpi, 2011; Badri et al., 2018). They are composed of repeating disaccharide units

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of aminosugar (D-galactose or D-glucosamine) and uronic acid (L-iduronic or D- glucuronic acid) or galactose sugar, with their availability and chemical proportions depending on the species (Gandhi & Mancera, 2008; Badri et al., 2018). The freshwater fishes have been investigated only in limited number of species on the composition (Dellias et al., 2004; Arima et al., 2013) and bioactivities (Nogueira et al., 2019; Santiago et al., 2024; Rodrigues et al., *in press*), where the identification and the accessibility to GAGs vary with tissue of origin that could limit the biochemical investigation, *e.g*., specific location of a GAG in tissue, extraction method, residue quality, age and developmental biology (Pfeiler et al., 1998; Souza et al., 2007; Zhang et al., 2009; Santiago et al., 2024).

The amount of fish waste biomass varies with the filled species (Moreira et al., 2001; Oetterer et al., 2014; Machado et al., 2020) and in total yield / type of GAG in analyzed tissue (Arima et al., 2013). In Brazil, freshwater fishes are widely cultured using different production systems (Moreira et al., 2001; Rodrigues et al., 2011, *in press*; Marques et al., 2023), including *Prochilodus brevis* Steindachner, 1875, a rheophilic species found in the semiarid region of Caatinga area, but its populational declines by predatory fishing has affected the reprodutive biology (Nascimento et al., 2012) motivating studies on sperm cryopreservation (Nascimento et al., 2021); and *Oreochromis niloticus* Linnaeus 1758, a Cichlidae species widely cultured on an important industrial/scientific level, that has nutritional richness and represents the main first-matter for the development of various food coproducts (Moreira et al., 2001). However, there is littler knowledge on their filleting waste as raw material for GAGs obtaining and biological implications (Nogueira et al., 2019; Pereira et al., 2021), since that the extraction of fish GAGs have been still challenger (Liu et al., 2025) and usually associated with the abundance and chemical distribution (Dellias et al., 2004; Souza et al., 2007; Salles et al., 2017; Santiago et al., 2024).

As the filleting of fish generates about 40-60% of residual mass (Moreira et al., 2001; Machado et al., 2020) and there is also a lack of knowledge for more specialized organ (*e.g*., intestine and gonad) that integrate the viscera (Nogueira et al., 2019), this study provided information on GAGs of skin (*P. brevis* and *O. niloticus*), eyeball, gonad and intestine (*O. niloticus*) by analysing enzymatically extracted GAGs (Pb.wasteGAGs and On.wasteGAGs) by comparative biochemical techniques, prospecting in these two freshwater fish species their compositional biology aiming at circular economy, since the quantity these residuals is discarded without suitable destination and devoid of commercial value (Machado et al., 2020; Cooney et al., 2023).

Material and Methods

Specimens of *P. brevis* / *O. niloticus* and tissue preparation

The adult specimens of cultured *P. brevis* (16 individuals) males were provided by the Fish Reproduction Biotechnology Laboratory located at the State University of Ceará (SUC), Brazil, while those of monosex *O. niloticus* (50 individuals) were obtained from the Aquaculture Station of the Federal University of Ceará (FUC) (Table 1, Figure 2).

All the fishes collected, from the each system of daily monitored cultivation (Table 1), were subjected to Marques et al. (2023) and Santiago et al. (2024)' slaughter procedure by percussive stunning, followeed by packed in ice (1:1 kg - biomass:ice ratio) using sialed isothermal boxes and then taken to the Marine Biochemical (MarBio) laboratory located at Aquaculture Biotechnology Center, Department of Fisheries Engineering, FUC. All the fishes were authorized through our registration with SISGEN-AA4816B (National System for the Management of Genetic Heritage and Associated Traditional Knowledge) and approved by the Ethical Committee of the SUC and FUC (protocols nº 09664402/2019 and 6974061020, respectively).

Upon arriving at the MarBio laboratory, the separatelly sampled fishes, in length and weight using a rudimentary ichthyometer and a commercial balance on a 1 g precision scale, respectively, were manually washed, submitted to scaling, evisceration and specific organs of *P. brevis* (skin)

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and *O. niloticus* (skin, eyeball, gonad and intestine) removed with knife, clamp and, when necessary, chicken cutter to obtain adherent parts-free fresh tissues (Figure 2) (Santiago et al., 2024; Rodrigues et al., *in press*).

Table 1. Zootechnical cultivation parameters of *P. brevis* and *O. niloticus* fishes.

Parameters	*P. brevis**	*O. niloticus***
n (individuals)	16	50
Total biomass (kg)	2.05	52.93
Lt (cm)	25.50 ± 21.00	28.46 ± 2.51
Weight (g)	182.80 ± 101.60	409.14 ± 95.66
Stocking density (fish L^{-1})	0.20	8.82
Feed rate (%)	1.00	3.5-3.30
Temperature (°C)	25.5	28.04 ± 2.21
Dissolved oxygen (mg L^{-1})	7.00	3.73 ± 0.89
pH	7.00	6.82 ± 0.43
Total ammonia (mg L^{-1})	0.25	5.00 ± 2.30
Cultivation system	rectangular fiber tank with aeration system	rectangular pond with recirculation water system

*Santiago et al. (2024); **Marques et al. (2023).

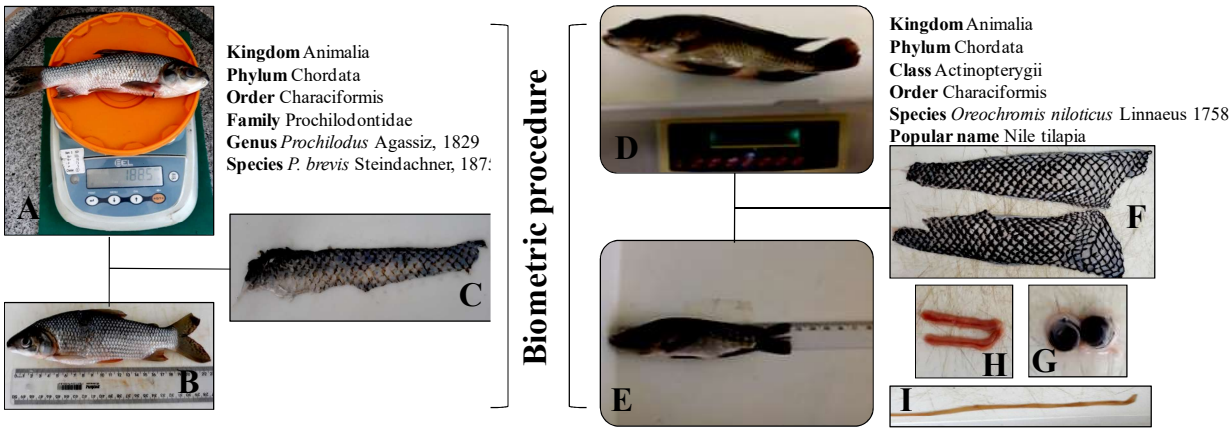

Figure 2. Biometry of *P. brevis* and *O. niloticus* cultured at the Aquaculture systems - SUC (in left) and FUC (in right). Steps of weighting (A, D), measurement (B, E) and wastes (skin - C, F; eyeball - G; gonad - H; intestine - I) separately removed from the fish body.

All the wastes separated from each fish body were extensively washed with destillated water for cleaning of particles and reduction of surface mucus; and then they taken to an oven with air circulation for dehydration (45°C, 6-48 h). The dehydrated fish wastes were weighed and quantified (%) based on total fish mass and, subsequently, they were kept in a closed recipient until GAGs extraction based on Santiago et al. (2024) and Rodrigues et al. (*in press*).

Enzymatic extraction of fish waste GAGs and metachromasia testing

The dehydrated wastes of both fish species (Figure 3*a*) were cut into small peaces and representative samples of about 10 g were further incubated in a thermostatic bath, with suspension in 100 mM sodium acetate buffer (pH 5.0) containing protease (papain) applying a digestion rate of 15% (w w^{-1}), in the presence of 5 mM EDTA and 5 mM cysteine. The extraction process followed

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at 60°C with duration of 24 h according to Rodrigues et al. (2011) followed by Salles et al. (2017) and Pereira et al. (2021) according to the scheme shown in figure 3*b*.

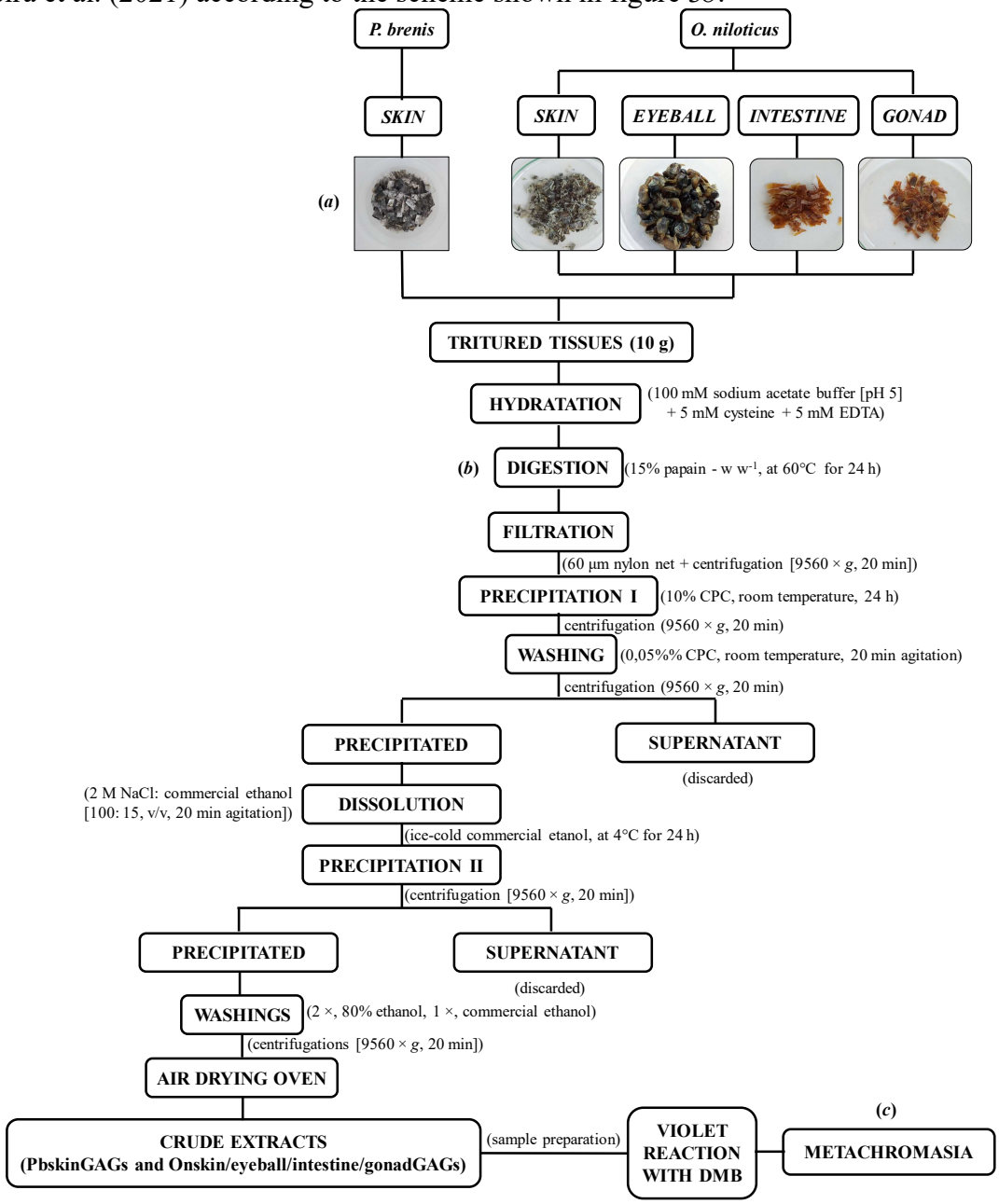

Figure 3. Tritured wastes of *P. brevis* and *O. niloticus* fishes (*a*) for extraction (*b*) and metachromasia checking (*c*) of GAGs samples.

After 24 h incubation, the mixture was continually filtered using a nylon net and the supernatants were saved and then centrifugated (9.560 × *g* for 20 min). GAGs that were present in medium were precipitated with 10 mL of 10% cetylpyridinium chloride (CPC) solution at room temperature (25-28°C) for 24 h period. The mixtures were then centrifuged at 9.560 × *g* for 20 min and the *pellet*s containing the GAGs were washed with 100 mL of 0.05% CPC solution, dissolved (under mechanical stirring for 20 min) in 100 mL of a 2 M NaCl: 92.8% ethanol (100:15 ratio, v:v) solution, followed by precipitation (24 h, 4°C) with addition of 100 mL of 92.8% ice-cold commercial ethanol. The recovered precipitate was centrifugated (9.560 × *g* for 20 min), washed twice with 100 mL of 80% ethanol, and once with the same volume of 92.8% commercial ethanol.

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After each centrifugation (9.560 × *g* for 20 min) among the mentionated steps, the partially purified material was dried using an oven with air circulation (60°C, 6-24 h) to obtain the crude extracts of GAGs. The extraction yield was calculated as the percentage (w w^{-1} %, n = 3) based on dehydrated fish matter (g) according to Rodrigues et al. (*in press*).

To verify the metachromasia in the extracted sample, a solution of crude GAGs was previously prepared and aliquots containing from 9 to 27 µg (w v^{-1}) were used in the presence of 1,9-dimethylmetilene (DMB) blue dye that is an indicator of colorimetric reaction based on complex formed (Farndale et al., 1976). The *in vitro* assay was performed following Santiago et al. (2024) and Rodrigues et al. (*in press*) for fish waste-derived GAGs, in triplicate, using glass tubes (24-28°C) and the violet property to be specific for sulfated GAGs (Figure 3*c*). The results were recorded by photographic images from a portable device after the assays.

Agarose gel electrophoresis (AGE)

This system initially characterized the GAGs from fish waste samples by charge density and homogeneity pattern (Dietrich & Dietrich, 1976). The test sample (~ 12 µg) was applied to a 0.5% agarose gel prepared in 0.05 M 1,3-acetate diaminopropane buffer at pH 9.0 and the run was carried out at constant voltage (100 V, 1 h). After that, the gel was treated with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution for ~ 24 h to fix the GAGs and then it was dehydrated using an oven with air circulation (55°C, ~ 6 h).

Polyacrylamide gel electrophoresis (PAGE)

The procedure of PAGE characterized the GAGs from fish waste samples by apparent molecular mass distribution and the presence of contaminants. Similary, the test sample (~ 12 µg) applied to a 6% polyacrylamide gel using a system of 0.02 M Tris/HCl buffer at pH 8.6, being the run performed at 500 mA for 1 h based on Santiago et al. (2024) and Rodrigues et al. (*in press*).

From both gels, the GAGs from fish waste samples were stained with 0.1% toluidine blue or Stains-All cationic dye for 30 min and 24 h, respectively. After that, the gels were destained with a solution containing absolute ethanol, distilled water and acetic acid or using distilled water only. As known markers of molecular mass, chondroitin-6-sulfate (C-6-S,~ 60 kDa), chondroitin-4-sulfate (C-4-S,~40 kDa), sulfated dextran (DexS,~8 kDa), dermatan sulfate (DS,~40 kDa) and/or HEP (~15 kDa) were applied as standards (Dietrich & Dietrich, 1976; Andrade et al., 2017).

Electrophoretic gels that revealed the GAGs from fish waste samples were separatelly scaned and the images saved in Windows file to construct the integrated figure.

Fourier Transform Infrared (FT-IR) spectroscopy

The structural features of GAGs from fish waste samples were recorded by FT-IR using a spectrometer (IRPrestige-21 Shimadzu, Japan). For this, each test sample was separatelly weighed (~10 mg) and then pressed in potassium bromide (KBr). The measurements were generated from *pellets* at a resolution of 4 cm^{-1}, with 64 scans min^{-1} at wavenumber 500-4000 cm^{-1}. The spectral value and the graphicals were assigned and represented using the Origin software version 8.0 as the Statistical Analysis Software (USA). All the graphicals of the analyzed polymer samples were separatelly saved in Windows file to construct the integrated figure.

Statistical analyses

All the values were expressed as mean ± standard deviation (n = 3). For yield comparison among the extracted samples, statistical analysis was conducted by one-way ANOVA, followed by Tukey' test, applying $p < 0.05$ as significant. The statistical analyses were performed using the GraphPad Prism® version 5.01 for Windows (GraphPad Software, 1992-2007, San Diego, CA; www.graphpad.com).

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Results and Discussion

Amount of dehydrated fish wastes

The total yield of dehydrated biomass of *P. brevis* and *O. niloticus* filleting (organ) wastes varied among the inedible parts removed of the fish body (Figure 4). The total yield of adherent parts-free solid tissues of the sampled individuals showed skin as a majority first matter (0.51 and 0.65% - w w^{-1}, respectively) than eyeball (0.29%), intestine (0.08%) and gonad (0.03%) considering the tilapia total biomass (Table 1), as well as in the percentage of total skin generated between both species. Clearly, there was a difference of skin waste of, at least, 17-fold higher this external barrier of defence of the fishes (Moreira et al., 2001) in relation to the amount of digestion and reproduction-related organs that integrate the visceras, which represent ~15% of this fish mass in the abdominal cavity of the animals (Nogueira et al., 2019). According to Machado et al. (2020), the inadequade discard of *in natura* skin and viscera wastes generated a ratio of 24 and 5%, respectively, from a fish market of the State of São Paulo, Brazil. These volumes of wastes are the most used for silage, tanning and fish meal production (Moreira et al., 2001).

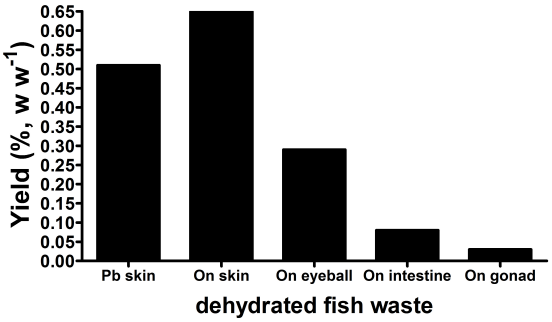

Figure 4. Total biomass, per dehydrated waste, obtained from cultured *P. brevis* and *O. niloticus*.

Obviously, there is a difference in number of sampled individuals (Table 1), but the total yield of waste could be impacted by the morphology between both species, since that the *P. brevis* fish has a fusiformis body that resulted in a difference of comparatively lower skin mass (~14% lower, w w^{-1}) *vs*. the *O. niloticus* fish that has a body shape considerating head and longitudinal length of species (Moreira et al., 2001), when also analyzed in figure 2. Additionally, the human operation and equipament contribute for such issues that determine the process of filleting in the fish industry (Moreira et al., 2001). Characterization of these fish wastes in dehydrated mass would indicate the volume of raw material prior to GAGs extraction (Rodrigues et al., *in press*) because the reduction of constitution water in the parts of fishes help to obtain relatively higher GAGs yields than in *in natura* process (Nogueira et al., 2019) during the discarded waste valorization for circular economy utilization (Cooney et al., 2023). The initial preparation of the first-matter reduces the microbial charge attached to tissue during the human handling for eco-friendly biopolymer obtaining (Ogawa & Maia, 1999; Oetterer et al., 2014).

Yield of total GAGs from different fish-derived waste samples and metachromasia evaluation

Total biomass of dehydrated fish waste was previously prepared (Figure 4) and the samples, per waste, digested with papain combined with both CPC and 92.8% ice-cold alcohol precipitations resulted in different GAGs masses (Table 2). This action denoted that this methodology isolated GAGs present in other inedible parts removed and prepared both *P. brevis* and *O. niloticus* fishes (Figures 1, 3), as already demonstrated for skins (Rodrigues et al., 2011; Salles et al., 2017; Nascimento et al., 2021) and gills (Santiago et al., 2024) using same species. Considering the range of content, the total extraction yield varied from 0.16 ± 0.03 to 0.40 ± 0.08% (w w^{-1}) reaching ~

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0.24% within the analyzed fish wastes, therefore, percentages of crude GAGs extracts very low (Table 2), but contrasted in volume of dehydrated skin mass representing highest amount (Figure 4), since that the use of papain digestion releases the matrix GAGs from protein cores to which they are covalently attached making proteoglycan-structure (Gandhi & Mancera, 2008; Badri et al., 2018).

Table 2. Yields of crude GAGs in comparison with fish wastes and protocols by other studies.

Species	Origin	Method	Waste (origin)	GAGs yield (%)	Reference
P. brevis	cultivation (fiber tank)	papain, CPC+alcohol precipitation/24 h		0.35 ± 0.03*a	this study
O. niloticus	cultivation (pond)	papain, CPC+alcohol precipitation/24 h		0.18 ± 0.02*b	this study
O. niloticus	cultivation (netcage)	papain, CPC+alcohol precipitation/6 h	skin (tritured)	0.09*	Rodrigues et al. (2011)
O. niloticus	cultivation (netcage)	papain, CPC+alcohol precipitation/24 h		0.10 ± 0.05*	Salles et al. (2017)
O. niloticus	cultivation (pond)	papain, CPC+alcohol precipitation/24 h		0.22 ± 0.00*	Nascimento et al. (2021)
O. niloticus	cultivation (pond)	papain, CPC+alcohol precipitation/24 h		0.12 ± 0.00*	Pereira et al. (2021)
"tambatinga"	cultivation (pond)	papain, CPC+alcohol precipitation/24 h		0.37 ± 0.03*	Rodrigues et al. (*in press*)
O. niloticus	cultivation (pond)	papain, CPC+alcohol precipitation/24 h	eyball (tritured)	0.16 ± 0.03*b	this study
O. niloticus	cultivation (pond)	papain, CPC+alcohol precipitation/24 h	intestine (tritured)	0.36 ± 0.10*a	this study
T. orientalis	caught	N Amano G, alcohol precipitation	intestine (minced)	28**	Arima et al. (2013)
O. niloticus	cultivation (pond)	papain, CPC+alcohol precipitation/24 h	gonad (tritured)	0.40 ± 0.08*a	this study

*Yield calculaded on the basis of dehydrated fish waste (%, w w^{-1}); **LiCl-fractionated material.

Teleost fishes are ectothermic organisms that regulate their physiological status according to water conditions. Skin mucus (collagens, multi-adhesive glycoproteins, elastin and GAGs - "called mucopolysaccharides") is a natural protective layer (between fish and aquatic environmental stress) produced by epidermal glands that also acts against pathogenic bacteria, but in adverse cultivation situations lead to depletion of body mucus influencing in osmotic balance and immunity of fish and, when to prolongated exposure, vulnerable animals to diseases or until mass mortality in aquaculture system (Moreira et al., 2001; Ali et al., 2023). In this study, the recovered yield of *P. brevis* skin GAGs was about 2-fold higher than that of *O. niloticus* one ($p < 0.05$), which was comparatively similar to other tilapia skin studies listed in table 2 (Rodrigues et al., 2011; Salles et al., 2017, Nascimento et al., 2021; Pereira et al., 2021), revealing a significant difference in GAGs to this analyzed external organ between both experimentally-monocultured species using different aquaculture systems in favorable zootechnical conditions (Marques et al., 2023; Santiago et al., 2024) based on cultured "tambatinga" fish skin that yielded 0.37 ± 0.03% of crude GAGs extract (w w^{-1}, table 2) (Rodrigues et al., *in press*). Factors as the nutrition and the species could influence on the fish skin mucus production (Moreira et al., 2001).

Bony fish eyeball is a sensitive organ similar to other vertebrates with functionality variable depending on the habitat that the species occurs (Moreira et al., 2001) integrating the head on both

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sides as a discarded raw waste during the filleting operation (Ogawa & Maia, 1999; Oetterer et al., 2014; Machado et al., 2020). Eyeball samples of *O. niloticus* fish (Figure 4) revealed a matrix tissue type susceptible to a partial proteolytic action capable of extracting similar level in GAGs ($p > 0.05$) compared with that found in tilapia skin as the external part of the animal (Table 2). This incomplete digestion by enzyme-contact period already observed in filtration procedure (Figure 3) could indicates the presence of a substantial lipid content (oils) in eyeball as a characteristic of its composition (Liu et al., 2025) common in some bony fish filleting-derived wastes (Ogawa & Maia, 1999; Machado et al., 2020), although it previously dehydrated (Figure 4).

Regarding viscera-integrating wastes, intestine and gonad were the main sources in GAGs (0.36 ± 0.10 and 0.40 ± 0.08%, respectively, $p > 0.05$) compared to yields achieved from other tilapia wastes, but similary yielded for that of *P. brevis* skin (Table 2). Related studies in fishes have involved digestive (gastrointestinal tract) and reproductive bases (Moreira et al., 2001) that could be discussed on the matrix GAGs secreted by glands for mucosal surface of the membrane that covers and protects the living cells of the epithelial barriers in these organs accounting for an average 0.11% of total fish weight (Figure 4). Nogueira et al. (2019) concluded that the yield in tissue containing specific GAG would lead to a comparatively lower amount that in viscera yielding a GAGs mixture. Similar to what was observed in the total yield of *T. orientalis* intestine/stomach-fractionated GAGs by Arima et al. (2013) (Table 2). These combined reports could explain the different yield values among the wastes found in this study. The global consumption of GAGs has been substantially demanded from bovine intestinal mucosa, although with extractable availabitily vey low and environmentally unsustainable (Badri et al., 2018). The fish gonad, to the best our knowledge, has been little understood on its biochemical scenario (gonadal development, gamete quality, fertilization, and larval survival), infering the use of skin GAGs as antioxidant supplement in the fish semen preservation (Nascimento et al., 2021; Pereira et al., 2021) and in anticoagulation of human plasma (Rodrigues et al., 2011; Salles et al., 2017).

Indirect analyses have reported on the distribution and location of GAGs in the different organs of freshwater fish (Souza et al., 2007). On the basis of GAGs yields in each mucosal site (Table 2), further step was to investigate their anionic character related to physical-chemical barrier of these molecules in each fish waste (Figure 5).

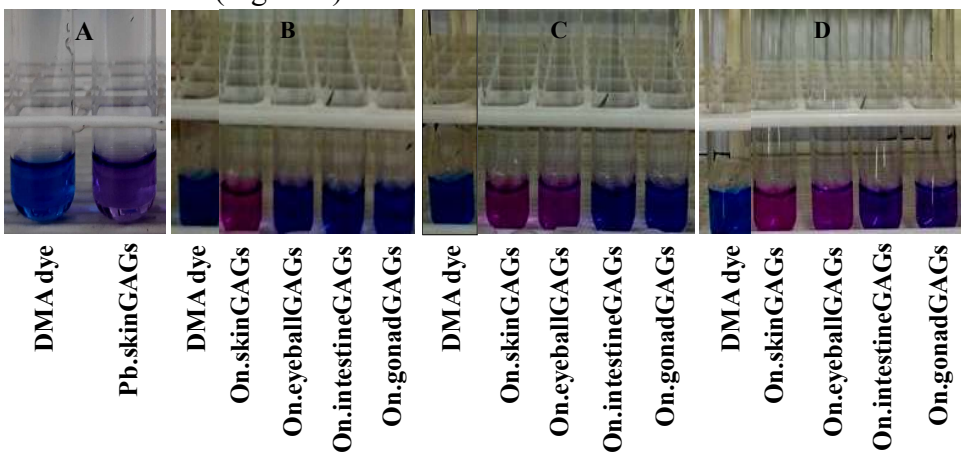

Figure 5. Metachromasia assay using 9 (B), 18 (C) or 27 µg (A, D) of *P. brevis* (Pb).wasteGAGs or *O. niloticus* (On).wasteGAGs in the presence of DMB dye.

The Farndale et al. (1976)' method clearly revealed that there was a distinct metachromatic property among the body wastes removed of fish (Figure 5). The external anatomy-related wastes (skin and eyeball) showed richness in negativelly charged GAGs composition due to a specific DMB dye-binding ability for the violet reaction from the samples (Santiago et al., 2024; Rodrigues

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et al., *in press*). Even to those extracted from the abdominal cavity (intestine and gonad) suggested a weak detection for sulfate groups in the chemical structures. Collectively, observations revealed a domain in charge density depending on the analyzed fish body tissue (Pfeiler, 1998).

These combined results implied on a heterogeneity in charge composition in the fish waste matrix (Pfeiler, 1998). The electrostatic environment observed for the external parts of fishes would favor as a more effective barrier in mucus against adverse impacts (*e.g.*, pathogenic bacteria) compared to other biological functions that could be attributed to the lack of sulfated GAGs chains in both intestine and gonad matrices (Moreira et al., 2001).

Souza et al. (2007) biochemically analyzed GAGs in samples of three eletric eel organs (*Electrophrus electricus*) and found metachromatic sulfated GAGs. More recently, Nogueira et al. (2019) exclusively detected sulfated GAGs in viscera of Nile tilapia, *O. niloticus*, however, the authors did not report for the possible presence of uncharged GAG in samples. Possibly due to the *in natura* viscera mass used to extract the material using alcalase protease, but without its isolated parts. In order to evaluate such questions, it was partially investigated by electrophoresis and infrared techniques the interface between such implications in terms of class and purity of GAGs, as relevant factors for industrial applications (Badri et al., 2018; Liu et al., 2025).

Partial biochemical identification by AGE / PAGE

The use of electrophoretic techniques was to identifying of GAG species in the various fish wastes for the determination of charge density and molecular mass using AGE / PAGE, respectively (Figure 6) (Dietrich & Dietrich, 1976; Santiago et al., 2024). The test samples were visualized in two well-known cationic dyes (toluidin blue and Stains-all) for their high sensivity to stain sulfated GAGs (Dellias et al., 2004) and uncharged sugar residues (Andrade et al., 2017) in small preparations, respectively.

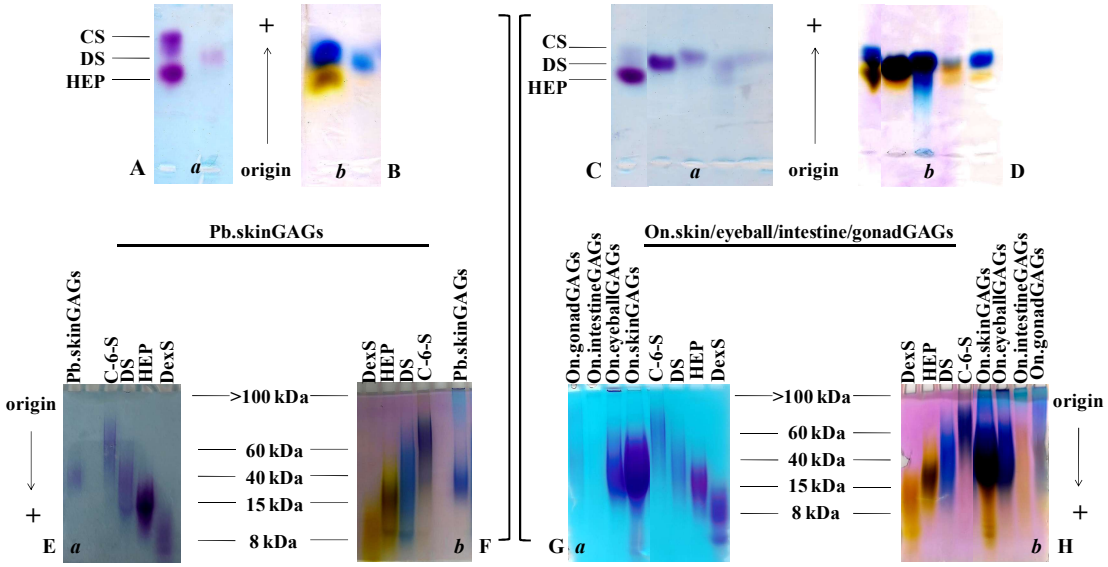

Figure 6. Agarose (A, B / C, D) / polyacrylamide (E, F/ G, H) gels electrophoreses of GAGs extracted from *P. brevis* skin and *O. niloticus* skin/eyeball/intestine/gonad and standards chondroitin-6-sulfate (C-6-S,~60 kDa), dermatan sulfate (DS,~40 kDa), dextran sulfate (DexS,~8 kDa) and/or heparin (HEP,~15 kDa) present on gels were stained with 0.1% toluidine blue (*a*) or Stains-All (*b*).

All the analyzed fish wastes contained GAGs-like components, but their charge density and polydispersivity differed among the samples examined by respective dye. Skin samples essentially judged an electrophoretic composition for DS-type GAGs, after toluidine blue treatment, for both *P. brevis* and *O. niloticus* bony fishes (Figures 6A, C) supporting the conserved hypothesis this GAGs species in fish skin (Dellias et al., 2004; Rodrigues et al., 2011, *in press*; Salles et al., 2017;

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Nascimento et al., 2021), but the homogeneous charge density differed between both cultured species, since that the sulfate groups in GAGs structure reacted with the diamine in run buffer (Dietrich & Dietrich, 1976). Results supported the slighly difference in the metachromasia assay by the Farndale et al. 1976)' method (Figure 5) two DS-type GAGs evaluating with differences in degree of sulfation (Dellias et al., 2004).

AGE analysis with other tilapia wastes denoted skin GAGs more highlighted than those of eyeball, intestine and gonad on the same electrophoretic profile (Figure 6C). Tilapia waste samples contained single or mixed GAGs, as CS in eyeball, DS or CS/HS in intestine and DS or CS/HS in gonad, presuming polymeric complexity in different compositions. Other animal sources revealed CS/DS/HA in Pacific bluefin tuna intestine/stomach (Arima et al., 2013), CS in squid eye (Liu et al., 2025); and CS/DS/HS in pacu and tilapia visceras (Nogueira et al., 2019). This study suggested metachromatic bands (purple) for DS/HS as the main sulfated GAGs classes in freshwater fishes, but GAGs in mixture would require high quality control during industrial separation, elevating the cost (Badri et al., 2018; Liu et al., 2025).

Stains-all staining of agarose gel revealed marked results of GAGs-containing samples after their fixation with the dye (Figures 6B, D). As expected, there were samples and standards with great intensity (Santiago et al., 2024; Rodrigues et al. *in press*), especially for intestine and gonad samples revealing other spots indetectable with toluidine blue dye alone (Figures 6A, E), as additional components in samples revealed by specific color. Samples of skin GAGs showed for purple (mainly tilapia); while those of eyeball, intestine and gonad for different color with basis in AGE-separated component, including dark blue+bright-blue (eyeball), yellow+bright-blue (intestine) and two well-separated band in dark blue / yellow, respectively (gonad). This molecular scenario allowed us to speculate, respectively, CS+HA, DS+HS and DS/CS+HS GAGs mixtures.

In spite of this color specificy, samples were also analyzed for their molecular masses that varied from ~ 8 to >100 kDa based on mobility of standards with known sizes on PAGE. Firstly, GAGs extracted from fish waste samples were examined in the toluidine blue dye indicating predominant masses of ~ 40 kDa with mobilities in the central portion of gel (Figures 6E, G). Curiously, analyzing by means of stains-all alone, except tilapia skin GAGs, the heterogeneous system of polydisperse blots had other components, at the top of the gel, in bright-blue (Figures 6F, H) and the confirmation of yellow blot in central region (gonad) (Figure 6H).

In fact, both techniques were complementary to characterization, at initial level, also for HA (uncharged GAG) (Figures 6F, H) based on its size > 100 kDa (Andrade et al., 2017), when coextracted DS/CS/HS from *P. brevis* skin / tilapia eye/intestine/gonad in samples revealing protein/DNA contamination (Andrade et al., 2017). LiCl-fractionated GAGs from Pacific bluefin tuna intestine/stomach revealed HA in mixture (Arima et al., 2013). The electrophoretic migration in mixture could explain the discrepancy in GAGs yield found in table 2 among the tilapia wastes, when separately examined. Our study suggests HA as an another GAG available in tilapia fish and add Nogueira et al. (2019) who firstly reported the CS/DS/HS GAGs composition for tilapia *in natura* visceras, in which integrate intestine and gonad. Complexity of these overall results led us to investigate the structural groups of each sample by infrared analysis.

Spectral analysis of various fish waste GAGs by FT-IR

The molecular identity of GAGs varied among the cultured fish wastes according to the FT-IR analysis, in the region of 4000 to 500 cm^{-1}, that specificity revealed for detection of different structures of GAGs also in mixture within preparations (Figure 7). Spectral values showed common characteristics for GAGs (at 1200-950 cm^{-1}) (Alcântara et al., 2023) with regions at 3404-3435 and at 2920-2931 cm^{-1} for -OH and C-H, respectively, presenting total sulfation (at 1238-1259 cm^{-1}), uronic acid (at 1409-1460 cm^{-1}) (Pereira et al., 2021; Santiago et al., 2024; Rodrigues et al., *in*

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press); and peaks related to amide I (at 1631-1647 cm^{-1}, C=O/N-H), amide II (at 1552-1564 cm^{-1}, N-H) and amide III (at 1309-1381 cm^{-1}, N-H) (Alcântara et al., 2023).

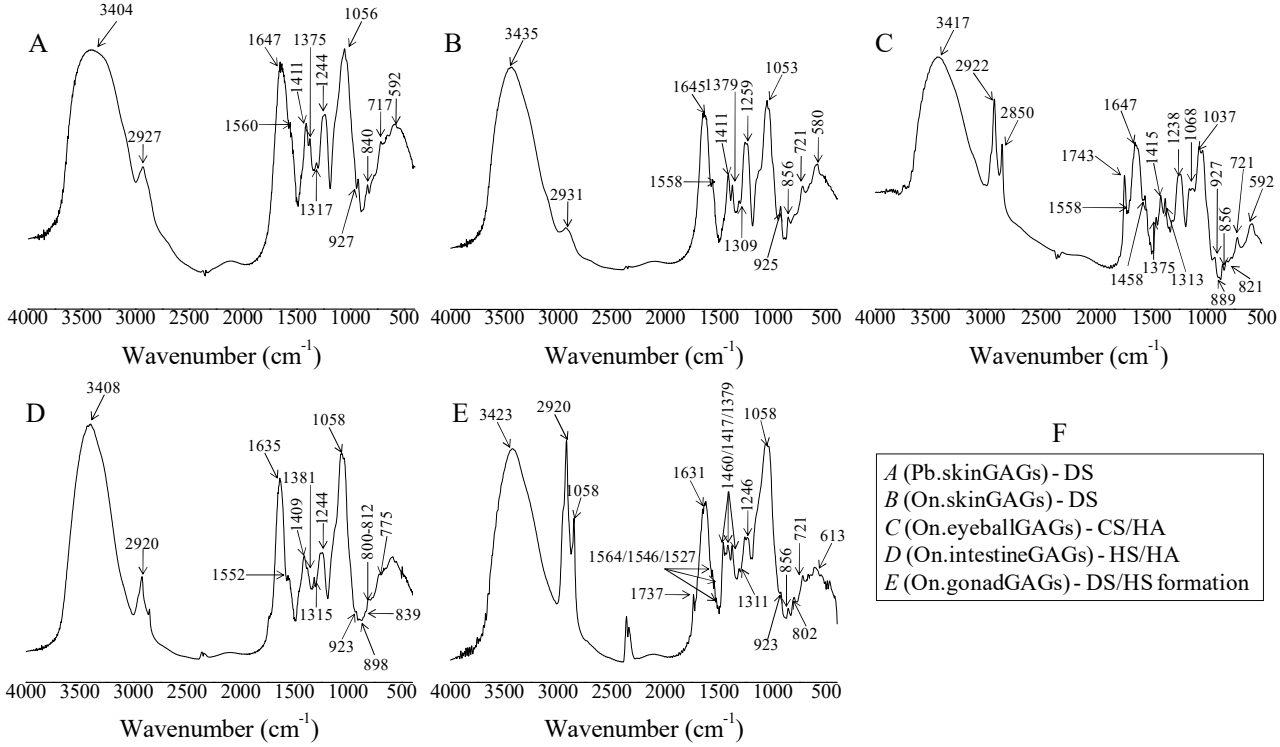

Figure 7. FT-IR spectra of the *P. brevis*.skinGAGs (A) and *O. niloticus*.wasteGAGs (skin-B, eyeball-C, intestine-D, gonad-E) obtained from kBr samples at 500-4000 cm^{-1}.

Dehydrated skin samples of *P. brevis* and *O. niloticus* had spectral values corresponding to DS GAGs with both chemical shifts at 840 and 856 cm^{-1}, respectively (Figures 7A, B), possibly due to the enzymatic extraction method (Figure 3) that infered structural variations in the polymer (Alcântara et al., 2023) similar to those seen previously in other DSs isolated from the skin of fishes (Pereira et al., 2021; Rodrigues et al., *in press*). High molecular mass composition in bright-blue band (Figure 6F) could explain the presence of proteins complexed to GAG chains as a result of peptides generated by papain digestion, but without HA in sample (Figure 7A). Combined results uniquely supported DS in fish skin (Dellias et al., 2004; Souza et al., 2007; Arima et al., 2013; Salles et al., 2017) as a protective barrier in the animal matrix (Moreira et al., 2001; Ali et al., 2023) and already used in fish semen croypreservation (Nascimento et al., 2021; Pereira et al., 2021).

Analysis by means of FT-IR showed that the eyeball of Nile tilapia (Figure 7C) contained two GAGs species at 856 and 821 cm^{-1} related to specific sulfation and at 1157-1037 and 889 cm^{-1} sensitive to crystallinity, common features for C-4/6-S and HA as those extracted of gill (Santiago et al., 2024) and of eyeball (Alcântara et al., 2023), respectively. Herein, GAGs preparation was found in lowest yield among all the wastes (Table 2) and partially separated electrophoreses the GAGs in sample into distinct polydisperse masses (Figures 5C, D, G, H), but could be laborious in industrial terms (Liu et al., 2025). CS and HA are well-known GAGs for therapeutic applicabilities, such as suplemments for the symptomatic treatment of osteoarthritis by reducing inflammatory event and use as antioxidant against other diseases, therefore, presenting great worldwide demand and putting the natural population balance of some species at risk (Volp, 2011; Badri et al., 2018; Liu et al., 2025). Zebrafish (*Danio rerio*) has also been a model organism for GAGs biological studies (Zhang et al., 2009).

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The molecular complexity was also found for intestine and gonad of Nile tilapia that integrate visceras (Nogueira et al., 2019). The spectral scenario showed, by FT-IR, the presence of HS/HA in fish intestine due to chemical elongation at 800-812 and vibration at 898 cm^{-1}, respectively (Figure 7D), as typical signals for sulfate and region related to that of eyeball HA already identified (Figure 7C). Regarding tilapia gonad, the clearly revealed experiment to a DS in this waste because there was a peak at 856 cm^{-1} (Figure 7E) similar to those spectral data for fish skin (Figures 7A, B) confirming eletrophorectic observations (Figure 6). Curiously, constrasting result was revealed between electrophoresis and FT-IR analyses for this GAG sample, since that it did not appear on gel as a violet metachromatic band (Figure 6C, G), but with polydispersivity stained with stains-all was observed co-migrating as standard HEP (Figure 6D, H), reflecting also the presence of non-sulfated sugars residues that reacted with the respective dye (Andrade et al., 2017; Santiago et al., 2024; Andrade et al., 2017; Nascimento et al., 2021).

The chemical vibration around 802 cm^{-1} would indicate HS-type GAG (Figure 7E) as a minor component with reduced charge density present in gonad. As HS is related to development and differentiation-associated events (Zhang et al., 2009), its participation could also be discussed, since that, according to Pfeiler (1998), HS composition can be quite variable depending on the origin, as in fact suggested here (Figures 6C, G, D, H). It has been described that HS modifications are dynamically modulated during fish development and its amount varies according to life stage by means of signaling events (Zhang et al., 2009). The different ratio of DS/HS in this study determined the monosex tilapia gonodal matrix composition, but how the biochemistry of HS formation would provide to a functionality is still unclear (Gandhi & Mancera, 2008; Badri et al., 2018). However, the mixture confirmed GAGs in amount and distribution (Table 2, Figures 7D, E), but in a complementary manner that found in tilapia visceras (Nogueira et al., 2019).

Overall, utilization of dehydrated fish wastes allowed us to a biochemical prospection of GAGs to access their available and distribution in different mucosal sites. Although with further challenge associated to laborious steps of purification of these GAGs (Figures 6, 7), this study brings a relevant contribution to ichthyoglycobiology to reduces environmental damage and economic loss of discarded biomass by aquaculture to subsequent biological testing aiming at circular economy (Cooney et al., 2023). The methodology could be employed in several types of fish filleting tissues for biotechnological destination.

Conclusion

Filleting of *Prochilodus brevis* and *Oreochromis niloticus* generated skin and eyeball in a substantial volume than dehydrated intestine and gonad. These wastes in papain digestion combined with organic solvent precipitations exhibited an inverse rate in yield of crude glycosaminoglycans based on respective raw biomass, but the sulfate density and size as preponderant to glycosaminoglycans derived from the external wastes of the fishes. Combined electrophoretic and infrared analyses showed that the metachromatic classes occured in mixture or not depending on the origin presenting protein contamination, with skin and eyeball rich in dermatan and chondroitin/hyaluronic acid, respectively; and intestine and gonad for heparan and hyaluronic acid, respectively, while heparan in minor concentration, as a molecular basis for circular economy.

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