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COMBINED FRACTIONATION FOR CHONDROITIN SULFATE - SELECTIVE CHARACTERIZATION FROM *Oreochromis niloticus* GILLS

FRACIONAMENTO COMBINADO PARA CARACTERIZAÇÃO SELETIVA DE CONDROITIM SULFATO DE BRÂNQUIAS DE *Oreochromis niloticus*

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Abstract Fish waste glycosaminoglycans (GAGs) processing has been challenger in structural biology. This study papain-extracted chondroitin sulfate-CS was separately fractionated with cethylpiridium chloride-CPC, alcohol-AL or acetone-AC for its selective characterization from the cultured Nile tilapia gills. Enzimatic-extraction resulted in five CS containing-(2, 4, 6, 8 or 10%) CPC fractions ($p > 0.05$), of which in 10% CPC one checked for strongest metachromasia by cationic dye, accounting for 0.89 ± 0.16% total yield (w w^{-1}) from the tritured gills arches. Regarding tritured whole gills, crude CS (0.91 ± 0.03% yield, w w^{-1}) precipitated with AL or AC yielded 0.10 ± 0.47 and 0.16 ± 0.00% for fractions FAL1 and FAC1 (w w^{-1}, $p < 0.05$), respectively, but they had no metachromasia. By contrast, FAL1 and FAC1 showed different structurally materials, without sulfation and presence of C-6-S *vs.* crude CS (C-4/6-S), by agarose gel electrophoresis using known GAGs and infrared technique, respectively. Therefore, gills of cultured Nile tilapia have CS and applied technique with organic solvents fractionated it into different molecules for the partial knowledge of its structural biology.

Key Words: Cichlidae, cartilage, solvents, sulfated carbohydrates, structure.

Resumo Processamento de glicosaminoglicanos (GAGs) tem sido desafiador na biologia estrutural de resíduo de peixe. Neste estudo, condroitim sulfato extraído com papaína das brânquias de tilápia do Nilo cultivada foi fracionado separadamente com cloreto cetilpiridínio-CCP, álcool-AL ou acetona-AC para sua caracterização seletiva. Extração enzimática resultou em cinco fractions CCP (2, 4, 6, 8 ou 10%) contendo CS, das quais na fração CCP 10%, por corante cariônico, deteve metacromasia importante, totalizando 0,89 ± 0,16% (m m^{-1}). Quanto às brânquias inteiras trituradas, CS bruto (0,89 ± 0,16%, m m^{-1}) precipitado com AL ou AC rendeu, respectivamente, 0,10 ± 0,47 e 0,16 ± 0,00% para frações FAL1 e FAC1 (m m^{-1}, $p < 0,05$), porém não tiveram metacromasia. Por contrário, a eletroforese em gel de agarose usando GAGs conhecidos e a técnica de infravermelho mostraram, para FAL1 e FAC1, materiais estruturalmente diferentes sem sulfatação e presença de C-6-S, respectivamente, *vs*. CS bruto (C-4/6-S). Portanto, brânquias de tilápia do Nilo cultivada possuem CS e a técnica aplicada com solventes orgânicos fracionou-os em moléculas diferentes para o conhecimento parcial de sua biologia estrutural.

Palavras-chave: Ciclídeo, cartilagem, solventes, carboidratos sulfatados, estrutura.

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Introduction

Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) processing industry is well-consolidated worldwide. The commercial production of this Cichlidae species in Brazil reached 534 thousand t in 2022 (Fao, 2022). This aquaculture species contributes to the socio-economic development and it is well-known by its high nutritional value from fillet that has a yield of ~ 32% of the total fish (Moreira et al., 2001). However, from processed fish, an important amount of solid wastes (*e.g.*, heads, gills, fins, frames, viscera and skin) is usually discarded in various ecosystems by food industry as non-edible materials impacting natural chemical levels, food chains and, until, human activities (Oetterer et al., 2014). It would represent available biomasses from fish filleting to usage as valuable platforms to explore versatile molecules (Arima et al., 2013), such as glycosaminoglycans (GAGs), for several applicabilities (Badri et al., 2018).

GAGs are highly complex polymers composed of linear heteropolysaccharides that play relevant roles in many biological processes in which interact through association with various proteins, making proteoglycans. They have viscous and lubricating properties to animal cell surfaces in the extracellular matrix and, sometimes, known as mucopolysaccharides, which are produced by secretor cells (Gandhi & Mancera, 2008). Their basic structures are made of repetitive di-saccharide units with a hexosamine (glycosamine or galactosamine) linked to a non-nitrogenated sugar, uronic acid (D-glycuronic acid or L-iduronic acid) or a galactose sugar, whose structural compositions depend on the tissue or organism. Chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin (HEP) and hyaluronic acid (HA) are the main GAGs entities known in the extracellular matrix of the animals, of which HA has not sulfation and may occur free in tissues, therefore, unlinked covalently to proteoglycan structure (Oliveira et al., 2015a). Several GAGs-related functions arise as therapeutic possibilities on coagulation, thrombosis, inflammation, cancer, viral infection, tissue development, tissue regeneration and in the development of antiviral and anti-tumour drugs (Valcarcel et al., 2017).

Biochemical analysis of GAGs from fish tissues has been challenge to establishes protocols due to different sources in which they are found (Arima et al., 2013). Traditionally, large-scale production of GAGs has increased in the last decades, including HEP (porcine intestine or bovine lung), CS (porcine or bovine trachea and shark cartilage) and HA (microbial fermentation) (Badri et al., 2018), but studies those derived from fish wastes are still scarces and limited at biological level due to variability of species and localization to access GAGs these tissues (Oetterer et al., 2014). The development of biology techniques to provide expertise in order to create alternative production approaches and to structure analyze became need to reduce the environmental impact generated by fish filleting wastes-uncontrolled discard (Oetterer et al., 2014; Liu et al., 2025) and huge risks (*e.g.*, virus and prions) associated to mammalian GAGs use (Oliveira et al., 2015a), as well as to animal protection due to the increased demand for commercial GAGs (Volpi, 2011).

The gill structure in teleostean fishes is formed by arches and filaments ocupping the opercular cavity. It is a respiratory organ playing a key role in the exchange of gases and ions, creating a diffusional barrier to ecological fluctuations (Baldisserotto, 2002). It has connective tissue of epithelial layer constituted mainly collagen and mucopolysaccharides (Li et al., 2022), preventing loss of water and acting as osmotic agents by matrix balancing the diffusion of small molecules and ions (Wasserman et al., 1972). Fish gills-derived GAGs have been poorly explored *vs*. other solid wastes of inedible parts (Arima et al., 2013; Salles et al., 2017; Nogueira et al., 2019). Previous studies on cartilage wastes revelead GAGs (HA, CS and HS) in *Cyprinus carpio* fish gill arches (Wasserman et al., 1972), *Carcinus maenas* crab gills (Regnault & Durand, 1998) and, recently, CS in *Prochilodus brevis* fish gills (Santiago et al., 2024). CS has medicinal use implying important biological functions (Volpi, 2011), as well as for the biochemical knowledge on the aquatic biology of fishes (Moreira et al., 2001). Although global CS demand has considerably increased (Volpi,

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2011; Oliveira et al., 2015b), there are methodological limitations in its practical quality and appropriate source-tissues analysis for the risk-free production compared to commercially availables (Badri et al., 2018). Animal-based raw materials have usually impurities, requiring meticulous adjustment of operating conditions and analytical accuracy for industrial and scientific production (Liu et al., 2025).

Based on these considerations, the present study applied to a combined technique of two-steps fractionation by precipitation procedures using organic solvents (cetylpiridinium chloride-CPC, alcohol-AL or acetone-AC) for CS-selective recovery and then its characterization by Fourier Transform Infrared (FT-IR) spectroscopy from Nile tilapia (*Oreochromis niloticus*) gills-extracted GAGs (OnGAGs) removed from filleted specimens, describing for the first time the partial structural biology of tilapia CS present in the gill extracellular matrix, and to evaluate its apparent quality and identity from the used tissue source, improving reusability and valorisation perspective.

Material and Methods

Source material and preparing the dehydrated tilapia gills

The fresh gills were removed from monosex Nile tilapia provide by the Aquaculture farm of the Federal University of Ceará (FUC), Brazil (Figure 1A), and then they were carefully transported in two isothermal boxes containing ice (1:1.1 kg ratio) for experimental unit of the Marine Biochemical (MarBio) laboratory located at the Aquaculture Biotechnology Center belonging to the Department of Fisheries Engineering of the FUC.

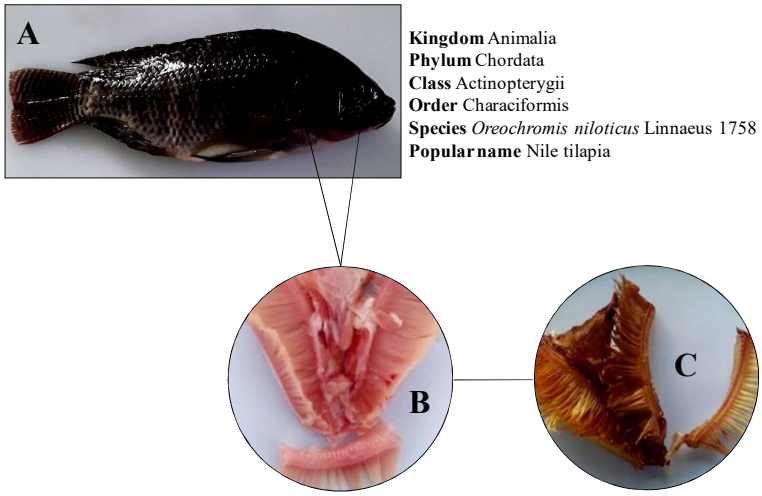

Figure 1. Nile tilapia cultured by the Aquaculture farm (A) and fresh whole gills removed from head and their arches separated (B) and dehydrated (C) from the original tissue.

In Marbio, a total of seventeen specimens was measured (28.46 ± 2.51 cm) and weighed (409.14 ± 95.66 g) using a rudimentary ichthyometer and a commercial balance on a 1 g precision scale, respectively. After biometric sampling, each fish was manually washed and then fresh whole gills removed from tilapia heads (opercular cavity) using knife, clamp and, when necessary, chicken cutter, being they washed with destillated water for cleaning blood (Figure 2B). So, some of the pretreated gills were then separated in arches from original tissue for the first-step of GAGs fractionation, while the restant of the whole gills for the second-step one. Both gill tissue preparations were separately dehydrated in an oven with air circulation (45°C, 48 h - Figure 2C) and further kept in two closed recipients until OnGAGs obtaining (Santiago et al., 2024). The specimens of Nile tilapia used in the study were registered in SisGen (National System for the

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Management of Genetic Heritage and Associated Traditional Knowledge) platform under code AA4816B and approved by the Animal Ethical Committee of the FUC (protocol nº 6974061020).

First-step: Extraction and differential precipitation with CPC for OnGAGs from gill arches samples and checking by metachromasia

The arches samples separated and dehydrated from Nile tilapia whole gills were cut into small peaces and then the tritured material (10 g) was overnight incubated, in reactor glass flask with a thermostatic bath in a continuous process, using proteolysis by the unspecific enzymatic treatment of crude papain in an extraction rate of 15% (w w^{-1}), in 100 mL of 100 mM sodium acetate buffer (pH 5.0) added of 5 mM EDTA, and 5 mM cysteine, with digestion process determined at 60°C based on Santiago et al. (2024), as shown in figure 2*a*.

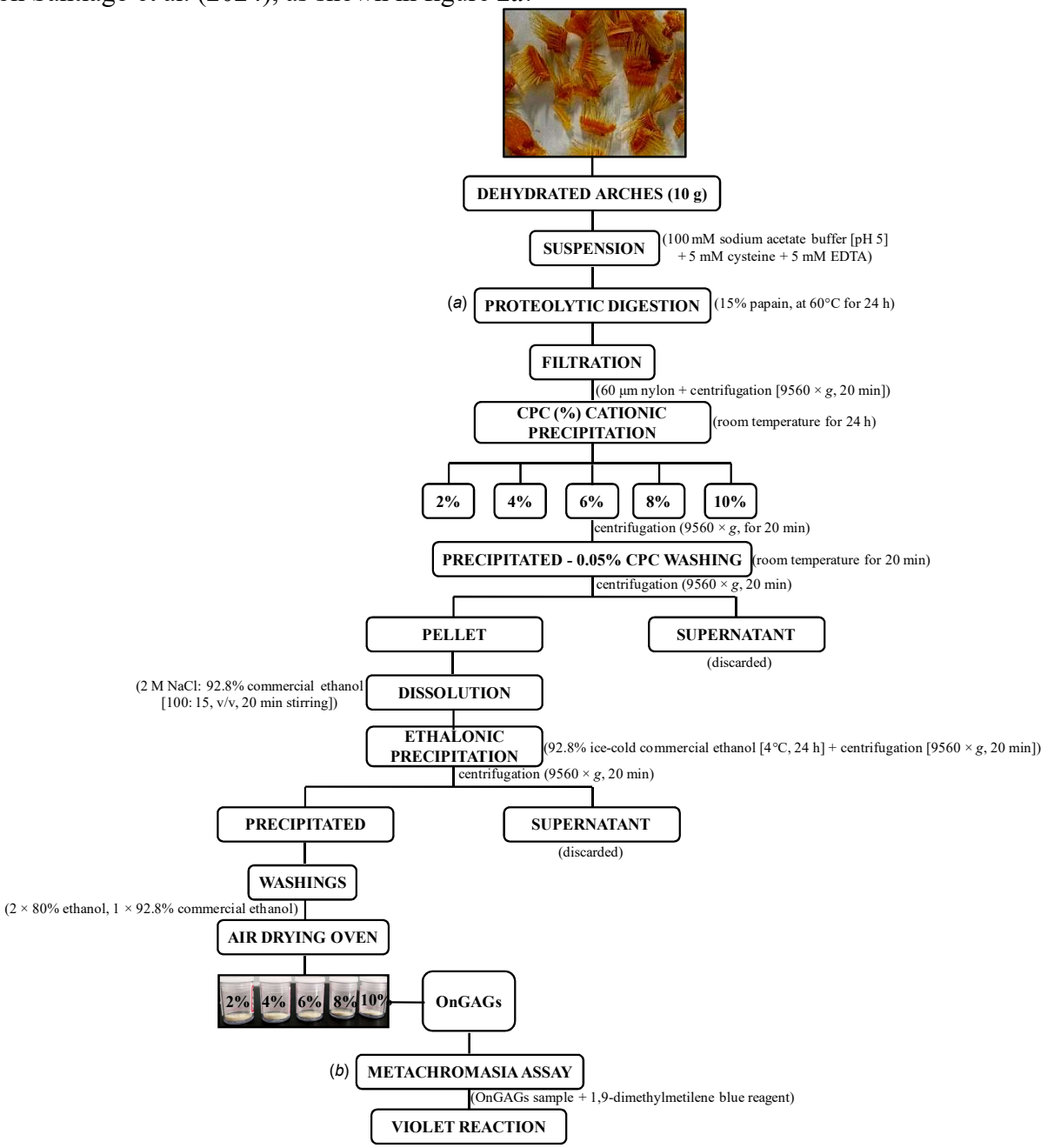

Figure 2. Scheme of CPC fractionation-combined extraction (*a*) and metachromasia (*b*) analysis of GAGs from cultured Nile tilapia gill arches.

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After extraction time, the suspension was continually filtered through nylon net and the supernatants were saved and centrifugated (9.560 × *g* for 20 min) to remove residual tissue particles. The filtrate (~ 100 mL) was separated in volumes of 20 mL in plastic tubes and the OnGAGs that were present in each one were precipitated with 2 mL of 2, 4, 6, 8 or 10% cetylpyridinium chloride (CPC) solution, respectively, at room temperature (at 25-28°C) for 24 h. The mixtures were then separately centrifuged at 9.560 × *g* for 20 min. The *pellet*s containing the partially fractionated OnGAGs were washed with 50 mL of 0.05% CPC solution, dissolved (under mechanical stirring for 20 min) in 25 mL of a 2 M NaCl: ethanol (100:15 ratio, v:v) solution, and then sujected to precipitation (24 h, 4°C) with addition of 50 mL of 92.8% ice-cold commercial ethanol. The precipitates obtained were centrifugated (9.560 × *g* for 20 min), washed twice with 50 mL of 80% ethanol, and once with the same volume of 92.8% commercial ethanol. After each centrifugation (9.560 × *g* for 20 min) among the separated steps, the materials were individually dried using a drying oven with air circulation (60°C, 24 h) to obtain the raw fractionated of OnGAGs. Finally, each fractionated extraction yield was calculated according to Eq. (*1*) and expressed as the percentage (w w^{-1} %, n = 3) of the dehydrated matter (g).

$$\text{Yield (\%)} = \text{OnGAGs WDM}^{-1} \times 100 \qquad (1)$$

where: OnGAGs the dry weight of crude GAGs and WDM the raw weight of dehydrated matter.

The metachromasia assay was performed to detect the OnGAGs from extracted and fractionated samples. For this, solutions were previously prepared and aliquots containing ~30 µg of OnGAGs were separately checked for metachromasia in the presence of 1,9-dimethylmetilene (DMB) blue dye used as an indicator of colorimetric reaction based on complex formed (Farndale et al., 1976). The assay was conducted, in triplicate, using glass tubes and the visualization of violet coloring would reveal to be specific for sulfated GAGs from the cultured Nile tilapia arches-isolated samples (Figure 2*b*) (Santiago et al., 2024).

Second-step: Extraction and combined fractionation with CPC and AL or AC precipitation of OnGAGs from the whole gill samples and checking by metachromasia

Dehydrated whole gills of cultured Nile tilapia (Figure 1C) were used to generate a tritured tissue prior to OnGAGs extraction, as already described in figure 2. After 24 h proteolytic incubation period (10 g of gill samples), the filtered was centrifuged for residual tissue elimination and then OnGAGs that were present essentially precipitated with 10% CPC (24 h, 25-28°C) followed by other steps to obtain the crude extract (CE, w w^{-1} %, n = 3) as calculated by Eq. (*1*) (Santiago et al., 2024).

Second-step recovery for CE selective precipitation, from the whole gills removed from cultured Nile tilapia, was combined with organic solvents (AL or AC) (Figure 3*a*). For this, CE sample was dissolved in destilated water (5 mg mL^{-1}) under constant agitation. After 1 h (at 25-28°C) time, it was added to material three volumes of 95% ice-cold AL or 95% ice-cold AC to once volume of the CE solution (3:1 ratio, v v^{-1}) to concentrate the material for 72 h at 4°C. Separately, both resulting AL and AC precipitates were recovered by centrifugation (20 min, 9.560 × *g*) and, then, they were partially purified by twice washing with 50 mL of respective solvent and centrifugation again. Finally, they were individually dried using a drying oven with air circulation (60°C, 3 h) to obtain the OnGAGs fractions.

The OnGAGs-containing AL or AC fractions (n = 3) were named FAL1-3 or FAC1-3 and, when detected mass, their yields were quantified (%) based on initial preparation of the sample.

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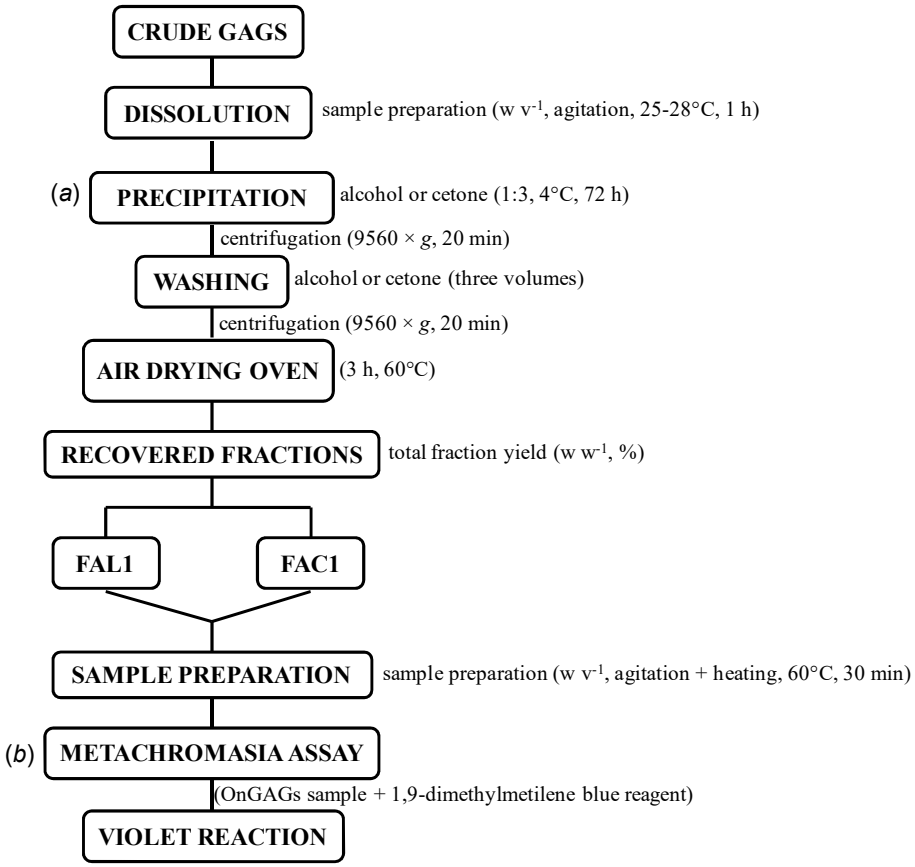

Figure 3. Combined protocol of fractionation (*a*) and metachromasia (*b*) checking of the GAGs obtained from cultured Nile tilapia gills.

The initial detection of OnGAGs from the recovered fractions (FAL1 or FAC1) was checked by metachromatic property (Figure 3*b*). This analysis was performed using previously prepared solutions (crude extract and fractions) and aliquots containing from ~ 30 to 90 µg of OnGAGs were assayed for metachromasia in the presence of DMB dye (Farndale et al., 1976). The test was conducted, in triplicate, using glass tubes and a possible violet-complex formation would indicate to be sulfated GAGs samples (Santiago et al., 2024). The results were recorded by a photographic image from a portable device after the assay.

Agarose gel electrophoresis

This analytical system characterized the OnGAGs-containing CE and its fractions (FAL1 or FAC1) isolated from gill samples by charge density as described by Dietrich & Dietrich (1976). Aliquot of 6 µL of each test sample (~ 30 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 OnGAGs and then dehydrated using a drying oven with air circulation at 55°C for ~ 6 h. Next, the dried gel was treated with a 0.1% toluidine blue solution to reveal the OnGAGs after ~30 min embedded in the respective dye. Then, it was destained with a solution containing absolute ethanol, distilled water and acetic acid until to visualization of metachromatic bands. Finally, gel that revealed the OnGAGs was scaned and the image saved in Windows file to obtain the illustrated figure.

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Characterization by Fourier Transform Infrared (FT-IR) spectroscopy

The structural features of OnGAGs (CE and fractions FAL1 or FAC1) from gills samples were determined by FT-IR and the spectra were obtained using a spectrometer (IRPrestige-21 Shimadzu, Japan). For each measurement, ~10 mg of each sample were initially prepared in potassium bromide (KBr) *pellets* and the spectral values recorded at a resolution of 4 cm^{-1}, with 64 scans min^{-1} at 500-4000 cm^{-1}.

Statistical analyses

Data were expressed as mean ± standard-deviation (n = 3). The yield by CPC fractionation was analyzed by one-way ANOVA, followed by Tukey' test, with $p < 0.05$ as statistically significant. Regarding solvent GAGs obtaining, the values were submitted to *t*-Studant test for unpaired analysis, considerating $p < 0.05$. The graphical representations of yield and FT-IR were constructed using the GraphPad Prism® version 5.01 for Windows (GraphPad Software, 1992-2007, San Diego, CA; www.graphpad.com) and the Origin software version 8.0 as the Statistical Analysis Software (USA), respectively.

Results and Discussion

First-step: amount of enzymatically extracted GAGs and then fractionated with CPC from tilapia gill arches

The use of crude papain for unspecific proteolytic extraction followed by CPC recovery for obtaining of gill arches GAGs of cultured Nile tilapia cartilage waste was to be a satifactory approach based on Santiago et al. (2024). As shown in the figure 4A, the average yields of OnGAGs from gill arches samples by extraction method adopted followed by fractionation by differential precipitation with 2→10% CPC were comparatively similar among the crude extracts recovered (0.17 ± 0.02, 0.18 ± 0.04, 0.17 ± 0.04, 0.17 ± 0.03 and 0.19 ± 0.02%, w w^{-1}, $p > 0.05$) from the raw waste source, therefore, accounting for 0.89 ± 0.16% from the dehydrated tissue. Collectivelly, these results revealed that the gill arches of Nile tilapia as a commercial value-devoid source for GAGs prospection, since that this species has already been described to other inedible parts containing GAGs (scales - Arima et al., 2013; skins - Salles et al., 2017; visceras - Nogueira et al., 2019) become this fish a valuable platform of bioactive GAGs with pharmacological interest due to their increased demand worldwide (Volpi, 2011; Badri et al., 2018). Use of proteases have been eco-friendly recommended for fish cartilage-derived GAGs extraction (Li et al., 2022).

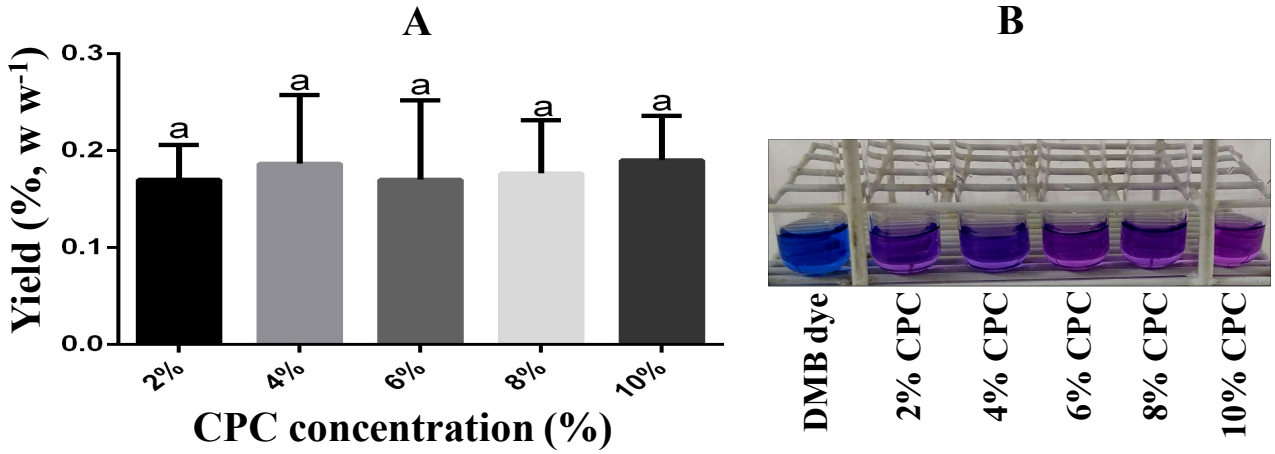

Figure 4. Yield of GAGs containing-CPC fractions from cultured Nile tilapia gill arches (A) and sulfation-positive fractions by metachromatic complex in the presence of DMB dye (B). Similar letters on the bars reaveling significative absence among the CPC concentrations at level of 5% (ANOVA, Tukey test, $p > 0.05$).

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Gill arches of teleostean fishes (Baldisserotto, 2002) have primarily two components (collagen and GAGs) that play osmoregulatory function depending on the water conditions (Moreira et al., 2001), of which GAGs consist of higher percentage in the total connective tissue in cartilaginous tissues (Li et al., 2022), therefore, there is still limited information on the composition about these compounds in this organ-related part (Santiago et al., 2024). In a previous study, Wasserman et al. (1972) studied gill arches of carp (*C. carpio*) dehydrated in acetone, dried in air, defatted and ground to a fine powder prior to papain-assisted extraction followed by precipitation with four volumes of alcohol. After combined procedures, carp gill arches-derived GAGs were precipitated by CPC complex using different NaCl (2.0, 1.5, 1.0, 0.5 and 0.1 M of salt) concentrations, revealing different percentages in acid sugar residues of the CPC fractions recovered from this process.

On the basis of CPC precipitation (Figure 4A), the amount of GAGs contained in the gill arches of cultured Nile tilapia for each fraction had no relation to concentration used, suggesting that the cationic reagent had no significant impact on the OnGAGs-fractionated yield, since that the development alternative GAGs production methods has been widely encouraged based on cost-benefit relation (Badri et al., 2018). This difficults to optimize OnGAGs separation from raw biological material could be a high lipid concentration undigested by protease and/or same sites of CPC complexation occuring with the molecule extracted from the connective tissue (Wasserman et al., 1972; Regnault & Durand, 1998), denoting perharps molecular homogeneity. Cartilage by-products of fish processing industry are also challengers to increase the process yield of GAGs obtaining due to low accessibility to tissue, structural variability, and heterogeneous composition of these polymers in different sources, affecting standartization and the adulteration risk of the products (Badri et al., 2018).

By contrast, analyzing the metachromatic property of the CPC fractions, it was demonstrated by Farndale et al. (1976)' method that the Nile tilapia gill arches extracellular matrix was rich in sulfated GAGs based on OnGAGs-DMB dye binding ability compared with other aquatic sources (Wasserman et al., 1972; Regnault & Durand, 1998; Santiago et al., 2024), as progressively visualized in violet with the increase of the concentration of CPC used for precipitation (Figure 4B). GAGs-like compounds were to be more intense for 10% CPC fraction, showing a highest sulfated GAGs complex due to a more specific affinity of the cationic dye with the ester sulfate groups present in the highly charged polysaccharide structure (Santiago et al., 2024). Such colorimetric property indicated that this respiratory organ secreted mucopolysaccharides by functional glands present in the connective tissue of the gills responsable for the natural protection of the fishes (Moreira et al., 2001; Santiago et al., 2024).

Because of this preliminary analysis of OnGAGs from the cartilage by-product (Figure 4), further step involved the whole gill of cultured Nile tilapia for total extraction of sulfated GAGs in order to explore this residue as an important reservoir of natural product combined with the alternative separation by organic solvents (AL or AC) for obtaining of GAGs fractions.

Second-step: amount of enzymatically extracted GAGs and their combined fractionation with 10% CPC and AL or AC from cultured Nile tilapia gills

Whole gills of cultured Nile tilapia susceptible to papain digestion followed by precipitation with 10% CPC solution yielded 0.91 ± 0.03% from the dehydrated tissue (w w^{-1}) comparing with other cartilage sources and protocols (Table 1). This yield was similar to that considerating gill arches-derived total CPC fractions (Figure 4A), suggesting gill arches as the majority part in GAGs based on Wasserman et al. (1972). The amount of GAGs in the whole gill of cultured Nile tilapia was also in according to other aquatic organisms that showed important yields of crude GAGs ranging from 0.3 to 1.54 ± 0.02% from the dry tissue (w w^{-1}). On a GAGs mass-to-gill mass basis, this amount represented ~1,7-fold lower than that found for cultured *P. brevis* whole gills, but was 3-fold higher

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based on total yield found in *T. orientalis* gill lamellas according to the literature, although considerating other extraction procedures by different authors (Table 1).

Table 1. Yield of GAGs extracted from cultured *O. niloticus* gills *vs.* other aquatic organisms.

origin	crude GAGs	extraction condition	tissue	yield (w w^{-1})	reference
O. niloticus (cultivation)	OnGAGs	papain + 10% CPC (pH 5, 24 h, 60°C)	tritured whole gills	0.91 ± 0.03% (9.1 ± 0.26 mg g^{-1})	this study
P. brevis (cultivation)	PbGAGs	papain + 10% CPC (pH 5, 24 h, 60°C)	tritured whole gills	1.54 ± 0.02%	Santiago et al. (2024)
T. orientalis (Sea of Japan)	ToGAGs	N-Aman (pH 7, 7 days, 55°C)	powder gill lamellas	~ 0.3%	Arima et al. (2013)
C. maena (intertidal)	CmGAGs	papain + CPC (pH 6, 18 h, 62°C)	tritured whole gills	7.52 ± 0.51 mg g^{-1}	Regnault & Durand (1998)

Shark cartilage is main source in CS-type GAGs for a market that had an estimated rate of 15% in 2011 (Badri et al., 2018), however, this animal demand by uncontrolled fishing is not a sustainable option (Badri et al., 2018; Li et al., 2022) and the quality control and cost required for production of commercially-important GAGs could also look up to other inedible parts of fishes (Arima et al., 2013). This scenario led us to further analyze the enzimatically-extracted product from cultured Nile tilapia-removed whole gills using alternative separation by organic solvents (AL or AC) for obtaining of GAGs fractions and their purity by electrophoresis, since that GAGs are industrially sujected to chemical processes (*e.g.,* chemical synthesis of low molecular weight HEP, known as Arixtra) that result in laborious steps and expensive obtaining (Badri et al., 2018; Liu et al., 2025).

After extraction step, prepared samples of crude GAGs solution, extracted from cultured Nile tilapia whole gills, were submitted to two organic solvents by adding of three volumes and the precipitates obtained under our experimental conditions (4°C, 24 h) generated only two fractions (FAL1 or FAC1), resulting in 0.10 ± 0.47% and 0.16 ± 0.00% (w w^{-1}), respectively, therefore, with a significant difference in terms of GAGs-containing mass, since that no product was recovered by other two volumes added to aqueous preparations for fractions obtaining. Results pointed out that the interaction between enzimatically extracted GAGs from cultured Nile tilapia gills with both solvent precipitations showed a higher efficiency with acetone than alcohol (usually used for CS precipitation – Li et al., 2022) because there is an important difference in polarity between these two organic reagents to polysaccharide recorevy (Masterton et al., 1990). It eco-friendly methodology could be an alternative of low-cost operation compared to the use of chromatographic procedures using commercial resins for GAGs separation, which are attributed to ionic bonding between GAGs chains and mobile solution (Liu et al., 2025), *e.g.*, using LiCl (Arima et al., 2013) or NaCl (Salles et al., 2017).

On a purity basis, analysis by mean of metachromasia assay suggested that both fractions FAL1 or FAC1 did not react with the DMB dye *vs*. CE (OnGAGs, Table 1) that presented a visible complex of violet coloring as demonstrated in figure 5A. Finding speculated that the respective fractions obtained by both organic solvents showed to a lack of ester groups in the GAGs structures of the samples recovered, contrasting those CPC fractions presenting metachromasia by Farndale et

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al. (1976)' method (Figure 4B) and in other cartilage sources (Wasserman et al., 1972; Regnault & Durand, 1998; Santiago et al., 2024).

In fact, investigating by 0.5% agarose gel electrophoresis procedure, it was not observed any metachromatic band for organic solvents-obtained fractions (FAL1 or FAC1) based on CE and standards of mammalian CS, DS and HEP (Figure 5B) applied on gel that were visualized by sulfated GAGs+toluidin blue dye complex because the diamine present in run buffer interacted with the sulfate groups occuring in polysaccharidic structures (Dietrich & Dietrich (1976). The disparity in anionic character is logically attributable to way by which each solvent (CPC, alcohol and acetone) act on the molecular structure (Masterton et al., 1990), therefore, impacting in the overral results, since that the sulfate level is a crucial factor that determines the quality and bioactivity of pharmaceutical and food preparations (Oetterer et al., 2014; Volpi, 2011; Valcarcel et al., 2017).

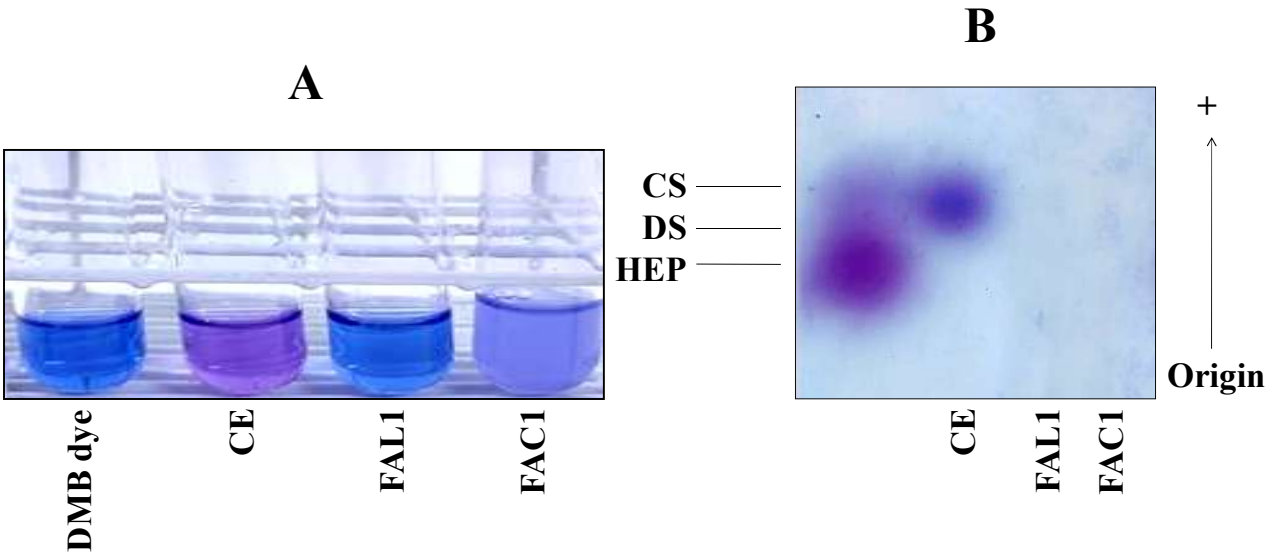

Figure 5. Metachromasia assay revealing sulfation-positive crude extract (CE, OnGAGs) in the presence of DMB dye (A) and 0.5% agarose gel electrophoresis of cultured *O. niloticus* whole gills GAGs (CE and fractions FAL1 and FAC1) and standards chondroitin sulfate (CS,~60 kDa), dermatan sulfate (DS,~ 40 kDa) and unfractionated heparin (HEP,~14 kDa) after treated with 0.1% toluidine blue dye (B).

On the other hand, it was noted that the cultured Nile tilapia gills preponderantly showed CE rich in GAGs by 10% CPC precipitation (Table 1) with metachromatic homogeneous band comigrating as a CS based on standard used (Figure 5B), therefore, it was also suggested the unique presence this GAG in *O. niloticus* cartilage (whole gill) by-product, as also recently found in *P. brevis* gills (Santiago et al., 2024). On a point of industrial view, it would be important by transformation process in cost reduction (Badri et al., 2018), since that studies revealed HA, CS and HS in gills arches of *C. carpio* fish (Wasserman et al., 1972) and in gills of *C. maenas* crab (Regnault & Durand, 1998); and CS, DS and HA in gill lamellas of *T. orientalis* fish (Arima et al., 2013). The analytical accuracy of animal-based raw materials requires meticulous adjustment in the industry to impurities/contaminants elimination (high-quality polymers) (Liu et al., 2025).

Furher step was to investigate the cultured Nile tilapia gills GAGs-related functional groups by FT-IR technique in comparison with other already reported for fish filleting-derived by-products.

Structural characterization of OnGAGs by FT-IR

The molecular scenario found in cultured Nile tilapia gills GAGs revealed to a structural basis by FT-IR analysis, which characterized the three samples (CE and fractions FAL1 / FAC1) as different chemically compounds among them, as illustrated in figure 6. Such results were revealed for the

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spectral values of FAL1 and FAC1 against those found for analyzed CE sample, not differing only by intensity of signals, but also by absence of some of them, especially on the range from 800 to 1250 cm^{-1} which were associated to uronic acid residues and sulfation peaks (Oliveira et al., 2015b; Santiago et al., 2024).

On a general view, sulfated GAGs were detected, but with variable intensity between CE and FAC1 by FT-IR analysis. The spectral window from 4000 to 500 cm^{-1} indicated regions from 3433 to 536 cm^{-1} for CE; from 3421 to 460 cm^{-1} for FAL1; and from 3286 to 700 cm^{-1} for FAC1, respectively. Collectivelly, these signals pointed out to obtaining of materials with different structures (Figure 6).

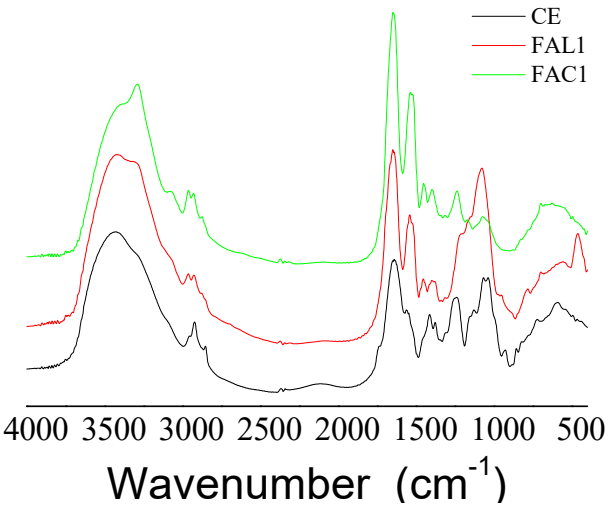

Figure 6. FT-IR spectra of CE, FAL1 and FAL1, obtained from *O. niloticus* gills GAGs, using KBr *pellets*.

In fact, by spectra obtained in figure 6, it was detected the occurence for CS structure-containing CE in *O. niloticus* gills, whose main chemical bands were 3433, 1639, 1558, 1377, 1128 and 1064 cm^{-1}, besides signals related to sulfation at 1242, 854 and 819 cm^{-1}. Of which at 854 and 819 cm^{-1} were attributed to the presence of C-4-S and C-6-S, respectively, suggesting both CS features in cultured Nile tilapia gills GAGs. Bones of *O. niloticus* (Oliveira et al., 2015b) and gills of *P. brevis* (Santiago et al., 2024) were demonstrated to have both C-4-S and C-6-S structures making part of the same backbone of GAGs. CS structures in tilapia gills arise as a new source for therapeutic applicabilities, since that CS is well-recognized as important suplemments, such as for the symptomatic treatment of osteoarthritis by reducing inflammatory event (Volp, 2011; Valcarcel et al., 2017; Li et al., 2022) and involved in other animal-physiological processes (Oliveira et al., 2015a).

Regarding fractions (Figure 6), comparative analysis by FT-IR between fractions FAL1 and FAC1 indicated amide (1651 and 1555 cm^{-1}, N-H), uronic acid, N-H COO-, glucuronic acid and glicosidic linkages, but without presenting sulfate ester (S=O) for FAL1. Even FAC1 showed peak at 1234 cm^{-1} (sulfation) as observed in CE; furthermore, CH at 2927 cm^{-1}, C-N, uronic acid C-O, N-H COO- of glucuronic acid at 1452 cm^{-1}, C-O-C, C-OH and C-C at 1070 cm^{-1}; and a discrete shoulder at 833 cm^{-1} (C-O-S), denoting a low concentration in C-6-S from the analyzed polymer based on Oliveira et al. (2015b) and reduced charge from the FAC1 sample.

These combined interpretations led us to explain the absence of both fractions by metachromasia and agarose gel analyses (Figure 5) and the FT-IR technique clearly evidenced as an important tool for more refined characterization of heterogeneous structures, when in low availability (Oliveira et al., 2015b). Therefore, use of acetone was a selective agent for sulfated GAGs obtaining than in

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alcohol-precipitated materials, confirming their differences in polarity to interact with the functional groups (Masterton et al., 1990) of GAGs extracted from *O. niloticus* gills (Figures 2, 3), as resumed in table 3 in terms of solvents and purity.

On the other hand, this degree of chemical difference between fractions *vs*. CE, although FT-IR analysis revealing an analytical accuracy in polysaccharidic structure (Figure 6), detecting components make an essential step for exploring structure-potency relationship to facilite industrial production (Badri et al., 2018; Liu et al., 2025).

Table 3. Spectral characteristics for GAGs CS.

samples	spectral signals (cm^{-1})	GAGs
CE	1242 (S=O) 854 (C-O-S) 819 (C-O-S)	C-4/6-S
FAL1	-	-
FAC1	1234 (S=O) 833 (C-O-S)	C-6-S

- material not identified as sulfated GAG.

Therefore, the introduction of a combined strategy on the two-steps fractionation by precipitation using three organic solvents for CS-selective recovery showed reusability and valorisation perspective from cartilage (fish gills) by-products, since that studies on the structural biology of animal GAGs are laborious because involve chemical methods (*e.g.*, alkali and acid) and time consuming leading to human and environmental risks (Liu et al., 2025). Although this investigation showing comparatively reduced sulfate levels (Figure 6, Table 3) because they would determine the quality of GAGs-based products, this reduction could be useful to impact solubility properties of GAGs (*e.g.*, gelling) by precipitation process with acetone-treated sample by affecting the total charge from the native molecule (Gandhi & Mancera, 2008; Badri et al., 2018). Additional studies must be conducted on the chemical biology of the Nile tilapia gills-isolated GAGs.

Conclusion

The combined methodology for selective precipitation of enzymatically extracted glycosaminoglycans from cultured Nile tilapia gills, followed by their structural characterization by infrared spectroscopy revealed to partial application. Gills arches rich in glycosaminoglycans, which they were preponderantly concentrated with 10% cetylpiridinium chloride, showed to be sulfated compounds by metachromasia assay. When in whole gills, molecular analyses by agarose gel electrophoresis and infrared procedures presumed a chondroitin-4/6-sulfate structure. Further treatment with alcohol or acetone revealed different structures, showing the acetone precipitated-fraction selective for chondroitin-6-sulfate obtaining, although in low amounts and reduced charge according to the biochemical assays.

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