KAZUKO OGURA

EXTRACTION OF GLYCOSAMINOGLYCANS (GAGS) FROM DISCARDED BY-PRODUCTS OF BLUEFIN TUNA (*THUNNUS THYNNUS*) FISHED OFF ALGARVE

UNIVERSIDADE DO ALGARVE
Instituto Superior de Engenharia
2023

KAZUKO OGURA

EXTRACTION OF GLYCOSAMINOGLYCANS (GAGS) FROM DISCARDED BY-PRODUCTS OF BLUEFIN TUNA (*THUNNUS THYNNUS*) FISHED OFF ALGARVE

Mestrado em Tecnologia de Alimentos

Trabalho realizado sob a orientação de:

Professor Doutor Jaime Miguel Costa Aníbal

Professor Doutor Eduardo Bruno Oliveira Esteves

UNIVERSIDADE DO ALGARVE
Instituto Superior de Engenharia
2023

EXTRACTION OF GLYCOSAMINOGLYCANS (GAGS) FROM DISCARDED BY-PRODUCTS OF BLUEFIN TUNA (*THUNNUS THYNNUS*) FISHED OFF ALGARVE

Declaration of authorship of the work

I hereby declare to be the author of this work, which is original and unpublished. Authors and works consulted are properly cited in the text and included in the reference list.

_____

(Kazuko Ogura)

ACKNOWLEDGEMENTS

Thanks to the support of many people, I was able to complete this master's thesis. Firstly, I would like to thank my supervisors, Professor Jaime Aníbal and Professor Eduardo Bruno Oliveira Esteves, for their patience and kind support. Thanks to the guidance of the professors, I have gained confidence in my ability to apply myself as a researcher in whatever area of research I will be doing in the future. I also cannot thank Mr. Hajime Tanaka, Ms. Maria Nunes, Ms. Cíntia Gonçalves, Ms. Inga Barata da Silva and any other people in Tunipex. S.A. who gave me invaluable knowledge, experience and valuable samples, which are irreplaceable. I will forever be grateful.

I would also like to express my sincere gratitude to Vera Margarida de Deus Nunes Gonçalves, and Neusa Maria da Silva Ferro Rodrigues who have always been kind and gentle in the laboratory and supported me. I would also like to express my gratitude to all other professors of the Food Technology Department of Instituto Superior Engenharia, University of Algarve for this learning opportunity. Finally, I must express my deepest gratitude to my husband and children. Without the support of my family, I would not have been able to achieve the results I have throughout the research and writing process of this thesis.

RESUMO

O onsumo global de pescado aumentou quase o dobro da taxa anual do crescimento da população mundial no mesmo período, de 1961 a 2019, tornando o consumo de pescado maior do que qualquer outro consumo de proteína animal. Por outro lado, a proporção dos mananciais de pescarias sustentáveis continua a diminuir, devido à sobrepesca e ao aumento da temperatura da água do oceano, criando a necessidade de aumentar a utilização eficaz dos subprodutos de resíduos marinhos. O objetivo deste estudo foi estabelecer uma metodologia para a extração e quantificação de glicosaminoglicanos (GAG) em globos oculares e brânquias de atum, os quais são subprodutos descartados de atum rabilho *(Thunnus thynnus)*. Pretendeu-se também estabelecer uma base para aumentar o valor de subprodutos descartados e facilitar a sua distribuição para as indústrias farmacêutica, cosmética e alimentar.

Os GAG são polissacáridos de cadeias longas, lineares, carregadas negativamente, e com uma estrutura dissacárida repetitiva de N-acetilglucosamina e ácido glucurónico, sendo o ácido hialurónico (HA) e o sulfato de condroitina (CS) dois exemplos. Os GAG são compostos bioativos como componentes-chave das matrizes celulares e extracelulares, e desempenham papéis importantes no crescimento celular, diferenciação, morfogénese, migração celular e infeção bacteriana/viral. Os GAG foram extraídos das brânquias e globos oculares de atuns rabilho, recolhidos na região do Algarve (Portugal), pela empresa Tunipex, S.A., através de um processo de desengorduramento, hidrólise enzimática e precipitação. Nos ensaios com as brânquias (14 amostras), a extração de GAG foi realizada utilizando as partes mais cartilagíneas das áreas externas da estrutura branquial, tendo-se descartado a parte óssea. Nos ensaios com os globos oculares (15 amostras), os GAG foram extraídos a partir do humor vítreo, contido no interior da cavidade ocular. A enzima papaína e um tampão acetato de sódio foram utilizados para a hidrólise enzimática, sendo a precipitação dos GAG efetuada através da adição de etanol.

A quantidade de GAG presente nas amostras foi estimada através da determinação dos hidratos de carbono totais pelo método de Dubois. As brânquias apresentaram um teor de GAG de 8,71±0,66 mg/g de massa seca precipitada pelo etanol, enquanto o valor correspondente no humor vítreo dos glóbulos oculares foi de 1,79±0,72 mg/g. A comparação com resultados de outros estudos de métodos de extração semelhantes confirmou que o atum rabilho contém tanto ou mais GAG do que outros organismos. O GAG médio das guelras por um indivíduo

de atum rabilho foi de 2,3867 g e o GAG médio de por um indivíduo de atum rabilho (2 globos oculares) foi de 0,2269 g. Comercialmente, à data de realização deste trabalho, o CS é vendido a um preço médio de 442 €/g e o HA a um preço médio de 18.737 €/g, a empresas médicas, institutos de investigação ou empresas farmacêuticas em Portugal. Com base nestes preços, e por cada atum rabilho, estima-se que o CS contido nas brânquias tenha um valor de 1.056 €, e o HA contido no globo tenha um valor de 4.252 €. Por extrapolação das capturas da empresa Tunipex S.A. (cerca de 1374 atuns rabilho por ano), calculou-se que as brânquias poderiam render € 1.450.998,96 € em CS e os globos oculares 5.842.577,76 € em HA.

Os mercados globais de CS e HA estão se expandindo rapidamente. Em escala industrial, o CS é extraído principalmente da cartilagem de tubarões e animais terrestres. No entanto, nos últimos anos, questões éticas e de sustentabilidade relacionadas a tubarões ameaçados foram levantadas e, em agosto de 2021, o governo do Reino Unido promulgou uma nova lei proibindo a importação e exportação de barbatanas de tubarão. Devido ao seu baixo custo, o HA é produzido principalmente por via microbiológica, no entanto devido a questões de segurança, as aplicações médicas geralmente usam HA extraído de animais terrestres ou marinhos. Além disso, devido à crescente consciencialização sobre a importância da rastreabilidade devido a questões como BSE, espera-se que a procura por CS e HA originários de organismos marinhos aumente ainda mais no futuro. Até ao momento, nenhum estudo foi realizado sobre a extração de GAG, CS ou HA, em brânquias ou globos oculares de atum rabilho. O atum rabilho é um dos principais predadores marinhos de topo e a uma das maiores espécies de atum presente em águas portuguesas. Devido ao seu grande tamanho é possível produzir quantidades apreciáveis de GAG relativamente à quantidade e peso dos subprodutos descartados.

Como resultado desta pesquisa, descobriu-se que os globos oculares e as brânquias do atum rabilho contêm grandes quantidades de GAG e que são materiais de alto valor, de utilização ecologicamente sustentável e baixo custo. A parte descartada do atum rabilho pode ser uma nova matéria-prima para extração de GAG, como HA e CS, e tem aplicações potenciais nas áreas médica, farmacêutica, alimentícia e cosmética. Este estudo pode servir de base para avaliar o uso comercial dos subprodutos descartados do atum rabilho.

No entanto, existem muitos desafios para estabelecer o potencial comercial. Além das questões técnicas, como a remoção do odor peculiar do pescado, também existem dificuldades devido às matérias-primas, como a flutuação da quantidade de pescado e dos ingredientes do peixe cru, dependendo da estação do ano e da manutenção do frescor. Nas extrações efetuadas a partir do globo ocular, não houve uma relação significativa entre o tamanho do indivíduo e a

quantidade de GAG, mas no caso dos ensaios com brânquias, quanto maior o tamanho da brânquia, maior a quantidade de GAG extraído (mg por g de peso seco) (p=0.04) e o teor de hidratos de carbono por mg de GAG bruto (p=0.03). Os GAG extraídos dos dois tipos de tecido em estudo, brânquia e humor vítreo, apresentaram cor, textura e propriedades diferentes. Para estabelecer o seu potencial comercial dos GAG extraídos, são necessários mais estudos para voltar a testá-los após novos processos de purificação e para analisar a potencial atividade biológica dos GAG isolados.

Palavras-chave: Valorização de subprodutos, Compostos bioativos, atum rabilho, glicosaminoglicanos

ABSTRACT

Social needs for effective utilization of marine discarded byproducts are increasing due to factors such as the decreasing proportion of sustainable fish resources. Therefore, with the aim of increasing the value of discarded by-products and providing a basis for promoting their distribution, we extracted glycosaminoglycans (GAGs) from the eyeballs and gills of bluefin tuna discarded by-products and estimated their content. GAGs are long linear polysaccharides that form a repeating disaccharide structure of N-acetylglucosamine and glucuronic acid, where hyaluronic acid (HA) and chondroitin sulfate (CS) are two examples. Crude GAG was extracted from the gills and eyeballs of bluefin tuna collected in the Algarve region, Portugal by the processes of acetone defatting, enzymatic hydrolysis with papain, and ethanol precipitation. Assuming that the amount of carbohydrate in the crude GAG is the amount of GAG, the average GAG per gills of one individual bluefin tuna was 2.3867 g, and the average GAG per one individual bluefin tuna (2 eyeballs) was 0.2269 g. In the experiments with gills, the larger the gill size, the higher the GAG yield (mg per g of dry pellet) (p=0.04) and the carbohydrate content per mg of crude GAGs (p=0.03). CS is sold at very high prices, average 442 €/g and HA at average 18,737 €/g, to medical, research institute or pharmaceutical companies in Portugal. Based on these prices, it is estimated that the gills could be the main ingredient of CS product worth €1,056 and the eyeball could be the main ingredient of HA product worth €4,252, per one bluefin tuna respectively. This study serves as a basis for the commercial utilization of bluefin tuna discarded by-products. However, to establish its commercial potential, further studies are needed to re-test it after further purification processes and to analyze the potential biological activity of the isolated GAGs.

Keywords: Valorization of by-products, Bioactive compounds, bluefin tuna, glycosaminoglycans

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

CS	Chondroitin Sulfate; Chondroitin Sulphate
CPC	Cetylpytridinium Chloride (Antiseptic/ Disinfectant)
GAGs	Glycosaminoglycans
KS	Keratan Sulfate
MW	Molecular Weight
HA	Hyaluronic acid; Hyaluronate; Hyaluronan.
HS	Heparan Sulfate
Hp	Heparin
SDGs	Sustainable Development Goals

1

INTRODUCTION

1.1 GLOBAL POPULATION AND SUSTAINABILITY IN FISHING INDUSTRY

Global populations are projected to increase by about three billion people in 2100, to a total of 10.9 billion individuals (United Nations, 2019). In addition to the explosive growth of the world's population, the deterioration of the global environment, such as global warming, gives us increasing concerns about the tight supply and demand of energy and food resources (FAO, 2017). Global seafood consumption increased at a rate almost twice that of annual world population growth for the same period, from 1961 to 2019, and higher than that of all other animal protein foods (meat, dairy, milk, etc.) for a variety of reasons, including the health benefits of fish products (FAO, 2022a). On the other hand, the proportion of sustainable fish stocks continues to decline due to overfishing and increasing water temperatures, suggesting the necessity to shift to a sustainable fishery industry.

In September 2015, with the participation of more than 150-member state leaders at the United Nations Summit, to create a society in which all people can continue to live affluently and peacefully toward 2030, the 2030 Agenda for Sustainable Development was signed (United Nations, 2015). In addition, "Sustainable Development Goals (SDGs)" consisting of 17 goals and 169 targets were set up. The fishery-related goal is "14. Conservation and sustainable use of marine and marine resources for sustainable development" is one of the most critical issues, because of the major disruptions in fish populations and aquatic food webs due to overfishing and increasing water temperatures. Goal 12 is “Ensure sustainable consumption and production patterns”, which also includes waste management and reduction as targets under Target 12.3, "Responsibility to Create Responsibility to Produce”.

1.2 INCREASING NEEDS TO UTILIZE FISH BY-PRODUCTS

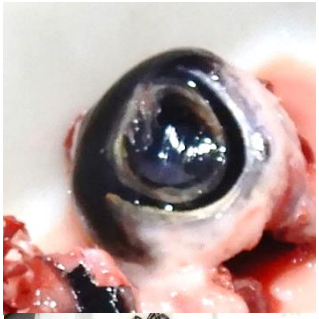
As the world's seafood supply increases dramatically, marine discards from fisheries have also increased, by-products discarded from the fishery account for 10.1% of the annual catch (FAO, 2020). As it has been suggested that the generation of marine discards could increase even more significantly in the future (eurostat Statistics Explained, 2020), there is an urgent need to review, not only the fishing methods, but also the distribution and commercialization methods (FAO, 2020). FAO has also required sustainable use and intensive technology promotion of fish resources, and to increase the use of fish by-products (FAO, 2014, 2021, 2022b; Save Food Initiative, 2021). The legal framework established by the Common Fisheries Policy (CFP) of the European Union, based on the essential objective of sustainability as a fundamental premise for the economic and social future of European fisheries, defined by-products as part of the catch, and proposed that harvested resources or biomass to be pursued to the maximum extent possible and the need to eliminate discards (European Commission, 2019). The phase-in period began in 2015, with a mandatory discard ban in all EU countries in January 2019.

In response to this growing social demand for the utilisation of discarded by-products of fish processing, we have initiated a project in collaboration with Tunipex. S.A. to maximise the value of discarded bluefin tuna by-products. Tunipex. S.A. is a fishing company that started operations in Olhão (Algarve) in 1994. Tunipex. S.A. caught approximately 300 tons, 1374 of bluefin tunas in 2022, of which 21.9 % (65.8 tons) is considered as discarded by-products such as viscera, tails, bones, etc. which are difficult-to-process for food. Currently, these discarded by-products are used as raw materials for fish flour, which feeds animals, and the selling price has been on the rise in recent years, at 30 €/ton in 2021, 60 €/ton in 2022 and 140 €/ton in 2023. However, fish residues are rich in various kind of more expensive bioactive ingredients such as polyunsaturated fatty acids such as DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) (Olsen et al., 2014), collagen, CS, chitin and chitosan (Govindharaj et al., 2019; Ozogul et al., 2021; Vázquez et al., 2019, 2020) There are a lot of possibility to utilize them in several areas, such as: food and nutrition (e.g. health foods), medical care (e.g. artificial skin and sutures), cosmetics (e.g. moisturizer), agriculture (e.g. biodegradable fertilizer), industry (e.g. food protection, drug delivery and textiles), and environmental fields (e.g. wastewater treatment) (el Knidri et al., 2018, Rameshthangam et al., 2020, Oliveira et al., 2021) (Figure 1.1).

HEADS

PUFA (DHA, olsenEPA), Fish Oil (Olsen et al., 2014)

Chondroitin sulfate (Abdallah et al., 2020)



EYES

Hyaluronic Acid (Abdallah et al., 2020)

FINS

Chondroitin sulfate (Abdallah et al., 2020)

Chitin / Chitosan (Olsen et al., 2014) a

SPINS

Ca, Mg (Välimaa et al., 2019)

Collagen (Olsen et al., 2014)

Isolate fish protein (Olsen et al., 2014)

VISCERA

Bioactive Peptide (Olsen et al., 2014)

Taurine (Ozogul et al., 2021)

Figure 1.1 Discarded By-Products of Bluefin Tuna and Expected Main Bioactive Ingredient of Each Part.

1.3 OBJECTIVE OF THIS STUDY AND ORGANIZATION OF THE DISSERTATION

The aim of this study was to stablish a methodology for the extraction and quantification of GAGs such as HA and CS in tuna eyeballs and gills of bluefin tuna (*Thunnus thynnus*) discarded by-products, thereby providing a basis for increasing the value of discarded by-products and facilitating the distribution of bluefin tuna discarded by-products to pharmaceutical, cosmetic and food manufacturers at a higher price.

Fish discarded by-products is a potential source of safer and more sustainable GAGs than the other sources. GAGs derived from marine by-products have the potential to be used not only in food, health, and beauty products, but also in the medical field, and will contribute to the realization of affluent lives and the extension of healthy life expectancy. In addition, compared to chemical refining, it can reduce water and energy usage, reduce marine waste and pollution, and contribute to the sustainable development of the circular economy and food industry through the rational use of resources as well as environmental conservation.

As extraction of active ingredients from fish discarded by-products is fraught with challenges such as fish-specific odours, countermeasures against spoilage, extraction costs, time requirements and variations in quality, technical improvements are essential to valorize fish discarded by-products. Tuna by-products have relatively good scalability due to the size of their parts. The main product of bluefin tuna caught by Tunipex. S.A. is fillets for raw consumption, the by-products are refrigerated immediately after fishing and then quickly frozen at -60℃ shortly thereafter, which may have better storage conditions and a positive impact on the by-product valorisation process. Few studies have researched about the valorisation of bluefin tuna discarded parts so far. It is anticipated that our study will provide valuable evidence to valorise bluefin tuna discarded by-products.

The overall objective of this work was to study the possibility of valorisation of bluefin tuna （*Thunnus thynnus*） discarded by-product. The specific objectives of the study were identified as:

1. To extract and quantify GAGs from the gills and eyeballs of bluefin tuna
2. To optimize the GAGs extraction method from the gills and eyeballs of bluefin tuna.
3. To estimate the potential value of gills and eyeballs of bluefin tuna as a source of GAG products such as HA and CS.

2 LITERATURE REVIEW

2.1 GLOBAL SITUATION IN TUNA FISHING INDUSTRY

Tuna is one of the bony fishes classified in the Order of Perciformes and Family Scombridae which are temperate/warmwater, pelagic, migratory, and large carnivorous fish (Collette et al., 2001). Tuna is rich in high-quality protein and n-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and has attracted attention for its health benefits (Mesías et al., 2015). It is traded internationally as canned fish, sashimi, and sushi material (Marie Lecomte et al., 2018). Due to its wide distribution and high economic value, tuna has become the predominant species in 85 fish markets worldwide and is part of the most valuable fishery resource (FAO, 2011, 2020; McKinney et al., 2020). It accounts for 61 % of the high seas catch by weight and contributes USD 40 billion to the global economy in 2018 (McKinney et al., 2020). Global catches of tuna and tuna-like species have increased more than 15 times in the last 60 years, with an increasing from less than 0.4 million tons in 1950 to 6 million tons in 2019 (Coulter et al., 2020). Currently, global tuna catches are skipjack (*Katsuwonus pelamis*) 57 %, yellowfin (*Thunnus albacares*) 29 %, bigeye tuna (*Thunnus obesus*) 8 %, albacore (*Thunnus alalonga*) 5 % and bluefin tuna (*Thunnus thynuss Atlantic*, or *T. orientalis Pacific*) 1 % (FAO, 2020; ISSF, 2020).

Although bluefin tuna is a small proportion of total tuna catches, it is one of the most highly valued tuna species in the world's sashimi markets and enjoys stable prices. As the top marine predator and the largest species of tuna, the bluefin tuna plays an important role in maintaining the balance of the marine ecosystem. However, contrary to increasing demand and fishing capacity, the bluefin tuna had experienced a decline in adult populations (ISSF, 2020; MacKenzie et al., 2009). Recent assessments estimate that the biomass of western Atlantic bluefin tuna stock declined to its lowest level in the mid-to-late 1970s and remained so for

more than 20 years thereafter; since the implementation of the International Commission for International Commission for the Conservation of Atlantic Tunas recovery plan in the late 1990s, biomass has gradually increased (ICCAT (International Commission for the Conservation of Atlantic Tunas), 2017, 2021), but has still been of great public concern from an international environmental protection perspective.

2.2 ATLANTIC BLUEFIN TUNA (*THUNNUS THYNNUS*) IN ALGARVE REGION

Atlantic bluefin tuna (*Thunnus thynnuss*) is caught in fisheries in more than 50 countries and the Mediterranean Sea is one of the spawning grounds for Bluefin tuna. The Algarve region, on the south side of Portugal, was once a thriving region for tuna fishing. Tuna is a migratory fish, and until the 1960s, the preferred route for Atlantic bluefin tuna to and from the warmer waters of the Mediterranean was close to the Algarve coastline. However, as the number of fishes migrating in the Algarve waters declined in the mid-1960s, the tuna fishery began to decline, and the tuna fishery, which had a history of more than 400 years, once ended in the early 1970s.

Tunipex. S.A. is a fishing company that started operations in Olhão (Algarve) in 1994, having started a project to rebuild the tuna fishery in Algarve region in the early 1990s. Two kinds of tuna were caught in the Algarve: 'In-coming tuna', which were mature tuna that enter the Mediterranean Sea from the Atlantic Ocean to spawn, and 'Out-going tuna', fishes returning to the North Atlantic after spawning. The first can be found from April to mid-June and the second from June to mid-August in Algarve region. Northern European bluefin tuna migrate from the Atlantic Ocean through the Strait of Gibraltar in search of suitable hatching areas in spring and return to their birthplace to breed in the warm, clear, and salty Mediterranean waters (Schaefer, 2001). 'Incoming tuna' have accumulated energy to migrate these thousands of kilometres and they are maturing. On the other hand, 'out-going tuna', which return to the Atlantic Ocean from their spawning area in summer, lost up to 35 % of their weight due to reproductive effort (García et al., 2010). This post-spawning 'out-going tuna' is leaner, has less fat, drier flesh and could not be immediately caught and sold due to low market demand. Therefore, the Tunipex. S.A. made use of the set net techniques, catching bluefin tuna returning from Mediterranean Sea in the post-spawning period, then store and feed them in enclosed areas until they reached the required quality (Esteves & Aníbal, 2019). These practises are contributing to rebuild the tuna fishery the Algarve region.

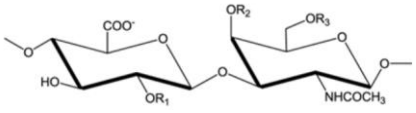
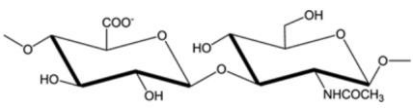
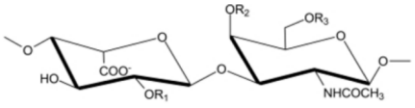
The market price of bluefin tuna is strongly correlated with the quality of the meat. Slaughter, processing, storage, and shipping must be of the highest standard. Therefore, bluefin tuna are

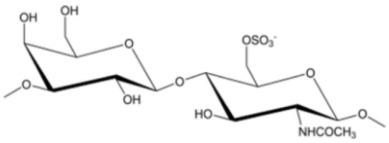
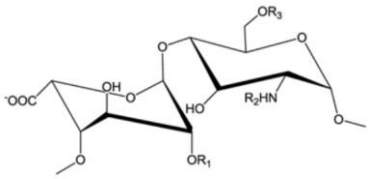
processed very quickly to obtain the best and freshest meat quality, and once the tuna are on board, discarded by-products such as guts and heads are immediately cut off and placed in ice water, and within an hour they are cut into pieces at a processing plant near the dock and ready for distribution. The caught tuna is sold to the Portuguese, European and Japanese markets, mainly as fillets for consumption as sushi.

2.3 GLYCOSAMINOGLYCANS (GAGS)

Glycosaminoglycans (GAGs) are long, linear, highly negatively charged, bioactive polysaccharides as key components of the extracellular matrix and cells. GAG play important roles in cell growth, differentiation, morphogenesis, cell migration and bacterial/viral infection. GAGs are composed of repeated disaccharide units comprising a uronic acid (D-glucuronic acid or L-iduronic acid) and a hexosamine (D-glucosamine or D-galactosamine) (Pomin, 2016) as shown Table 2.1. Uronic acid is the generic term for carboxylic acids, which are derivatives obtained by oxidation of monosaccharides in which the hydroxymethyl group (-CH_2OH) at the end of the main chain has been converted to a carboxy group (-CO_2H). Hexosamine is the generic term for amino sugars in which the hydroxyl group of the hexose is replaced by an amino group. GAGs include chondroitin sulfate (CS), hyaluronic acid (HA), heparan sulfate (HS), heparin (Hp), keratan sulfate (KS), which differ in the configuration, position, and linkages formed by the functional groups. Chondroitin sulphate and hyaluronic acid contain D-Glucuronic acid. Chondroitin sulphate contains D-Galactosamine and HA contains D-Glucosamine.

Table 2.1. Composition of Glycosaminoglycans (Abdallah et al., 2020; Gandhi & Mancera, 2008; Myron et al., 2014; Pudełko et al., 2019; Rudd & Yates, 2010).

Type of GAGs	**Uronic acid**	**Hexosamine**	**Linkages between Uronic acid Hexosamine**	**Chemical structure of the disaccharide or trisaccharide units**
Chondroitin Sulfate	D-Glucuronic acid	D-Galactosamine	The linkage is β 1→3.	
Hyaluronic acid	D-Glucuronic acid	D-Glucosamine	The linkage is β 1→3.	
Dermatan sulfate	L-Iduronic acid	D-Galactosamine	The linkage is β 1→3.	

Type of GAGs	Uronic acid	Hexosamine	Linkages between Uronic acid Hexosamine	Chemical structure of the disaccharide or trisaccharide units
Keratan sulfate	D-Galactose	D-Glucosamine	The linkage is β 1→3.	
Heparin Heparan sulfate	D-Glucuronic acid	D-Glucosamine	The linkage is β 1→4.	

2.3.1 CHONDROITIN SULFATE (CS)

CS is a GAG formed by repeating structures of disaccharides (D-Glucuronic acid and D-Galactosamine), the repeats of which are shorter than those of HA. CS is mainly found in the extracellular matrix and cell membrane of tissues (Haylock-Jacobs et al., 2011). CS has many variations in structure based on differences in length and sulphate position (Malavaki et al., 2008). CSs usually exist as proteoglycans covalently bound to core proteins called core proteins (Figure 2.1). Proteoglycans are particularly abundant in the extracellular matrix of cartilage as proteoglycans called aggrecans (Park et al., 2016).

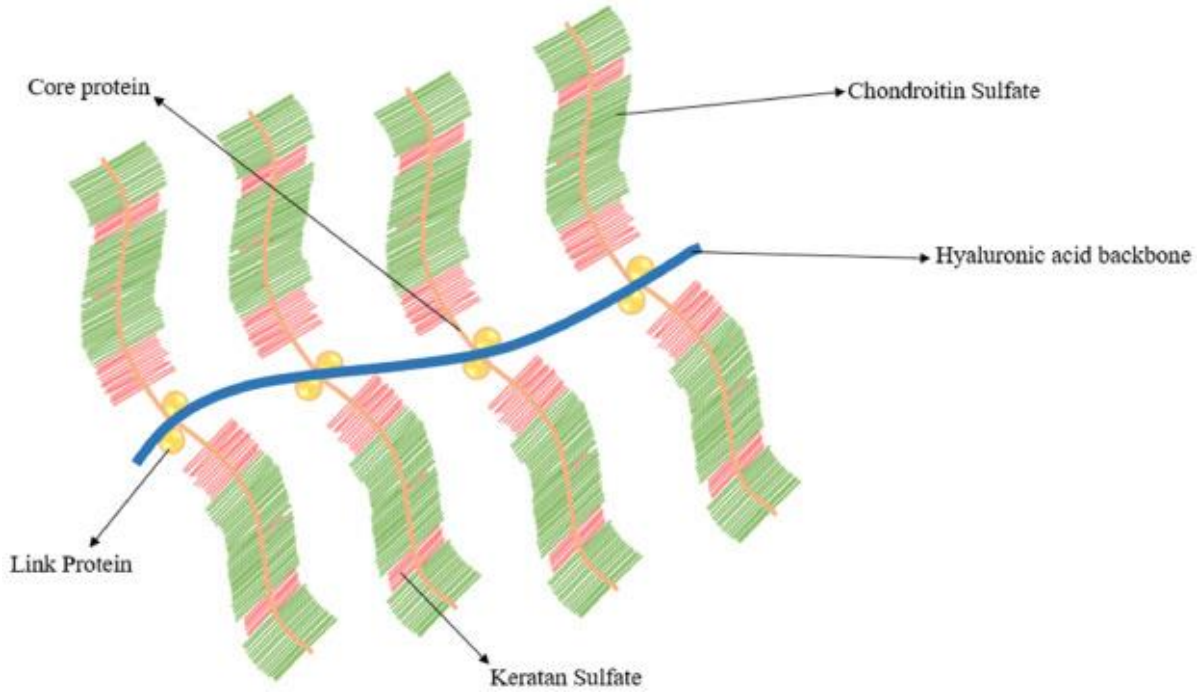


Figure 2.1. Overall Configuration of Protein with Attached GAGs (Sahu et al., 2023).

CS has been reported to have anticoagulant (ben Mansour et al., 2017), antiproliferative (Krichen et al., 2018), antioxidant (Campo et al., 2006), anti-inflammatory (Hu et al., 2019), and antiviral activity (Kato et al., 2010) and to exert a variety of pharmacological effects CS has also been widely employed in the development of health foods, cosmetics, and nonprescription drugs (DiNubile, 2018; Min et al., 2020; Rodriguez-Merchan, 2018). Annual sales of CS as a dietary supplement have exceeded $1 billion to date, with an estimated annual growth rate of 15 %, highlighting the excellent market potential of CS-derived products (Restaino et al., 2019).

2.3.2 HYALURONIC ACID (HA)

HA is a polysaccharide formed from disaccharide repeating units consisting of D-glucuronic acid and N-acetyl-D-glucosamine. HA is the only GAG that is not sulphated, is not bound to a core protein, and therefore does not constitute a proteoglycan (Lamberg & Stoolmiller, 1974). In contrast to other GAGs that are smaller in size, it is usually composed of 100-20 000 repeat units and has a molecular weight of 10^5-10^8 Da and has very long linear structures up to 2.4 mm (Laurent & Fraser, 1992; Sadhasivam et al., 2013). HA retains water and acts as a lubricant. The presence of carboxylic acid groups makes hyaluronic acid anionic, forming hydrogen bonds between the carboxylate groups of glucuronic acid and the acetamide groups of N-acetyl-D-glucosamine. Through that hydrogen bond, it attracts water molecules (Figure. 2.2). Positive dipoles in water are attracted to the negatively charged carboxylic acid groups, while negatively charged oxygen in water is attracted to the nitrogen group of the acetamide functional group. Water can fit between all HA subunits, making HA excellent at retaining water (Fallacara et al., 2018).

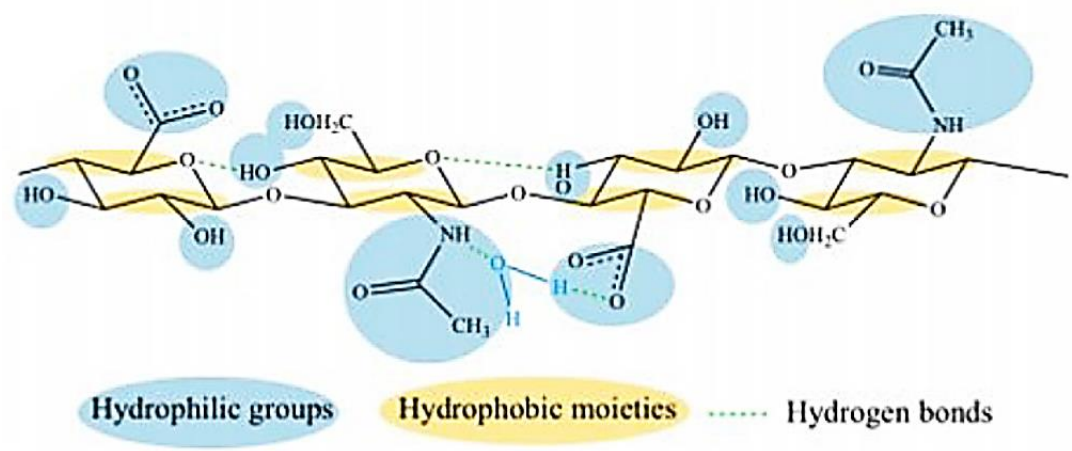


Figure 2.2. Hyaluronic Acid Bounding to Water (adapted from Dovedytis et al., 2020).

HA which is one of the natural biopolymers, like starch, cellulose, and chitosan, is considered the most influential biopolymer due to its unique viscoelasticity, hygroscopicity, non-immunogenicity, and biocompatibility, and is widely used in the medical, cosmetic, food, and healthcare fields (J.Necas et al., 2008; Kogan et al., 2007; Kumari et al., 2006).

2.4 EYEBALLS AND GILLS AS MATERIAL FOR GAG EXTRACTION

2.4.1 FISH GILL

Internal gills of fish consist of a great many vascularized plates and the primary gill lamellae, attached minute and tightly packed secondary gill lamellae perpendicularly and transversely. The structure of the enormous number of secondary lamellae provide a large surface area, occurring gas exchange because of the exceptionally short diffusion distance between water and blood (Bond, 1996; Liem & Walker, 2001) (Figures 2.3 and 2.4).

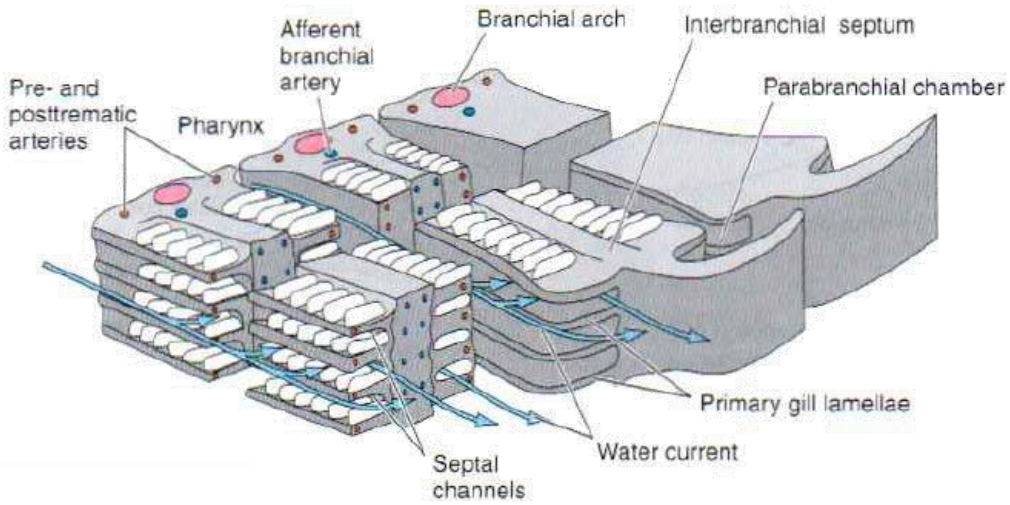


Figure 2.3. Gill of a Dogfish (Liem & Walker, 2001).

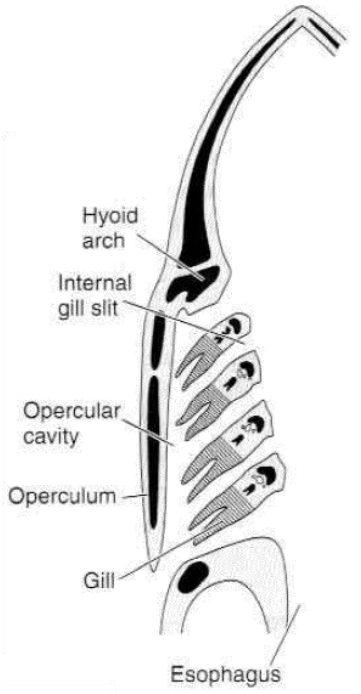


Figure 2.4. Frontal Sections of Aseptal Gills of a Teleost (Liem & Walker, 2001).

As these gill tissues are cartilaginous and CS is known to be widely distributed in the extracellular matrix of cartilage, gills may be efficient for CS extraction (Figure 2.5).

Figure 2.5. Surface and Cross-Section of the Gills of the Bluefin Tuna.

2.4.2 FISH EYEBALL

The structure and function of fisheyes differ slightly from those of land animals. For example, the curvature of the corneal portion is greater than the curvature of the posterior portion of the eye. The Crystalline lens protrudes farther toward the cornea than the iris, making it easier for light to enter the eyeball (Figure 2.6 and Figure 2.8).

Figure 2.6. Lateral and Anterior Surfaces of the Eye of the Bluefin Tuna.

The fish eyeball is composed of three core layers: an outer supportive bundle (Sclera), a middle nourishing vascular bundle (Choroid), and an inner Retina containing photoreceptor cells (Figure 2.7 and Figure 2.8). A ring of cartilage or sclerotic bones develop in the sclera of many vertebrates. A gelatinous vitreous humor with high HA content exists between the iris, lens, and sclera/retina (Bond, 1996; Liem & Walker, 2001; Walls, 1942).

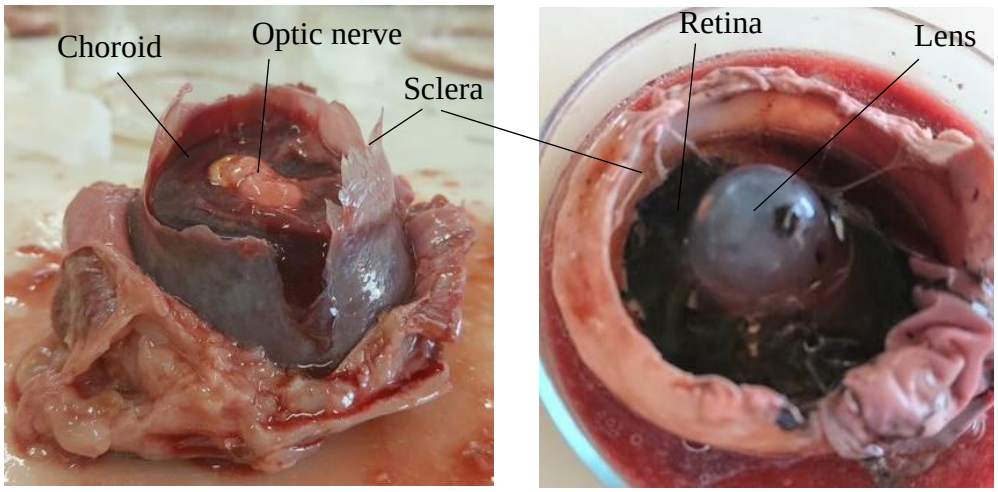


Figure 2.7. Inside the Bluefin Tuna Eyeball.

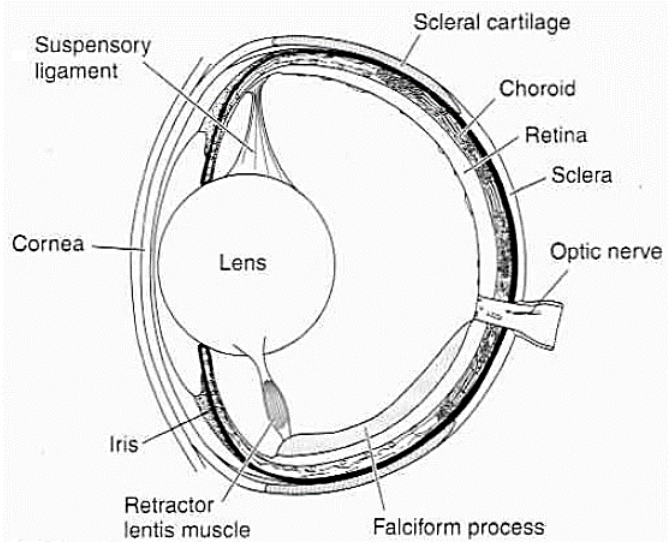


Figure 2.8. Diagram of Vertical Section of Teleost Fish Eye (Liem & Walker, 2001).

The vitreous humor is a highly transparent, viscoelastic semi-solid colloid (Yu et al., 2022) (Figure 2.9). The gel phase of the vitreous is mainly composed of collagen intertwined by HA, and the liquid phase is mainly composed of HA in aqueous solution (da Silva Fernandes et al., 2017). It plays an important role in maintaining the shape of the eyeball and normal intraocular pressure (16 ± 3 mmHg), regulating the partial pressure of oxygen, and maintaining the natural position of the retina (Kokavec et al., 2016). An important part of the ocular tissue, about 2/3 of the total eyeball is 98~99 % water, with the remaining 1~2 % being collagen, GAGs such as HA, heparin sulphate (HS) and CS and salt (Käsdorf et al., 2015). A rabbit study found a total content of 58 ng of vitreous GAGs with 13 % CS and 0.5 % HS (Kamei & Totani, 1982). A recent study of bovine vitreous found a predominance of HA (96.2%), CS 3.5 % and HS 0.3 % (Peng et al., 2018). HA concentration increases from anterior to posterior (Bettelheim & Zigler, 2004).

Figure 2.9. Vitreous Humor of Bluefin Tuna and Its Viscosity.

2.5 EXTRACTION METHODS OF GAGS

In general, GAG extraction from tissues of animal origin involves the processes of defatting with acetone or petroleum (Chascall et al., 1994), scientific hydrolysis and protein removal. Extraction solvents, previously commonly consisting of a mixture of chloroform and water (E.A. Balázs & R.W. Jeanloz, 1979), have been replaced by mixtures of water and organic solvents such as ethanol or acetone or enzymatic hydrolysis (Prescott, 2003), due to concerns about the negative environmental impact of chloroform and other chemicals. Organic solvents enable protein removal by promoting dehydration, reducing the dielectric constant of the medium, increasing electrostatic interactions and causing intra- and intermolecular aggregation of proteins and other substances. Enzymatic hydrolysis using papain, trypsin, pepsin, pronase, alcalase and actinase E has been most used these days. Enzymatic hydrolysis methods provide higher extraction yields than chemical methods, as the enzymes specifically interact with the proteins of the protein-carbohydrate macro-complex, preventing the breakdown of GAGs so that it allows the separation of undamaged GAGs molecules (Giji & Arumugam, 2014; Maccari et al., 2015; Volpi & Maccari, 2003) Among enzymes, papain in particular is the most commonly used to perform enzymatic extraction due to its low cost and high efficiency in digesting cartilage tissue. The extraction yield of GAGs from animal sources depends on the source and extraction conditions, such as organic solvent, enzyme, salt type,

pH and enzyme action time (Abdallah et al., 2020). For example, the decrease in pH can negatively affect the HA concentration (Balazs, 1987). Enzymatic hydrolysis times of 24 hours or more in GAG extraction from cartilage tissue (Maccari et al., 2015; Thomas et al., 2021) are mostly performed, while extraction from the vitreous decreases with longer times and the highest extraction was obtained at 6 hours (Sumogod et al., 2020). Precipitation is used in the GAG extraction process as well as it is effective in removing most proteins and other contaminants. Organic solvents such as ethanol, methanol, acetone, and propanol are most commonly used to remove proteins by promoting dehydration, reducing the dielectric constant of the medium, increasing electrostatic interactions and causing intra- and intermolecular aggregation (McDowall, 1989). Cetylpyridinium chloride (CPC) precipitation is mainly used for the extraction of GAGs from vitreous bodies, as it precipitates polysaccharides from aqueous solutions and thus yields high molecular weight HA in high yield (Mizuno et al., 1991; Amagai et al., 2009) .

In some reports, further various purification methods have been employed after extraction to increase the purity of GAGs, such as dialysis, cation exchange columns (Higashi, Takeuchi, et al., 2015), nultrafiltration-diafiltration (Kim et al., 2012), anion exchange chromatography (S. Chen et al., 2011; Choi et al., 2014; Lignot et al., 2003; Maccari et al., 2015; Souza et al., 2007). Dialysis can remove small molecules such as peptides hydrolysed by collagen in cartilage and salt by dissolving the precipitate in water and dialysing it. Ultrafiltration-diafiltration is a highly applicable method for purification and is a size-based separation for removing impurities and concentrating HA and CS in solution (Choi et al., 2014; Lignot et al., 2003; Opdensteinen et al., 2019). Anion exchange chromatography is used to increase the purity of GAGs by separating proteins (Chen et al., 2011; Maccari et al., 2015; Souza et al., 2007).

3

MATERIAL AND METHODS

3.1 RAW MATERIALS AND CHEMICALS

Bluefin tuna were collected from Tunipex. S.A., Olhão, Faro, Portugal (latitude 37°03′N; longitude -7°83′W) in May 2022. The eyeballs and the gills were dissected out freshly just after collection and stored at -60 ℃. After being transferred to the laboratory in the Departament of Food Engineering, Institute of Engineering, University of Algarve, the tissues were stored at -20 °C until further use. Papain was obtained from Drasanvi (papaína 30 cápsulas vegetais – Nutrabasics Drasanvi). All the solvents and reagents for the analysis were of analytical grade.

3.2 GAG EXTRACTION FROM GILLSS

GAGs extraction from tuna gills was performed as per methodology by Kanchana et al., (2013), with slight modifications (Figure 3.11).

3.2.1 PREPARATION

Fifteen individual gills were weighed in the frozen state and thawed at room temperature overnight. They were washed to get rid of blood, immersing in water and changing the water three times, and placed on a colander for 30 minutes, and placed kitchen paper over the top and bottom of the gills and press gently three times to wipe off the water. The raw gills were weighed before and after washing, defined as the overall raw weight for bluefin tuna size estimation. After washing the gills, the bone between the left and right gills and the hard bony part at the base of each gill were removed and weighed again. This weight was subtracted from

(a) the overall raw weight and defined as (b) raw weight for price value estimation (Figure 3.1).

(a) Weight for Estimating Size of Each Bluefin Tuna

(b) Weight for Estimating the Price Value of Gills per Each Bluefin Tuna

Figure 3.1. Two Types of Definition of Gill Weight per One Individual Bluefin Tuna.

3.2.2 MILLING AND DEGREASING PROCESS

The tissue was taken from a point 1 cm from the outside of the center of each sample and cut into small pieces with scissors and homogenized with a blender (Figure 3.2). 20 g of the tissue was weighed out per each individual gill and defatted with 20 mL of acetone at 4 ℃ for 24 h (Mizuno et al., 1991). Decanting the acetone, the precipitate was dried in an oven for 24 h at 60 ℃.

Figure 3.2. Part Used for Extraction of Glycosaminoglycans (GAGs).

3.2.3 ENZYMATIC DIGESTION FOR GILL

A sample of 5.0 g of the dry defatted gills (in the ratio of 1 g dry tissue to 20 ml) was hydrated in 100 mL of digestion buffer (pH=5,5 sodium acetate 100 mM, containing cysteine 5.0 mM and EDTA disodium 5.0 mM) for 24 h at 4 ℃ (Balbinot-Alfaro et al., 2022; da Rosa et al., 2012). 500 mg of papain (in the ratio of 1 mg of papain to 10 mg of dry tissue) was added. The mixture was incubated for 24 h at 60 °C in a stirrer (Figure 3.3). After boiling the mixture for 10 min, the mixture was centrifuged at 5000×g for 15 min, and the supernatant was recovered.

(a) (b)

Figure 3.3. Bluefin Tuna Gills Before (a) and After (b) Enzymatic Hydrolysis.

3.2.4 ETHANOL PRECIPITATION

Ethanol saturated with sodium acetate was made by adding sodium acetate into ethanol little by little stirring. Three volumes of ethanol saturated with sodium acetate were added to the supernatant and stored at 4 °C for 24 h (Figure 3.4). The precipitate was recovered by centrifugation at 5,000×*g* for 15 min. The precipitate was dried at 60 °C for 12 h (crude GAGs) (Figure 3.5).

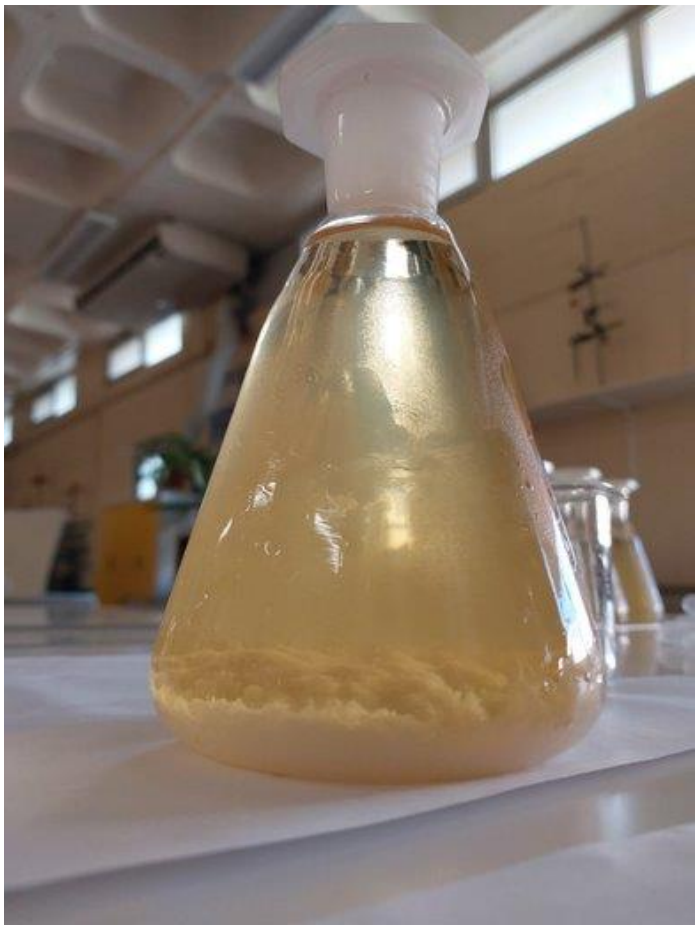


Figure 3.4. Ethanol Precipitation of Bluefin Tuna Gill Glycosaminoglicans (GAGs).

Figure 3.5. Crude Glycosaminoglycans (Crude GAGs) Extracted from Bluefin Tuna Gill.

3.3 GAG EXTRACTION FROM EYEBALLS

The GAGs extraction from eyeballs was performed as per methodology by (Alcântara et al., 2022) with slight modifications (Figure 3.11).

3.3.1 EYEBALL PREPARATION

Fifteen (15) eyeballs were weighed in frozen state and thawed at 4 ℃ over 2 days. The eyeballs were cleaned up from surrounding tissues such as fat and muscle (Figure 3.6). The eyeballs were weighed before and after washing. Vitreous humor was carefully removed through an incision from the posterior part of the eye (Figure 3.7), and weight and measured the volume.

Figure 3.6. Bluefin Tuna Eyeball After Thaw.

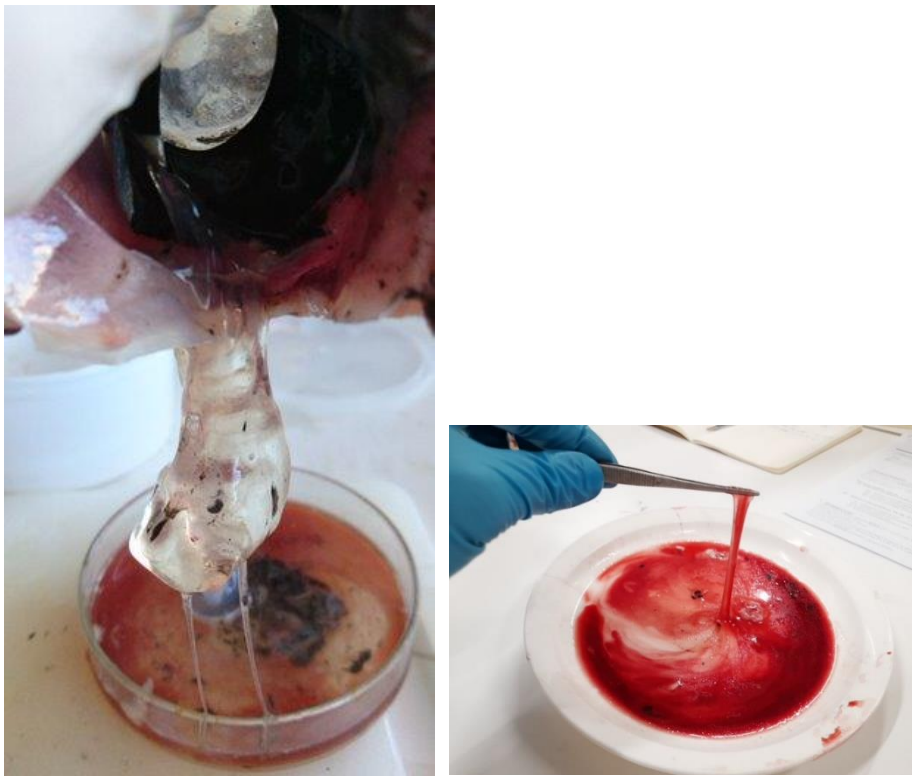


Figure 3.7. Vitreous Humor of Bluefin Tuna.

3.3.2 VITREOUS HUMOR DEGREASING

The samples were submitted to degreasing by immersing in a ratio of 100 g sample in 100 mL acetone (Mizuno et al., 1991) for 24 h at 4 °C and then dried at 60 °C for 2 h.

3.3.3 ENZYMATIC DIGESTION OF THE VITREOUS HUMOR

A sample of 20 g of the gel-like portion from vitreous humor after evaporation of acetone were weighed off and added 100 mL of 100 mM sodium acetate buffer pH 5.5 (containing 5 mM EDTA and 5 mM cysteine) and 500 mg of papain, and the solution was incubated for 6 h at 60 ºC with gently stirring (Figure 3.8). After boiling the mixture for 10 min, the mixture was centrifuged at 5000×g for 15 min, and the supernatant was recovered, and the pellet was discarded.

(a) (b)

Figure 3.8. Bluefin Tuna Vitreous Humor Before (a) and After (b) Enzymatic Hydrolysis.

3.3.4 ETHANOL PRECIPITATION

Three volumes of ethanol saturated with sodium acetate were added to the supernatant and stored at 4 °C for 24 h (Figure 3.9). HA is a very sticky substance and the precipitate stuck to the bottom of the flask, which was carefully scraped off and the mixture transferred between

centrifuges. The precipitate was recovered by centrifugation at 5,000×*g* for 15 min. The precipitate was dried at 60 °C for 12 h (crude GAGs) (Figure 3.10).

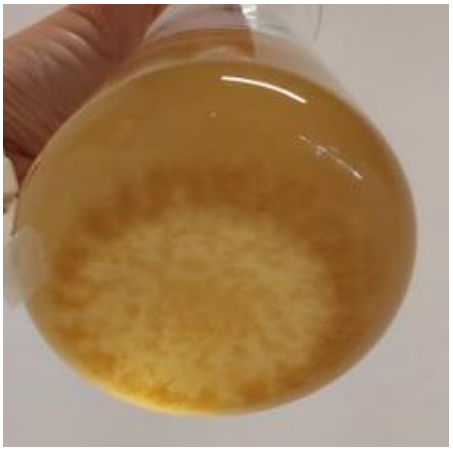


Figure 3.9. Ethanol Precipitation of Bluefin Tuna Vitreous Humor Glycosaminoglycans (GAGs).

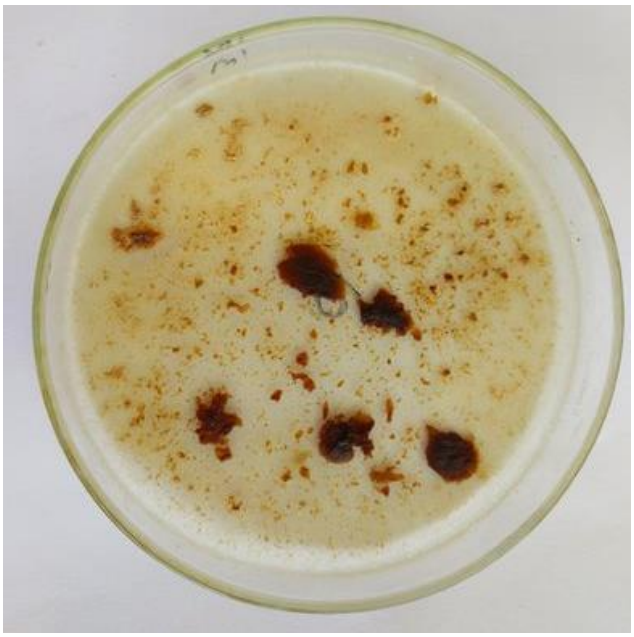


Figure 3.10. Crude GAG Extracted from Vitreous Humor.

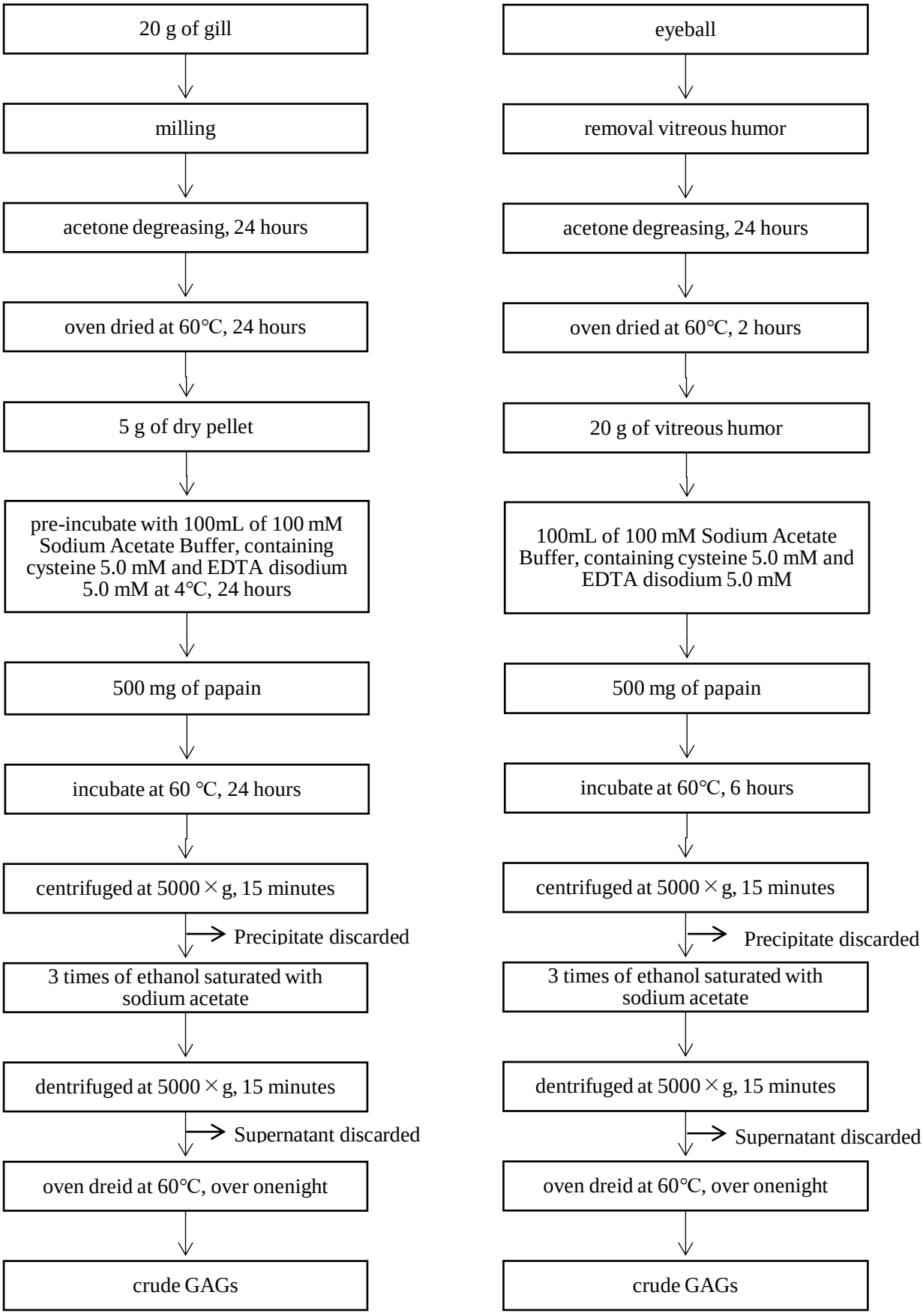


Figure 3.11. Glycosaminoglycans Extraction Procedure from Gills and Eyeballs.

3.4 THE YIELD OF GAGS

The yield was calculated according to the following equation:

1) GAG Yield for Gills

$$\text{GAG Yield (mg per g of dry pellet)} = \text{GAGs} / \text{WDP} * 100$$

where GAGs is the dry weight of crude extracted GAGs (mg) or the amount of carbohydrate (mg) in the crude GAG determined by the Dubois methods (Dubois et al., 1956); WDP: Weight of the dry pellet (g).

2) GAG Yield for Eyeballs

$$\text{GAG Yield (mg per g of vitreous humor)} = \text{GAGs} / \text{WVH} * 100$$

where GAGs is the dry weight of crude extracted GAGs (mg) or the amount of carbohydrate (mg) in crude GAGs determined by the Dubois method (Dubois et al., 1956). WVH: Weight of the vitreous humor after degreasing (g).

3.5 ANALYSIS OF CARBOHYDRATE CONTENT

The amount of carbohydrate in crude GAGs was determined by the Dubois method (Dubois et al., 1956). Triplicates were analyzed per each crude GAG sample. 2 mg of crude GAG from gill or crude GAG from vitreous humor were placed into a heat resistant (borosilicate) tube and 2 ml of distilled water was added. The tubes were placed on an ice tray, 1 ml of 5 % phenol was added rapidly and directly on sample and then 5 mL of concentrated sulfuric acid was added. The samples were left to stand for 10 minutes and shook at the end of that time. The samples were incubated at 25-30 ºC for 10 minutes. The samples were measured their absorbance at 485 nm. Calibration curve were prepared from a sucrose solution with a concentration of 0.5 g/L.

3.6 ESTIMATED THE VALUE OF THE TISSUE

To assess the potential value of a gill and an eyeball as a material for chondroitin sulphate or HA, the price of CS or HA at which they are sold for the dietary supplement products, cosmetics products, or medical, research and pharmaceutical production were searched. Prices of the product were searched in some online shopping platforms in Portugal, using the

keywords "ácido hialurónico" or "sulfato de condroitina". If other shops listed the same product, the price of the first shop searched for was quoted in order of relevance. For products sold on temporary sale at a lower price, the price before the sale was quoted. Products containing a mix of other nutrients such as glucosamine were excluded and only products containing chondroitin sulphate as the main ingredient were picked up. Where the same product was sold in different weight patterns, the price of the heaviest product was used.

3.7 ESTIMATE THE SIZE OF WHOLE BLUEFIN TUNA

Gill tissue was defined as the sum of the left and right gills and the condition including the bony tissue between them as one gill, and its weight was used to estimate the size of the whole tuna population. Eyeballs were defined as one eyeball with the surrounding lipid and muscle removed and were used to estimate the size of the solid. The association between gill and eye size and the content of crude GAGs, the amount of carbohydrate estimated by the Dubois method and GAG purity (the amount of carbohydrate in mg of crude GAG) was then investigated.

$$\text{GAG Purity (mg per mg of crude GAGs)} = \text{carbohydrate} / \text{crude GAGs}$$

where carbohydrate (mg) in crude GAGs determined by the Dubois method (Dubois et al., 1956) and crude GAGs is the dry weight of crude extracted GAGs (mg).

3.8 DATA ANALYSIS

In the experiment with gills, one sample was lost due to an instrument cracking during the experiment and GAG extraction and analysis was performed from n=14 gill samples. GAG extractions and analysis were performed from n=15 eyeball samples.

IBM SPSS software was used to analyse the collected data. All the values were expressed as mean ± SD. Simple linear regression model was used to test if the size of the tissues predicted dry pellet yield, crude GAG yield, GAG (carbohydrate) yield, or carbohydrate concentration among crude GAG. The t-test (for independent samples) was used to compare the carbohydrate concentration in the crude GAG extracted from the eyeball and gills. The level of significance α was set at 0.05.

4 RESULTS AND DISCUSSION

4.1 EXTRACTION GAGS FROM BLUEFIN TUNA GILL

4.1.1 RAW MATERIAL WEIGHT OF THE BLUEFIN TUNA GILL

The average weight of the bluefin tuna gills, including the bony part, were 2366.67±1078.20 g in the frozen state, 2157.00±942.00 g before washing after thawing, and 2102.87±943.16 g after washing and thawing (Table 4.1). The weight of the useful part for GAG extraction without the bone between the left and right gills, i.e. the arched bone that supports the gills and the fat, was 1055.07±511.88 g, 50.17 % of whole sample tissue after thaw and washing (Table 4.1).

Table 4.1. Raw Tissue Weight of Bluefin Tuna Gills (n=14)

	Mean±SD	Min	Max
One whole tissue weight in frozen state (g)	2366.67±1078.20	792	4686
One whole tissue weight after thaw			
before washing (g)	2157.00±942.00	750	4140
after washing (g)	2102.87±943.16	748	4066
One tissue (only useful part for GAG extraction) (g)	1055.07±511.88	289	1958

4.1.2 CRUDE GAG YIELD FROM THE BLUEFIN TUNA GILL

Crude GAGs were extracted from the bluefin tuna gills by degreasing, extensive protease digestion, and ethanol precipitation. The average dry pellet yield by defatting with acetone was 27.26±1.46 % (Mean±SD) of raw tissue weight after thawing and washing. The average crude GAG yield from bluefin tuna gill was 121.31±12.41 mg per g of dry pellet, that was 12.13 % of dry pellet. The yield from other sources extracted by the similar methods with our

study including enzymatic hydrolysis and ethanol precipitation were widely ranged, such as from the cartilage of *Acipenser schrenckii* was 25 % (Wang et al., 2020), and *Buffalo trachea* was 62.05 mg/g, nasal was 60.47 mg/g, joint cartilages 60.76 mg/g (Sundaresan et al., 2018).

4.1.3 CARBOHYDRATE ANALYSIS OF THE GAG EXTRACTED FROM THE BLUEFIN TUNA GILL

The yield of GAGs varies widely depending on the purification method as well as the kind of raw material (Table 4.2). In some previous studies, additional techniques such as ion-exchange chromatography (de Moura et al., 2021; Higashi, Okamoto, et al., 2015; Higashi, Takeuchi, et al., 2015), and ultrafiltration-diafiltration (Blanco et al., 2015; Murado et al., 2010; Vázquez et al., 2016, 2018, 2019) have been used for further purification (Table 4.2). Therefore, the amount of GAGs was further estimated more precisely based on the carbohydrate content of the crude GAGs obtained by the Dubois methods. The average of carbohydrate was 8.71±0.66 mg per g of dry pellet of gill. The GAG yield estimated by analysis of carbohydrate was very similar to the other articles with further purification methods, such as ion-exchange chromatography, and ultrafiltration-diafiltration. The yield for GAGs extracted from gills of yellow fin tuna was 8.5 % (Thomas et al., 2021), tilapia was 8.6 % (de Moura et al., 2021), heads of silver-banded whiting fish was 8.0 % (Ticar et al., 2020). The low percentage of carbohydrate content in the crude GAG indicates that other compounds such as proteins and minerals remain in the crude GAG extract. Therefore, if a high purity GAG is required, a further additional purification step is necessary, such as limited filtration or dialysis.

Table 4.2. Yields of Extracted GAGs from Different Marine Sources using Different Methods.

Material	**Yield of GAG**	**Extraction Method**	**Purification Method**	Reference
Bluefin tuna gill	**0.87% GAG (Dubois method basis)**	**Papain**	**None**	**This study**
Rabbit fish	6 %	Alkalase Papain Actinase E	Ultrafiltration-diafiltration	(Vázquez et al., 2019)
Bone of Salmon, Monkfish, codfish, spiny dogfish, and tuna	(% CS w/w) in bones of: Salmon 0.10 % Monkfish 0.34 % Codfish 0.011 % Dogfish 0.28 % Tuna 0.023	Papain	Dialysis followed by anion exchange chromatography	(Maccari et al., 2015)
Head (Fish processing discard) from ***Labeo rohita*** (L. rohita) and ***Piaractus brachypomus*** (P. brachypomus)	0.05 % GAG	Papain	None	(Gavva et al., 2020)
Heads of silver-banded whiting fish	0.8% GAG	Alkaline process	Dialysis	(Ticar et al., 2020)

Material	**Yield of GAG**	**Extraction Method**	**Purification Method**	Reference
Tilapia	0.86 % GAG	Papain	Ion exchange chromatography	(de Moura et al., 2021)
Sea cucumber	0.98 % Fucosylated	Papain	anion exchange chromatography	(S. Li et al., 2021)
European eel skin	1.22 % sulfated GAG	Alcalase®	None	(Sila et al., 2018)
Spotted dogfish Cartirage	1.5 % weight of CS on dry basis.	Papain	anion exchange chromatography	(Gargiulo et al., 2009)
Octopus	1.66 mg GAG/g of dry tissue	Actinase E	Dialysis, anion exchange chromatography	(Higashi, Okamoto, et al., 2015)
Sea snake Skins and Meat	10.1 % sulfated groups	Papain and Trypsin	Dialysis followed by ion exchange chromatography	(Bai et al., 2018)
Blue shark Fins	12.08 % CS (w/w dry cartilage)	Alcalase	Ultrafiltration-diafiltration	(Vázquez et al., 2016)
Carp Scales	157.37 µg CS/mg	Actinase	Ion exchange chromatography	(Toshihisa et al., 2000)
Sea cucumber	2.87% crude sufated GAG	Diastase	anion exchange chromatography	(Yang et al., 2018)
Cartilage of ***Acipenser schrenckii***	25% CS	Papain & Trypsin	anion-exchange chromatography	(Wang et al., 2020)
Chinese sturgeon Cartilage	26.51% CS	acid protease neutral protease alkaline protease pancreatin pepsin	anion exchange chromatography	(Zhao et al., 2013)
Skate Crtilage	3.37~16.62 % CS	Alcalase	Ethanol purification	(Jeong, 2016)
Blackmouth Catfish Cartilage	3.5-3.7 % CS of wet weight	Alcalase	Ultrafiltration-diafiltration	(Vázquez et al., 2018)
Small-spotted catshark、Head, skeleton and fins	4.8 % CS in head, 3.3 % CS in fins, 1.5 % CS in skeleton	Alcalase	Ultrafiltration-diafiltration	(Blanco et al., 2015)
Thornback skate Cartilage by-products,	41 g/L of extracted CS for Skate Cartilage, 15 % w/w CS extracted for Thornback skate	Alkaline process	Ultrafiltration-diafiltration	(Murado et al., 2010)
Skate Cartilage	47.44 % CS (w/w)	Alcalase	Protein removal by centrifugation	(Song et al., 2017)
Squid Cornea	5% CS (w/w) fuming	Papain	Ion exchange chromatography	(Karamanos et al., 1991)
Heads of red salmon	6 to 7 mg/g GAG		None	(Fuming Zhang et al., 2014)
Ray Cartilage, Shark Fins	7.49 % CS ray cartilage 15.05 % CSshrk fins	Papain	Dialysis	(Garnjanagoonchorn et al., 2007)
Gills of ***Thunnus albacares***	8.5±0.22 mg GAG g-1	Papain	None	(Thomas et al., 2021)
Squid Fins, arms, skin, head, eyes and mantl	CS (mg/g dry tissue) Fin 2.973 Arms 1.555 Skin 3.482 Head 2.475 Eyes 2.29	Actinase	Dialysis	(Tamura et al., 2009)
Different fish species Fins, head and skeleton	**S.canicula fins 3.9 %** **S. canicula head 5.8 %** S. canicula skeleton 1.9 % P. glauca head 12.1 % R. clavata skeleton 13.7 % (CS w/w dry cartilage)	Alcalase	Dialysis followed by ultrafiltration-diafiltration	(Novoa-Carballal et al., 2017)

Material	Yield of GAG	Extraction Method	Purification Method	Reference
Sea cucumbers	P. graeffei 11.0 % CS H. vagabunda 6.3 % CS S. tremulus 7.0 % CS I. badionotus 9.9 % CS.	Papain	Dialysis followed by anion exchange chromatography	(S. Chen et al., 2011)
Shark Fins	Total GAG amount **(mg/g dry weight),** **As a result, 7.7~44.9 mg GAG/g of dry tissue were reovered.** **blue shark 44.9** **shortfin mako shark 7.71** **Birdbreak dogfish 12.2** **Cloudy catshark 11.7** **Small tooth sand tiger 9.85** **Red stingray 43.8** **Frilled shark 16.6** **Silver Chimaera 22.0** **Spotless smooth-hound 39.8** **Kitefin shark 8.46** **Goblin shark 37.3**	Actinase	Dialysis, anion exchange chromatography	(Higashi, Takeuchi, et al., 2015)

4.1.4 POTENTIAL VALUE ANALYSIS OF GILL OF ONE INDIVIDUAL BLUEFIN TUNA

CS sold on the market is mainly extracted from sources such as bovine, porcine, chicken or squid cartilage and shark fins; CS is sold alone or in pharmaceutical-grade products or dietary supplements, often in combination with glucosamine. The market price of six chondroitin sulphate dietary supplements without glucosamine sold in Portugal was 0.43 €/g ± 0.13 €/g of chondroitin sulphate. According to previous studies, the purity of chondroitin sulphate varies greatly depending on the purification method and material. However, the information of the purity of chondroitin sulphate in commercial dietary supplements is not indicated on each product. The price quotations for 8 CS or chondroitin sulphate sodium products sold to medical institutions, pharmaceutical companies and research institutes were 442.47 €/g ± 509.89 €/g of CS or chondroitin sulphate sodium. Prices for CS products vary widely. One of the reasons for this is that CS products are used in a variety of applications, and the required purity and purification methods differ greatly depending on whether they are injectables, oral reagents, or laboratory reagents. The potential value per gill of one bluefin tuna assessed from the price sold to medical institutions, pharmaceutical companies and research institutes for medical use or as a raw material for various CS products is estimated to be 1056.04 € ± 524.34 € per gills of one bluefin tuna on a GAG basis by Dubois method. In a direct extrapolation, it is 1 450 998.96 € worth of CS products could be produced from gills of 1,374 bluefin tunas collected by Tunipex. S.A. in 2022.

The global chondroitin sulphate market size was valued at USD 1.25 billion in 2022 and is anticipated to expand at a compound annual growth rate (CAGR) of 3.5 % from 2023 to 2030 (Grand View Research, 2023). On an industrial scale, CS is extracted mainly from shark, pig, chicken, and bovine cartilage (Vázquez et al., 2018; Volpi, 2019; Volpi et al., 2021). However, in recent years, ethical and sustainability issues have been raised for endangered shark species, and the UK Government has enacted new legislation banning the import and export of shark fins in August 2021 (the UK Government, 2021). Despite this growing demand from the nutraceutical and medical sectors, a stable source of CS that is sustainable and free from risks such as viruses and BSE remains scarce and a challenge (Badri et al., 2018). Sulphation and other modifications of GAGs vary widely between species, aquatic species tend to contain more structural diversity in their GAGs than terrestrial animals, with over-sulphated complex structures identified in many marine sources (Yamada et al., 2011). It has also been reported that highly modified GAGs exhibit characteristic physiological activities such as a protective role against pathogens that do not involve the immune system (Jinno & Park, 2015). Extraction of CS of course requires vast quantities of ethanol and many expensive reagents such as enzymes and acetone but given the price quotations for CS products and the size of the demand, the potential for commercialization is high enough.

4.1.5 Association Between Size of Gill and GAG Yield or Carbohydrate of the GAG.

The relationship between weight of the whole gills, including the inside bone, and the GAG yield was investigated. Examining the relationship between the gill t size and dry pellet yield (%) per g of raw sample, no significant relationship was found between tuna size and dry pellet yield (linear regression, p=0.78) (Figure 4.1).

No significant correlation was found between crude GAG yield and raw sample size of the bluefin tuna gills (linear regression, p=0.78) (Figure 4.2).

However, there was a significant correlation between the estimated GAG weight based on the amount of carbohydrate estimated by the Dubois method and size of individual bluefin tuna estimated by raw gill weight after washing (linear regression, p=0.04) (Figure 4.3). It was suggested that the amount GAGs may increase as the size of the whole bluefin tuna increases. Moreover, the amount of carbohydrate in 1 mg GAG was significantly correlated with raw sample weights (linear regression, p=0.03) (Figure 4.4). This meant that as the size of the tuna increased, it was suggested that a higher purity of GAG could be obtained. One of the reasons

though there was no correlation with the total amount of crude GAG, but a significant correlation with carbohydrate content or concentration could be due to the presence of many undigested impurities such as collagen and minerals in the crude GAG. It should be studied again using further purification methods such as ion-exchange chromatography.

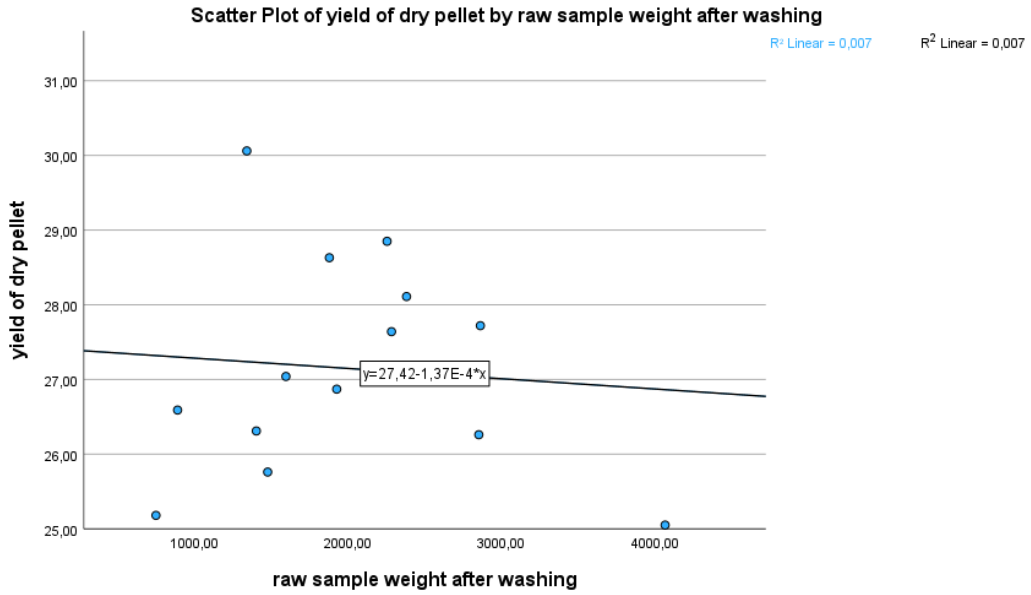


Figure 4.1. Raw Sample Weight and Dry Pellet Yield

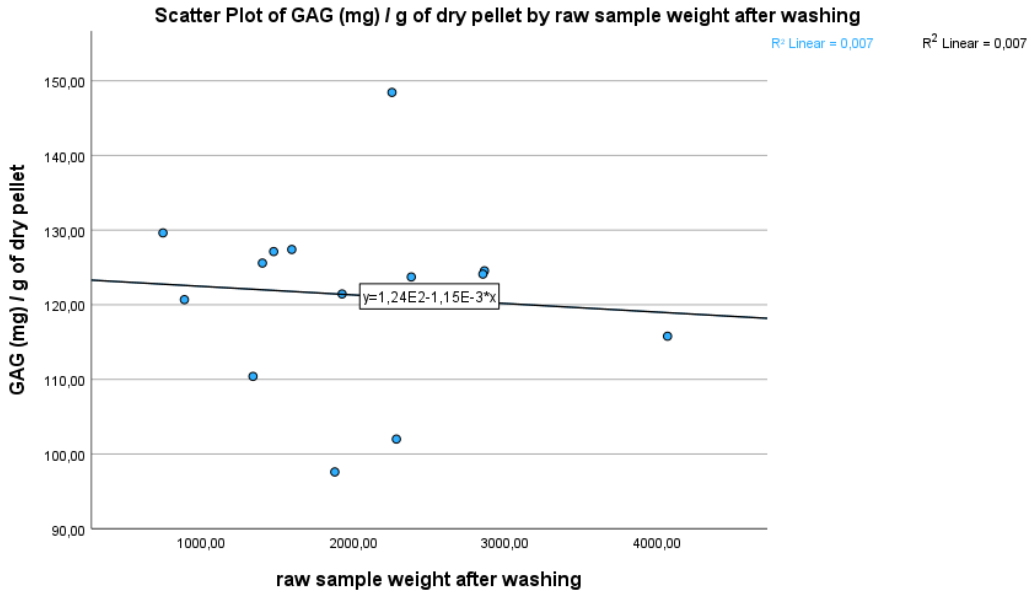


Figure 4.2. Crude GAG Yield and Raw Sample Weight

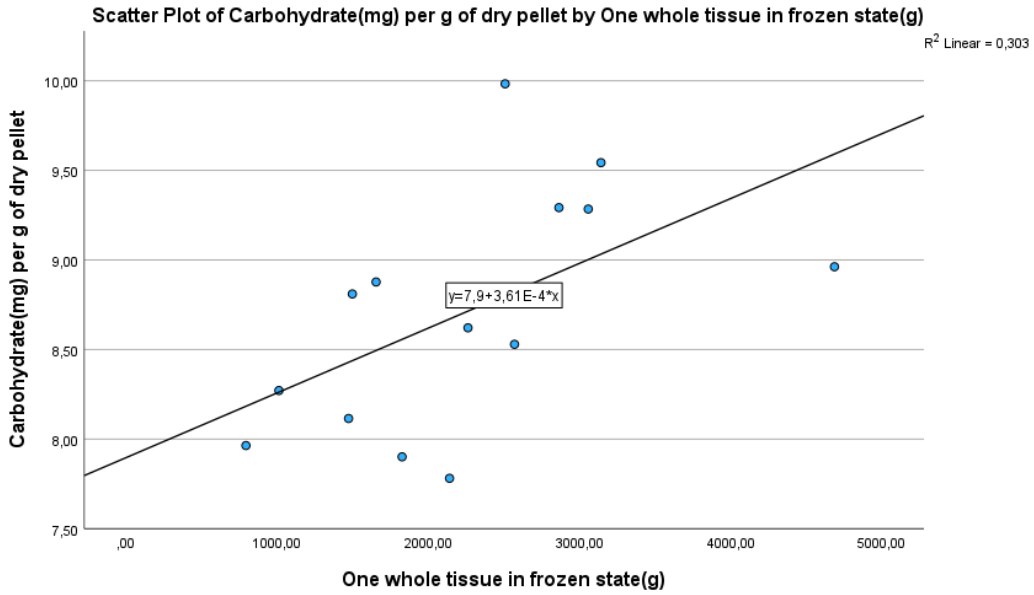


Figure 4.3. GAG Yield Estimated from Carbohydrate Analysis and Raw Sample Weight.

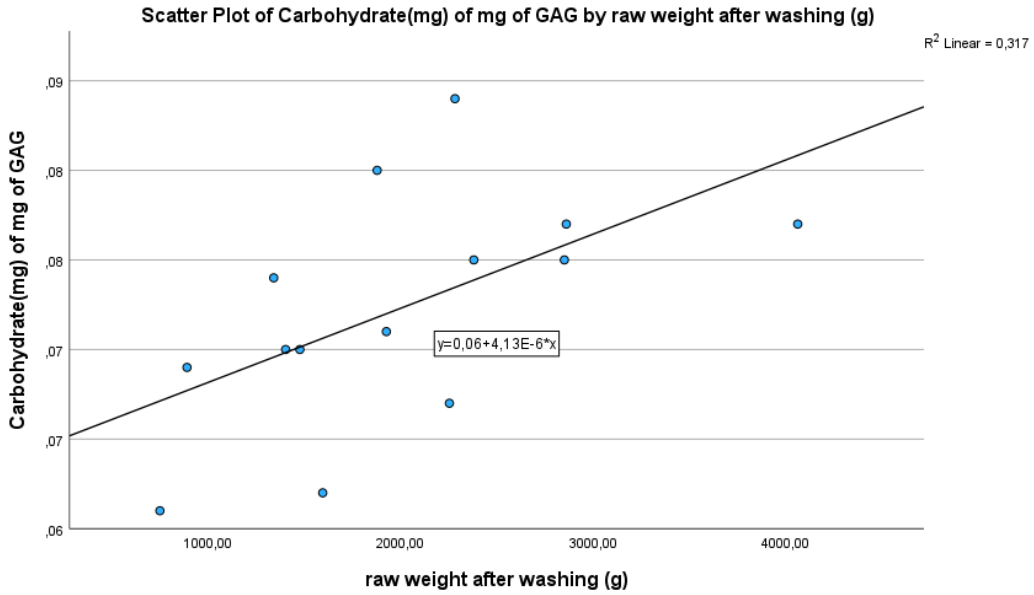


Figure 4.4. GAG Purity Estimated from Carbohydrate Analysis and Raw Sample Weight.

As far as I know, there are no reports on CS content and size or age in marine organisms, though it has been reported that CS levels in bovine intervertebral disc model tails decline with aging (Collin et al., 2017). In addition, the sulfation pattern of CS in horse articular cartilage and synovial fluid varies with age (Brown et al., 1998) and the sulfation pattern of chondroitin

sulfate disaccharides from human normal articular cartilage varies with the age of the specimen (Bayliss et al., 1999). As the function of CS varies according to its sulphate structure, further studies, including sulphate structure analysis of CS, are needed to determine whether CS function and purity differ according to age.

4.2 EXTRACTION GAGS FROM BLUEFIN TUNA EYEBALLS

4.2.1 YIELD OF THE CRUDE GAG FROM THE BLUEFIN TUNA EYEBALLS

The average weight of bluefin tuna eyeballs was 377.67 ± 139.24 g in frozen state, 250.77 ± 54.62 g before thawing and washing, and 161.01 ± 32.65 g after thawing and removal of surrounding tissue, and the weight of vitreous was 64.43 ± 11.60 g (Table 4.3).

Table 4.3. Sample weight of bluefin tuna eyeballs (n=15).

	Mean±SD	Min	Max
One whole tissue in frozen state (g)	377.67±139.24	199	628
One whole tissue weight after thaw			
Whole eyeball (g)	250.77±54.62	181	369
Whole eyeball removed surrounding tissues(g)	161.01±32.65	114	224
Vitreous humor (g)	64.43±11.60	44	90

4.2.2 CRUDE GAG YIELD FROM THE BLUEFIN TUNA EYEBALLS

Crude GAG was extracted from the vitreous humor from bluefin tuna eyes by degreasing, extensive protease digestion and ethanol precipitation. The mean crude GAG yield from the vitreous of bluefin tuna was 25.19 ± 12.29 mg crude GAG/g (Mean±SD). The yield of HA from different sources vary widely, as shown in Table 4.4. The results of the present study were higher than the other sources, such as the vitreous humor of bovine (469.9 μg HA/g) or owl (291.8 μg HA/g), yellow fin tuna eyeballs HA yielded (4.78 mg uronic acid per kg raw eyeball), and HA from the eyeballs of bigeye tuna (0.42 g HA/dm^3 of vitreous humor). However, the purification method may influence the yield, so future comparisons are needed after further purification methods such as filtration-diafiltration or dialysis.

4.2.3 CARBOHYDRATE ANALYSIS OF THE GAG EXTRACTED FROM THE BLUEFIN TUNA EYEBALLS

To estimate the crude GAG more accurately, the carbohydrate content obtained by the Dubois method was measured and the mean carbohydrate content was 1.79 ± 0.72 mg per g of vitreous humor. Defining the carbohydrate content of mg of crude GAG by the Dubois method as the amount of GAG, it was found to be more than many other materials such as eyeballs of bigeye tuna (0.42 g HA/dm^3 of vitreous humor), though less than yellowfin tuna eyeballs (4.78 mg uronic acid per kg raw eyeball) as shown Table 4.4. However, the low percentage of carbohydrate content in the crude GAG indicates that other compounds such as proteins and minerals remain in the crude GAG extract. Therefore, if a high purity GAG is required, a further additional purification step is necessary, such as limited filtration or dialysis.

Table 4.4. Extraction of GAG from Eyeball of Different Sources.

Material	**Yield**	**Extraction method**	**Purification method**	**Reference**
Bluefin tuna eyeballs	1.79 mg GAGs (Dubois method basis)	Papain	None	This study
Eyeballs of Nile Tilapia	0.054 mg HA/g	Papain	Dialysis	(Alcântara et al., 2022)
Yellow fin tuna Eyeballs	4.78 mg uronic acid/kg raw eyeball	Papain	Ultrafiltration-diafiltration	(Sumogod et al., 2020)
Swordfish eyeballs, shark eyeballs,	Swordfish vitreous humor 0.055 g HA/L (g per liter or kg) Shark vitreous humor 0.283 g HA/L (g per liter or kg) Veal vitreous humor 0.258 g HA/L (g per liter or kg)	Alkaline process	Ultrafiltration-diafiltration and protein electrodeposition	(Murado et al., 2012)
Eyeball of bigeye tuna	0.42 g HA/dm3 of vitreous humor	Mycolysin	Dialysis	(Amagai et al., 2009)
Vitreous humor of Bovine, Owl Monkey	469.9 μg HA/g bovine vitreous humor 291.8 μg HA/g Owl monkey vitreous humor	Use of organic sodium salt	Dialysis	(Gherezghiher et al., 1987)
Owl monkey eyes	0.394 mg HA/g	Pronase	Chloroform treatment	(E.A. Balázs & R.W. Jeanloz, 1979)

4.2.4 POTENTIAL VALUE ANALYSIS OF EYEBALLS OF ONE INDIVIDUAL BLUEFIN TUNA

Examining the prices of HA products sold through some online market in Portugal, we found that 2.96 €/kg ± 2.32 €/kg for HA dietary supplements (mean ± SD), and 184.57 €/mL ± 191.97 €/mL for 10 HA cosmetics liquid ample (mean ± SD). According to previous studies, the purity of HA varies greatly depending on the purification method and material. However, for HA dietary supplements, the purity is unknown as there is no indication of purity in any of the products. Therefore, the prices of HA sold for medical, research and pharmaceutical production field were investigated, so that the price quotations for 28 HA or hyaluronic acid sodium salt were 18737.37 € ± 41042.78 € per g of HA or Hyaluronic acid sodium salt. Most HA used as raw material for commercial dietary supplements and cosmetics is made from sodium hyaluronate, which is easily soluble in water. The potential value bluefin tuna eyeballs assessed from the price sold to medical, research institutions, pharmaceutical companies are estimated to be 4252.24 € ± 2118.04 € per one bluefin tuna (two eyeballs) on a GAG basis estimated by the Dubois method. It calculated that 5 842 577.76 € worth of HA products could be produced from eyeballs of 1374 bluefin tunas collected in Tunipex. S.A. in 2022.

Market prices for HA products were highly diverse. The commercial price of HA obtained from animal sources such as vitreous humor is much higher than that obtained by fermentation. HA is mainly microbiologically produced by fermentation using micro-organisms such as *Streptococcus* due to its low cost (Chong et al., 2005). However, it is difficult to obtain HA with the same molecular weight properties as HA of animal origin and that these products can commonly only be used in the superficial wound care and cosmetic industries due to concerns that has possibility to contain impurities such as nucleic acids, proteins, and microbial toxins (W. Y. Chen et al., 2009; J. Li et al., 2020). Due to very high purity requirements for HA in medical applications, where medical treatments require HA injections, HA extracted from terrestrial or marine animals is used (Nicholls et al., 2022; Shikina et al., 2022). Therefore, animal sources have remained the main alternative for supplying the growing HA industry.

The global HA market is valued at USD 9.6 billion in 2020 and is projected to reach USD 16.6 billion by 2027, at a compound annual growth rate (CAGR) of 8.1 % over this period (Grand View Research, 2020). It was confirmed that HA is marketed at very high prices, and that bluefin tuna eyeballs could be traded at high prices as a raw material for them. GAGs extracted from marine discarded by-products would serve safer alternatives for people with religious problem or concern about risks associated with the transmission of zoonotic diseases such as bovine spongiform encephalopathy (BSE), Creutzfeldt-Jakob disease and foot and mouth disease, GAGs from terrestrial animal sources. Extraction of HA of course requires huge

quantities of ethanol and many other expensive reagents such as enzymes and equipment costs but given the price quotations for HA products and the size of the demand, the potential for commercialisation is high enough. Therefore, animal sources have remained the main alternative for supplying the growing HA industry. Bluefin tuna eyeballs can be one of the promising options for extracting HA.

4.2.5 Relationship between Size of Bluefin Tuna Eyeball and GAG Yield or Carbohydrate of the GAG.

The relationship between eyeballs of bluefin tuna and the GAG yield was investigated. No significant correlation was found between crude GAG yield and the raw eyeballs weight (linear regression, p=0.10) (Figure 4.5).

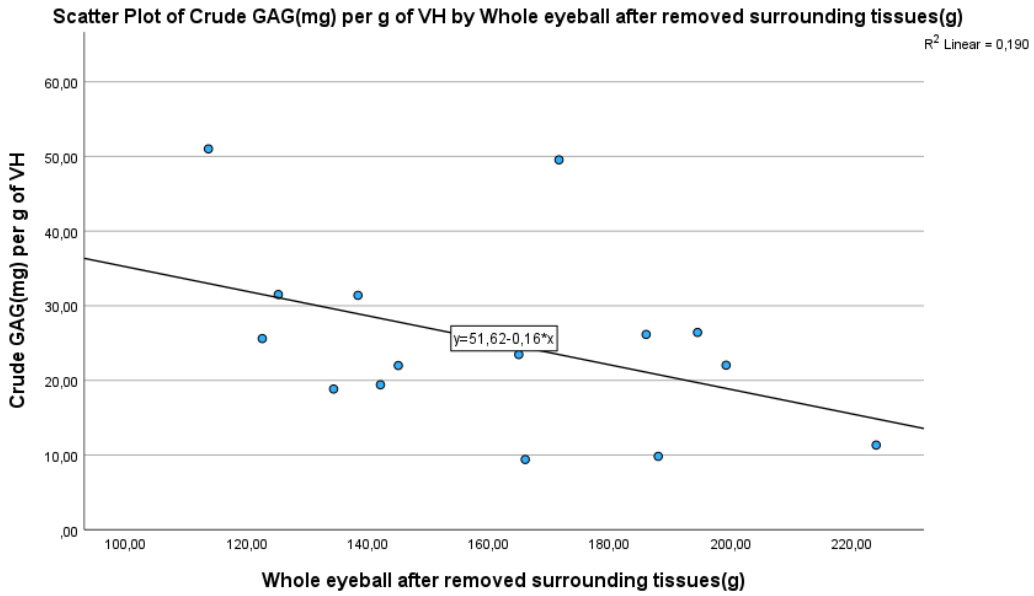


Figure 4.5. Crude GAG Yield and Raw Sample Weight

There was no significant correlation between the estimated GAG weight based on the amount of carbohydrate analysis by the Dubois method and raw eyeball weight (linear regression, p=0.20) (Figure 4.6).

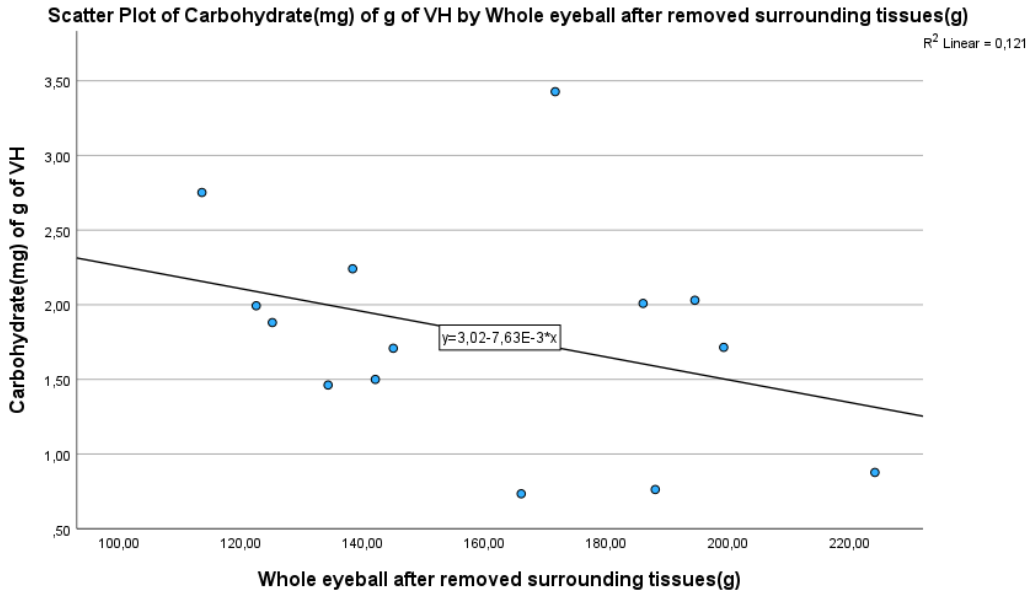


Figure 4.6. GAG Yield Estimated from Carbohydrate Analysis and Raw Sample Weight.

And there was a trend towards more the amount of carbohydrate in 1 mg GAG the heavier the raw sample weight, however, this trend was not statistically significant (linear regression, p=0.048) (Figure 4.7).

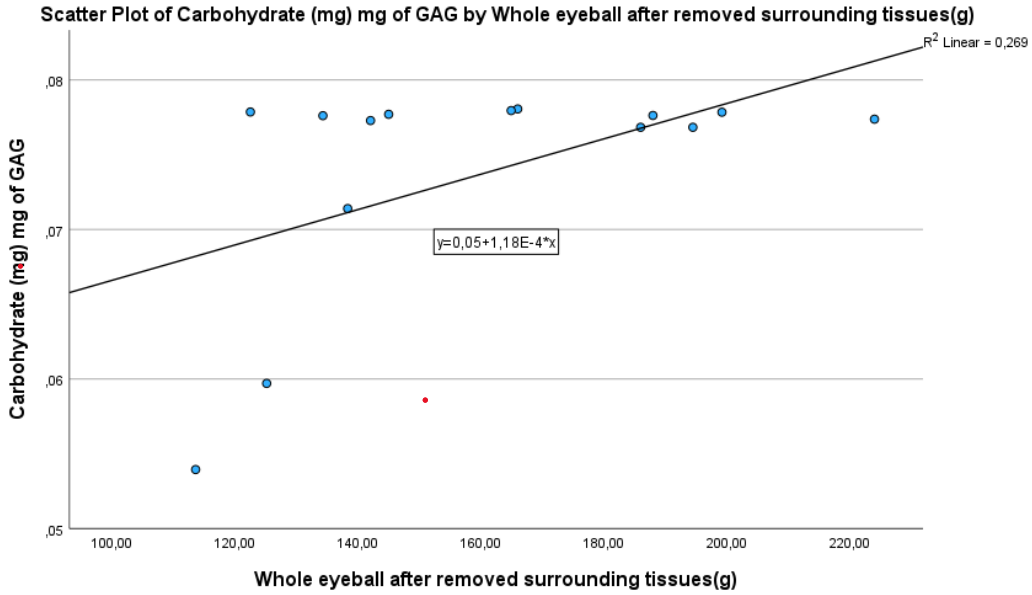
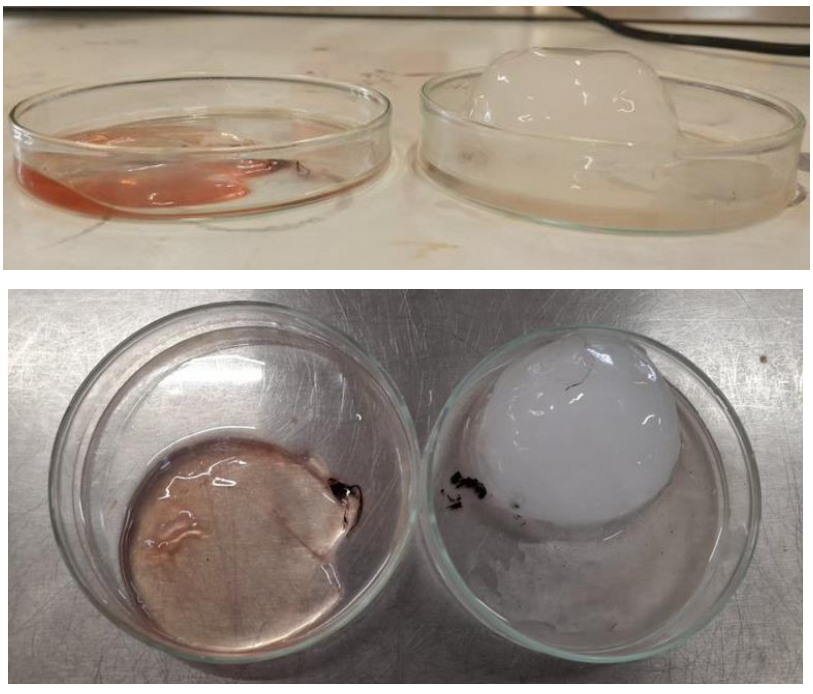


Figure 4.7. GAG Purity Estimated from Carbohydrate Analysis and Raw Sample Weight.

However, HA is a highly hygroscopic substance, absorbing huge amounts of water, up to around 1,000 times of its own volume (Mero & Campisi, 2014). When it is brought to room temperature from oven drying, it absorbs atmospheric humidity and continue to increase rapidly in weight. In our study, 20 minutes time delay was set between each sample processing so that the time between removal from the dryer and weighing was constant, but it is still possible that the weighing was not homogeneous and correct. In addition, very large molecular weight and very high viscoelasticity of HA (Jabbari et al., 2023) may have made it difficult to accurately measure the amount of carbohydrate.

Some of the eyeballs examined in this study had holes or cracks. This may have caused the vitreous to leak out of the eyeball or blood or drips to enter the retina, resulting in an inaccurate measurement of the weight of each eyeball. However, when vitreous humor was removed from the eyeball using the method reported by Amagai et al. (2009) using a frozen eyeball, no blood contamination was observed and a larger amount of colorless and transparent vitreous humor could be obtained, with improvements in both quality and quantity (Figure 4.8). It was found desirable to extract HA from the vitreous extracted in this frozen state for further research in the future. In addition, acetone was used to degrease the eyeballs in this study, but further improvements are needed in the timing and method of removing the lipid-containing acetone.



A B

Figure 4.8. Vitreous Humor Removed from Eyeballs After Thaw (A) and Removed from a Frozen Eyeball (B).

As far as I know no reports in marine sources have been found, a human vitreous study found that older samples were stiffer than the vitreous of younger individuals, with a positive linear correlation with increasing age, and vitreous dehydration caused by a decrease in HA concentration with aging has been proposed as a cause of solid phase hardening of the vitreous gel (Itakura et al., 2009). It has been reported that the decreased synthesis and increased degradation of HA as the human body ages may be responsible for symptoms of aging such as dry eye syndrome (Zhu et al., 2021). It was reported that in rhesus monkeys (*Macaca mulatta*) the concentration and molecular size of sodium hyaluronate did not show significant age-related changes, but the amount of liquid vitreous that could be collected increased with age (Denlinger et al., 1980). The reason for this discrepancy is currently unknown and needs to be re-compared with further purified GAG amounts in future studies.

In this study, no significant association was found between HA content or concentration and size of bluefin tuna. This could be an advantage for fishing companies as it means that bluefin tuna can be used for marketing as a source of HA for the functional food, cosmetic and pharmaceutical industries, without being sorted by size or age.

4.3 Difference Between GAGs Extracted From Gill and From Vitreous Humor

The yield of GAGs per g of raw material was significantly higher in gills than vitreous humor (Table 4.5). This is perhaps due to differences in water content in the raw samples. No difference was found in the amount of carbohydrate per mg of crude GAG obtained by extraction (Table 4.5).

Table 4.5. The Difference between GAGs from Gills and GAGs from vitreous humor

	Tissue		Mean	SD	*t*	*p*
Crude GAG (mg) per g of raw tissue	gill	n=14	32.90	3.62	*2.325*	*p*=0.033
	vitreous humor	n=15	25.19	12.29		
Carbohydrate (mg) per g of raw tissue	gill	n=14	2.36	0.23	*2.932*	*p*=0.008
	vitreous humor	n=15	1.79	0.72		
Carbohydrate (mg) per mg of crude GAG	gill	n=14	0.07	0.01	*0.274*	*p*=0.548
	vitreous humor	n=15	0.07	0.01		

In the gill experiments, during the ethanol precipitation process, spreading white precipitate occurred immediately after the addition of sodium acetate saturated ethanol. However, in the

vitreous humor experiment, no such obvious changes were observed during ethanol precipitation, but the precipitate was found to stick to the bottom of the flask.

The color and shape of the crude GAG after drying was milky white for the GAG extracted from the gills, while the GAG extracted from the vitreous was a darker brown colour (Figure 4.9) and was a very viscous object, with a tendency to stick easily to metal or glass tools. It also looked moist with a high moisture content even after 12 hours of drying and remained moist with a high moisture content even after drying at 60°C for a further three days. GAGs extracted from vitreous humor was also less soluble in water than the GAG extracted from the gills. Each GAGs extracted from two types of tissue, gill, and vitreous humor, clearly showed different properties. Though it has been reported that GAGs extracted from vitreous humor are rich in HA (Table 4.4) and GAGs extracted from fish gills are rich in CS (Thomas et al., 2021), analysis of the composition of GAGs from these tissues is required for commercialisation in the future.

(a) (b)

Figure 4.9. GAGs Extracted from Gills (a) and GAGs Extracted from Vitreous Humor (b)

5 CONCLUSIONS AND FUTURE WORK

The results showed that an average 2.3867 g and 0.2269 g of GAGs could be extracted from the gills and eyeballs of one bluefin tuna, respectively. The raw materials and extraction purification protocols have a significant impact on the yield and purity of GAGs. Comparison with results from other studies of similar extraction methods confirmed that bluefin tuna contains as much or more GAG than other organisms. Furthermore, this study calculated the potential value of gills and eyeballs of bluefin tuna as discarded by-products and showed that bluefin tuna eyeballs could be sold to the medical, cosmetic and food industries at very high prices. CS was sold in Portugal at very high prices, average 442 €/g and HA at average 18,737 €/g, to medical institutions, research institutes and pharmaceutical companies in Portugal. Based on these prices, it can be estimated that gill per one bluefin tuna could be the main raw material for a CS product with a value of 1056 € and eyeball per one bluefin tuna (2 eyeballs) could be the main raw material for an HA product with a value of 4252 €. Over the past decade, an ever-increasing number of applications for GAGs have been reported (Abdallah et al., 2020) and its commercial demand has spread to a variety of markets, including cosmetics, medicine, biotechnology, food, and textiles. In addition, demand for marine-derived CS and HA is expected to increase further in the future due to the ageing of the population and increasing health consciousness, as well as growing awareness of the importance of traceability due to issues such as BSE. To our knowledge, no studies had been conducted to date on the extraction of GAGs, CS or HA from bluefin tuna gills or eyeballs, and giving the high price and demand for GAGs, the study of GAG extraction from bluefin tuna eyeballs is a highly valuable research topic. The bluefin tuna is the top marine predator and the largest species of tuna. Due to its size and scalability, it is possible to produce large quantities of GAGs in proportion to the amount and size of discarded by-products. It also has the advantage in production that tissues such as vitreous and gills can be easily extracted, compared to extraction from small raw materials.

Nevertheless, there are many challenges. In addition to technical challenges such as the removal of odours specific to seafood, there are seasonal or daily fluctuations in the catch and composition of the raw fish, as well as difficulties arising from the raw material, such as maintaining freshness. In the extraction of GAGs from the eyeballs, there were no differences according to the estimated size of the bluefin tuna, but in the extraction of GAGs from the gills, as the estimated size calculated from the size of the bluefin tuna gills increased, the GAG yield increased, and the concentration of GAGs estimated by the Dubois method in 1 mg of crude GAG tended to increase. To realise the valorisation of extracted GAGs in the future, it is necessary to improve the method of degreasing and add further purification, etc., and then re-evaluate the results. As each GAGs extracted from two types of tissue, gill, and vitreous humor, clearly showed different properties, further structural elucidation is also warranted.

The results of this study showed that bluefin tuna eyeballs and gills contain many GAGs and are of high value as sustainable, environmentally friendly, and inexpensive GAG material. Discarded parts of bluefin tuna represent a new source for the extraction of GAGs such as HA and CS, with potential applications in the medical and pharmaceutical, food and cosmetic sectors. Harnessing them for commercial production of GAGs and using them for a variety of applications adds value to the fishery and helps reduce waste to some extent. This study could serve as a basis for valorising the commercial utilization of bluefin tuna discarded by-products. However, further studies on the purification process and the bioactive potential of isolated GAGs are needed to establish their commercial potential.

6 REFERENCES

Abdallah, M. M., Fernández, N., Matias, A. A., & Bronze, M. do R. (2020). Hyaluronic acid and Chondroitin sulfate from marine and terrestrial sources: Extraction and purification methods. *Carbohydrate Polymers*, *243*, 116441. https://doi.org/10.1016/J.CARBPOL.2020.116441

Alcântara, L. O., de Sousa, J. R., Andrade, F. K., Teixeira, E. H., Cerqueira, M. Â., Silva, A. L. C., Filho, M. d S. M. S., & Souza, B. W. S. (2022). Extraction and characterization of hyaluronic acid from the eyeball of Nile Tilapia (Oreochromis niloticus). *International Journal of Biological Macromolecules*. https://doi.org/10.1016/J.IJBIOMAC.2022.12.016

Amagai, I., Tashiro, Y., & Ogawa, H. (2009). Improvement of the extraction procedure for hyaluronan from fish eyeball and the molecular characterization. *Fisheries Science*, *75*(3), 805–810. https://doi.org/10.1007/S12562-009-0092-2/FIGURES/7

Badri, A., Williams, A., Linhardt, R. J., & Koffas, M. A. (2018). The road to animal-free glycosaminoglycan production: current efforts and bottlenecks. *Current Opinion in Biotechnology*, *53*, 85–92. https://doi.org/10.1016/J.COPBIO.2017.12.018

Bai, M., Han, W., Zhao, X., Wang, Q., Gao, Y., & Deng, S. (2018). Glycosaminoglycans from a Sea Snake (Lapemis curtus): Extraction, Structural Characterization and Antioxidant Activity. *Marine Drugs*, *16*(5). https://doi.org/10.3390/MD16050170

Balazs, E. A. , L. F. , L. A. , L. A. (1987). Chemically modified hyaluronic acid preparation and method of recovery thereof from animal tissue. *United States Patent 4713448*. chrome-extension://dnkjinhmoohpidjdgehjbglmgbngnknl/pdf.js/web/viewer.html?file=https%3A%2F%2Fpatentimages.storage.googleapis.com%2F30%2Fa3%2Fa1%2F3eb31339128af8%2FUS4713448.pdf

Balbinot-Alfaro, E., Novello, C. R., Düsman, E., Alfaro, A. T., Barddal, H. P. O., Almeida, I. v., Vicentini, V. E. P., & Martins, V. G. (2022). Bioactive properties of

glycosaminoglycans extracted from Turkey (Meleagris gallopavo) by-products. *Food Bioscience*, *46*, 101545. https://doi.org/10.1016/J.FBIO.2022.101545

Bayliss, M. T., Osborne, D., Woodhouse, S., & Davidson, C. (1999). Sulfation of Chondroitin Sulfate in Human Articular Cartilage: THE EFFECT OF AGE, TOPOGRAPHICAL POSITION, AND ZONE OF CARTILAGE ON TISSUE COMPOSITION. *Journal of Biological Chemistry*, *274*(22), 15892–15900. https://doi.org/10.1074/JBC.274.22.15892

ben Mansour, M., Balti, R., Ollivier, V., ben Jannet, H., Chaubet, F., & Maaroufi, R. M. (2017). Characterization and anticoagulant activity of a fucosylated chondroitin sulfate with unusually procoagulant effect from sea cucumber. *Carbohydrate Polymers*, *174*, 760–771. https://doi.org/10.1016/J.CARBPOL.2017.06.128

Bettelheim, F. A., & Zigler, J. S. (2004). Regional mapping of molecular components of human liquid vitreous by dynamic light scattering. *Experimental Eye Research*, *79*(5), 713–718. https://doi.org/10.1016/J.EXER.2004.07.011

Blanco, M., Fraguas, J., Sotelo, C. G., Pérez-Martín, R. I., & Vázquez, J. A. (2015). Production of chondroitin sulphate from head, skeleton and fins of Scyliorhinus canicula by-products by combination of enzymatic, chemical precipitation and ultrafiltration methodologies. *Marine Drugs*, *13*(6), 3287–3308. https://doi.org/10.3390/MD13063287

Bond, C. E. (1996). *Biology of fishes*. 750.

Brown, M. P., West, L. A., Merritt, K. A., & Plaas, A. H. K. (1998). Changes in sulfation patterns of chondroitin sulfate in equine articular cartilage and synovial fluid in response to aging and osteoarthritis. *American Journal of Veterinary Research*, *59*(6), 786–791.

Campo, G. M., Avenoso, A., Campo, S., Ferlazzo, A. M., & Calatroni, A. (2006). Antioxidant Activity of Chondroitin Sulfate. *Advances in Pharmacology*, *53*, 417–431. https://doi.org/10.1016/S1054-3589(05)53020-5

Chascall, V., Calabro, A., Midura, R. J., & Yanagishita, M. (1994). [24] Isolation and characterization of proteoglycans. *Methods in Enzymology*, *230*(C), 390–417. https://doi.org/10.1016/0076-6879(94)30026-7

Chen, S., Xue, C., Yin, L., Tang, Q., Yu, G., & Chai, W. (2011). Comparison of structures and anticoagulant activities of fucosylated chondroitin sulfates from different sea cucumbers. *Carbohydrate Polymers*, *83*(2), 688–696. https://doi.org/10.1016/J.CARBPOL.2010.08.040

Chen, W. Y., Marcellin, E., Hung, J., & Nielsen, L. K. (2009). Hyaluronan Molecular Weight Is Controlled by UDP-N-acetylglucosamine Concentration in Streptococcus

zooepidemicus. *Journal of Biological Chemistry*, *284*(27), 18007–18014. https://doi.org/10.1074/JBC.M109.011999

Choi, S., Choi, W., Kim, S., Lee, S. Y., Noh, I., & Kim, C. W. (2014). Purification and biocompatibility of fermented hyaluronic acid for its applications to biomaterials. *Biomaterials Research*, *18*(1), 1–10. https://doi.org/10.1186/2055-7124-18-6/FIGURES/5

Chong, B. F., Blank, L. M., Mclaughlin, R., & Nielsen, L. K. (2005). Microbial hyaluronic acid production. *Applied Microbiology and Biotechnology*, *66*(4), 341–351. https://doi.org/10.1007/S00253-004-1774-4/FIGURES/6

Collette, B. B., Reeb, C., & Block, B. A. (2001). Systematics of the tunas and mackerels (Scombridae). *Fish Physiology*, *19*(C), 1–33. https://doi.org/10.1016/S1546-5098(01)19002-3

Collin, E. C., Carroll, O., Kilcoyne, M., Peroglio, M., See, E., Hendig, D., Alini, M., Grad, S., & Pandit, A. (2017). Ageing affects chondroitin sulfates and their synthetic enzymes in the intervertebral disc. *Signal Transduction and Targeted Therapy*, *2*. https://doi.org/10.1038/sigtrans.2017.49

Coulter, A., Cashion, T., Cisneros-Montemayor, A. M., Popov, S., Tsui, G., Le Manach, F., Schiller, L., Palomares, M. L. D., Zeller, D., & Pauly, D. (2020). Using harmonized historical catch data to infer the expansion of global tuna fisheries. *Fisheries Research*, *221*. https://doi.org/10.1016/J.FISHRES.2019.105379

da Rosa, C. S., Tovar, A. F., Mourão, P., Pereira, R., Barreto, P., & Beirão, L. H. (2012). Purification and characterization of hyaluronic acid from chicken combs. *Ciência Rural*, *42*(9), 1682–1687. https://doi.org/10.1590/S0103-84782012005000056

da Silva Fernandes, M., Sanches Lima, F., Rodrigues, D., Handa, C., Guelfi, M., Garcia, S., & Ida, E. I. (2017). Evaluation of the isoflavone and total phenolic contents of kefir-fermented soymilk storage and after the in vitro digestive system simulation. *Food Chemistry*, *229*, 373–380. https://doi.org/10.1016/J.FOODCHEM.2017.02.095

de Moura, H. C., Novello, C. R., Balbinot-Alfaro, E., Düsman, E., Barddal, H. P. O., Almeida, I. V., Vicentini, V. E. P., Prentice-Hernández, C., & Alfaro, A. T. (2021). Obtaining glycosaminoglycans from tilapia (oreochromis niloticus) scales and evaluation of its anticoagulant and cytotoxic activities: Glycosaminoglycans from tilapia scales: anticoagulant and cytotoxic activities. *Food Research International*, *140*, 110012. https://doi.org/10.1016/J.FOODRES.2020.110012

Denlinger, J. L., Eisner, G., & Balazs, E. A. (1980). Age-related changes in the vitreus and lens of rhesus monkeys (Macaca mulatta). *Experimental Eye Research*, *31*(1), 67–79. https://doi.org/10.1016/0014-4835(80)90091-3

DiNubile, N. (2018). Glucosamine and chondroitin sulfate: What has been learned since the glucosamine/chondroitin arthritis intervention trial. *Orthopedics*, *41*(4), 200–207. https://doi.org/10.3928/01477447-20180511-06

Dovedytis, M., Liu, Z. J., & Bartlett, S. (2020). Hyaluronic acid and its biomedical applications: A review. *Engineered Regeneration*, *1*, 102–113. https://doi.org/10.1016/J.ENGREG.2020.10.001

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/AC60111A017/ASSET/AC60111A017.FP.PNG_V03

E.A. Balázs, & R.W. Jeanloz. (1979). *US4141973A - Ultrapure hyaluronic acid and the use thereof - Google Patents*. https://patents.google.com/patent/US4141973A/en

el Knidri, H., Belaabed, R., Addaou, A., Laajeb, A., & Lahsini, A. (2018). ag. *International Journal of Biological Macromolecules*, *120*, 1181–1189. https://doi.org/10.1016/J.IJBIOMAC.2018.08.139

Esteves, E., & Aníbal, J. (2019). Muxama and other traditional food products obtained from tuna in south Portugal and Spain: Review and future perspectives. *Journal of Ethnic Foods*, *6*(1), 1–12. https://doi.org/10.1186/S42779-019-0022-6/FIGURES/5

European Commission. (2019). *European Commission Discarding and the Landing Obligation Fisheries*.

eurostat Statistics Explained. (2020). *Fishery statistics*. https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Fishery_statistics&oldid=551016#cite_ref-4

Fallacara, A., Baldini, E., Manfredini, S., & Vertuani, S. (2018). Hyaluronic Acid in the Third Millennium. *Polymers 2018, Vol. 10, Page 701*, *10*(7), 701. https://doi.org/10.3390/POLYM10070701

FAO. (2011). Tuna and tuna-like species. *Review of the State of World Marine Fishery Resources, Food & Agriculture Org*.

FAO. (2014). *The State of World Fisheries and Aquaculture (SOFIA) Italy, Rome (2014)*.

FAO. (2017). *The future of food and agriculture: Trends and challenges.* .

FAO. (2020). *The State of World Fisheries and Aquaculture*.

FAO. (2021). *Code of Conduct for Responsible Fisheries*.

FAO. (2022a). *The state of world fisheries and aquaculture.Towards Blue Transformation.* https://www.fao.org/3/cc0461en/cc0461en.pdf

FAO. (2022b). *GLOBEFISH - Information and Analysis on World Fish Trade (2022)*.

Fuming Zhang, Jin Xie, & Robert J Linhardt 3. (2014). Isolation and structural characterization of glycosaminoglycans from heads of red salmon (Oncorhynchus nerka) - PubMed. *Jacobs J Biotechnol Bioeng*, *1*(1), 002. https://pubmed.ncbi.nlm.nih.gov/26918243/

Gandhi, N. S., & Mancera, R. L. (2008). The Structure of Glycosaminoglycans and their Interactions with Proteins. *Chemical Biology & Drug Design*, *72*(6), 455–482. https://doi.org/10.1111/J.1747-0285.2008.00741.X

García Vargas Enrique Alberto, & Florido del Corral David. (2010). The origin and development of Tuna Fishing Nets (Almadrabas). *Universidad de Cádiz - Servicio de Publicaciones*, 205–228.

Gargiulo, V., Lanzetta, R., Parrilli, M., & De Castro, C. (2009). Structural analysis of chondroitin sulfate from Scyliorhinus canicula: A useful source of this polysaccharide. *Glycobiology*, *19*(12), 1485–1491. https://doi.org/10.1093/GLYCOB/CWP123

Garnjanagoonchorn, W., Wongekalak, L., & Engkagul, A. (2007). Determination of chondroitin sulfate from different sources of cartilage. *Chemical Engineering and Processing: Process Intensification*, *46*(5), 465–471. https://doi.org/10.1016/J.CEP.2006.05.019

Gavva, C., Patel, K., Kudre, T., Sharan, K., & Chilkunda, D. N. (2020). Glycosaminoglycans from fresh water fish processing discard - Isolation, structural characterization, and osteogenic activity. *International Journal of Biological Macromolecules*, *145*, 558–567. https://doi.org/10.1016/J.IJBIOMAC.2019.12.189

Gherezghiher, T., Koss, M. C., Nordquist, R. E., & Wilkinson, C. P. (1987). Analysis of vitreous and aqueous levels of hyaluronic acid: Application of high-performance liquid chromatography. *Experimental Eye Research*, *45*(2), 347–349. https://doi.org/10.1016/S0014-4835(87)80156-2

Giji, S., & Arumugam, M. (2014). Isolation and Characterization of Hyaluronic Acid from Marine Organisms. *Advances in Food and Nutrition Research*, *72*, 61–77. https://doi.org/10.1016/B978-0-12-800269-8.00004-X

Govindharaj, M., Roopavath, U. K., & Rath, S. N. (2019). Valorization of discarded Marine Eel fish skin for collagen extraction as a 3D printable blue biomaterial for tissue engineering. *Journal of Cleaner Production*, *230*, 412–419. https://doi.org/10.1016/J.JCLEPRO.2019.05.082

Grand View Research. (2020). *Hyaluronic Acid Market Size, Share & Trends Analysis Report By Application (Dermal Fillers, Osteoarthritis (Single Injection, Three Injection, Five Injection), Ophthalmic, Vesicoureteral Reflux), By Region, And Segment Forecasts, 2020-2027*. https://www.grandviewresearch.com/industry-analysis/hyaluronic-acid-market

Grand View Research. (2023, January 11). *Chondroitin Sulfate Market Size, Share & Trends Analysis Report By Source (Bovine, Shark, Swine), By Application (Nutraceuticals, Pharmaceuticals), By Region (APAC, North America), And Segment Forecasts, 2023 - 2030*. https://www.grandviewresearch.com/industry-analysis/chondroitin-sulfate-market

Haylock-Jacobs, S., Keough, M. B., Lau, L., & Yong, V. W. (2011). Chondroitin sulphate proteoglycans: Extracellular matrix proteins that regulate immunity of the central nervous system. *Autoimmunity Reviews*, *10*(12), 766–772. https://doi.org/10.1016/J.AUTREV.2011.05.019

Higashi, K., Okamoto, Y., Mukuno, A., Wakai, J., Hosoyama, S., Linhardt, R. J., & Toida, T. (2015). Functional chondroitin sulfate from Enteroctopus dofleini containing a 3-O-sulfo glucuronic acid residue. *Carbohydrate Polymers*, *134*, 557–565. https://doi.org/10.1016/J.CARBPOL.2015.07.082

Higashi, K., Takeuchi, Y., Mukuno, A., Tomitori, H., Miya, M., Linhardt, R. J., & Toida, T. (2015). Composition of Glycosaminoglycans in Elasmobranchs including Several Deep-Sea Sharks: Identification of Chondroitin/Dermatan Sulfate from the Dried Fins of Isurus oxyrinchus and Prionace glauca. *PLOS ONE*, *10*(3), e0120860. https://doi.org/10.1371/JOURNAL.PONE.0120860

Hu, S., Wang, J., Xu, Y., Yang, H., Wang, J., Xue, C., Yan, X., & Su, L. (2019). Anti-inflammation effects of fucosylated chondroitin sulphate from: Acaudina molpadioides by altering gut microbiota in obese mice. *Food and Function*, *10*(3), 1736–1746. https://doi.org/10.1039/C8FO02364F

ICCAT (International Commission for the Conservation of Atlantic Tunas). (2017). *Report of the 2017 ICCAT Bluefin Stock Assessment Meeting*.

ICCAT (International Commission for the Conservation of Atlantic Tunas). (2021). *Report of the 2021 Western Bluefin Stock Assessment Meeting*.

ISSF. (2020). *Status of the World Fisheries for Tuna International Seafood Sustainability Foundation, Washington, D.C., USA*.

Itakura, H., Kishi, S., Kotajima, N., & Murakami, M. (2009). Decreased Vitreal Hyaluronan Levels with Aging. *Ophthalmologica*, *223*(1), 32–35. https://doi.org/10.1159/000165682

J. Necas, L. Bartosikova, P. Brauner, & J. Kolar. (2008). Hyaluronic acid (hyaluronan): A review. *Veterinární Medicína*, *53*(8), 397–411.

Jabbari, F., Babaeipour, V., & Saharkhiz, S. (2023). Comprehensive review on biosynthesis of hyaluronic acid with different molecular weights and its biomedical applications. *International Journal of Biological Macromolecules*, *240*, 124484. https://doi.org/10.1016/J.IJBIOMAC.2023.124484

Jeong, K.-S. (2016). Development of High Purity Purification Method of Chondroitin Sulfate Extracted from Skate Cartilage. *Journal of the Korea Academia-Industrial Cooperation Society*, *17*(6), 9–17. https://doi.org/10.5762/KAIS.2016.17.6.9

Jinno, A., & Park, P. W. (2015). Role of Glycosaminoglycans in Infectious Disease. *Methods in Molecular Biology (Clifton, N.J.)*, *1229*, 567. https://doi.org/10.1007/978-1-4939-1714-3_45

Kamei, A., & Totani, A. (1982). Isolation and characterization of minor glycosaminoglycans in the rabbit vitreous body. *Biochemical and Biophysical Research Communications*, *109*(3), 881–887. https://doi.org/10.1016/0006-291X(82)92022-8

Kanchana, S., Arumugam, M., Giji, S., & Balasubramanian, T. (2013). Isolation, characterization and antioxidant activity of hyaluronic acid from marine bivalve mollusc Amussium pleuronectus (Linnaeus, 1758). *Bioactive Carbohydrates and Dietary Fibre*, *2*(1), 1–7. https://doi.org/10.1016/J.BCDF.2013.06.001

Karamanos, N. K., Manouras, A., Tsegenidis, T., & Antonopoulos, C. A. (1991). Isolation and chemical study of the glycosaminoglycans from squid cornea. *International Journal of Biochemistry*, *23*(1), 67–72. https://doi.org/10.1016/0020-711X(91)90010-K

Käsdorf, B. T., Arends, F., & Lieleg, O. (2015). Diffusion Regulation in the Vitreous Humor. *Biophysical Journal*, *109*(10), 2171–2181. https://doi.org/10.1016/J.BPJ.2015.10.002

Kato, D., Era, S., Watanabe, I., Arihara, M., Sugiura, N., Kimata, K., Suzuki, Y., Morita, K., Hidari, K. I. P. J., & Suzuki, T. (2010). Antiviral activity of chondroitin sulphate E targeting dengue virus envelope protein. *Antiviral Research*, *88*(2), 236–243. https://doi.org/10.1016/J.ANTIVIRAL.2010.09.002

Kim, S. B., Ji, C. il, Woo, J. W., Do, J. R., Cho, S. M., Lee, Y. B., Kang, S. N., & Park, J. H. (2012). Simplified purification of chondroitin sulphate from scapular cartilage of shortfin mako shark (Isurus oxyrinchus). *International Journal of Food Science & Technology*, *47*(1), 91–99. https://doi.org/10.1111/J.1365-2621.2011.02811.X

Kogan, G., Šoltés, L., Stern, R., & Gemeiner, P. (2007). Hyaluronic acid: A natural biopolymer with a broad range of biomedical and industrial applications. *Biotechnology Letters*, *29*(1), 17–25. https://doi.org/10.1007/S10529-006-9219-Z

Kokavec, J., Min, S. H., Tan, M. H., Gilhotra, J. S., Newland, H. S., Durkin, S. R., Grigg, J., & Casson, R. J. (2016). Biochemical analysis of the living human vitreous. *Clinical & Experimental Ophthalmology*, *44*(7), 597–609. https://doi.org/10.1111/CEO.12732

Krichen, F., Bougatef, H., Sayari, N., Capitani, F., ben Amor, I., Koubaa, I., Maccari, F., Mantovani, V., Galeotti, F., Volpi, N., & Bougatef, A. (2018). Isolation, Purification and Structural Characterestics of Chondroitin Sulfate from Smooth hound Cartilage: In vitro Anticoagulant and Antiproliferative Properties. *Carbohydrate Polymers*, *197*, 451–459. https://doi.org/10.1016/J.CARBPOL.2018.06.040

Kumari, K., Baggenstoss, B. A., Parker, A. L., & Weigel, P. H. (2006). Mutation of Two Intramembrane Polar Residues Conserved within the Hyaluronan Synthase Family Alters Hyaluronan Product Size *. *Journal of Biological Chemistry*, *281*(17), 11755–11760. https://doi.org/10.1074/JBC.M600727200

Lamberg, S. I., & Stoolmiller, A. C. (1974). Glycosaminoglycans. A biochemical and clinical review. *Journal of Investigative Dermatology*, *63*(6), 433–449. https://doi.org/10.1111/1523-1747.EP12680346

Laurent, T. C., & Fraser, J. R. E. (1992). Hyaluronan 1 . *The FASEB Journal*, *6*(7), 2397–2404. https://doi.org/10.1096/FASEBJ.6.7.1563592

Li, J., Qiao, M., Ji, Y., Lin, L., Zhang, X., & Linhardt, R. J. (2020). Chemical, enzymatic and biological synthesis of hyaluronic acids. *International Journal of Biological Macromolecules*, *152*, 199–206. https://doi.org/10.1016/J.IJBIOMAC.2020.02.214

Li, S., Zhong, W., Pan, Y., Lin, L., Cai, Y., Mao, H., Zhang, T., Li, S., Chen, R., Zhou, L., Wang, W., Cui, Q., Yin, R., Huang, S., & Zhao, J. (2021). Structural characterization and anticoagulant analysis of the novel branched fucosylated glycosaminoglycan from sea cucumber Holothuria nobilis. *Carbohydrate Polymers*, *269*, 118290. https://doi.org/10.1016/J.CARBPOL.2021.118290

Liem, K. F., & Walker, W. F. (2001). *Functional anatomy of the vertebrates : an evolutionary perspective*. 703. https://www.nhbs.com/functional-anatomy-of-the-vertebrates-book

Lignot, B., Lahogue, V., & Bourseau, P. (2003). Enzymatic extraction of chondroitin sulfate from skate cartilage and concentration-desalting by ultrafiltration. *Journal of Biotechnology*, *103*(3), 281–284. https://doi.org/10.1016/S0168-1656(03)00139-1

Maccari, F., Galeotti, F., & Volpi, N. (2015). Isolation and structural characterization of chondroitin sulfate from bony fishes. *Carbohydrate Polymers*, *129*, 143–147. https://doi.org/10.1016/J.CARBPOL.2015.04.059

MacKenzie, B. R., Mosegaard, H., Rosenberg, A. A., & Brian MacKenzie, C. R. (2009). Impending collapse of bluefin tuna in the northeast Atlantic and Mediterranean. *Conservation Letters*, *2*(1), 26–35. https://doi.org/10.1111/J.1755-263X.2008.00039.X

Malavaki, C., Mizumoto, S., Karamanos, N., & Sugahara, K. (2008). Recent advances in the structural study of functional chondroitin sulfate and dermatan sulfate in health and disease. *Connective Tissue Research*, *49*(3–4), 133–139. https://doi.org/10.1080/03008200802148546

Marie Lecomte, Julien Rochette, Renaud Lapeyre, & Yann Laurans. (2018). *Tuna: fish and fisheries, markets and sustainability | IDDRI*. https://www.iddri.org/en/publications-and-events/report/tuna-fish-and-fisheries-markets-and-sustainability

McDowall, R. D. (1989). Sample preparation for biomedical analysis. *Journal of Chromatography B: Biomedical Sciences and Applications*, *492*(C), 3–58. https://doi.org/10.1016/S0378-4347(00)84463-1

McKinney, R., Gibbon, J., Wozniak, E., & Galland, G. (2020). Netting Billions 2020: A Global Tuna Valuation. *Security Research Hub Reports*. https://digitalcommons.fiu.edu/srhreports/iuufishing/iuufishing/105

Mero, A., & Campisi, M. (2014). Hyaluronic Acid Bioconjugates for the Delivery of Bioactive Molecules. *Polymers 2014, Vol. 6, Pages 346-369*, *6*(2), 346–369. https://doi.org/10.3390/POLYM6020346

Mesías, M., Holgado, F., Sevenich, R., Briand, J. C., Márquez Ruiz, G., & Morales, F. J. (2015). Fatty acids profile in canned tuna and sardine after retort sterilization and high pressure thermal sterilization treatment. *Journal of Food and Nutrition Research, 54*(2), 171–178.

Min, D., Park, S., Kim, H., Lee, S. H., Ahn, Y., Jung, W., Kim, H. J., & Cho, Y. W. (2020). Potential anti-ageing effect of chondroitin sulphate through skin regeneration. *International Journal of Cosmetic Science*, *42*(5), 520–527. https://doi.org/10.1111/ICS.12645

Mizuno, H., Iso, N., Sawairi, H., Saito, T., Saito, M., & Ogawa, H. (1991). Characterization of Hyaluronic Acid of Yellowfin Tuna Eyeball. *Nippon Suisan Gakkaishi (Japanese Edition)*, *57*(3), 517–519. https://doi.org/10.2331/SUISAN.57.517

Murado, M. A., Fraguas, J., Montemayor, M. I., Vázquez, J. A., & González, P. (2010). Preparation of highly purified chondroitin sulphate from skate (Raja clavata) cartilage by-products. Process optimization including a new procedure of alkaline hydroalcoholic hydrolysis. *Biochemical Engineering Journal*, *49*(1), 126–132. https://doi.org/10.1016/J.BEJ.2009.12.006

Murado, M. A., Montemayor, M. I., Cabo, M. L., Vázquez, J. A., & González, M. P. (2012). Optimization of extraction and purification process of hyaluronic acid from fish eyeball. *Food and Bioproducts Processing*, *90*(3), 491–498. https://doi.org/10.1016/J.FBP.2011.11.002

Myron, P., Siddiquee, S., & Al Azad, S. (2014). Fucosylated chondroitin sulfate diversity in sea cucumbers: A review. *Carbohydrate Polymers*, *112*, 173–178. https://doi.org/10.1016/J.CARBPOL.2014.05.091

Nicholls, M., Niazi, F., Nelson, W. W., Lau, E., Kurtz, S. M., & Ong, K. L. (2022). Association Between Bio-Fermentation Derived Hyaluronic Acid and Healthcare Costs Following Knee Arthroplasty. *ClinicoEconomics and Outcomes Research*, *14*, 575–585. https://doi.org/10.2147/CEOR.S347512

Novoa-Carballal, R., Pérez-Martín, R., Blanco, M., Sotelo, C. G., Fassini, D., Nunes, C., Coimbra, M. A., Silva, T. H., Reis, R. L., & Vázquez, J. A. (2017). By-products of Scyliorhinus canicula, Prionace glauca and Raja clavata: A valuable source of predominantly 6S sulfated chondroitin sulfate. *Carbohydrate Polymers*, *157*, 31–37. https://doi.org/10.1016/J.CARBPOL.2016.09.050

Oliveira, V. de M., Assis, C. R. D., Costa, B. de A. M., Neri, R. C. de A., Monte, F. T., Freitas, H. M. S. da C. V., França, R. C. P., Santos, J., Bezerra, R. de S., & Porto, A. L. F. (2021). Physical, biochemical, densitometric and spectroscopic techniques for characterization collagen from alternative sources: A review based on the sustainable valorization of aquatic by-products. *Journal of Molecular Structure*, *1224*, 129023. https://doi.org/10.1016/J.MOLSTRUC.2020.129023

Olsen, R. L., Toppe, J., & Karunasagar, I. (2014). Challenges and realistic opportunities in the use of by-products from processing of fish and shellfish. *Trends in Food Science & Technology*, *36*(2), 144–151. https://doi.org/10.1016/J.TIFS.2014.01.007

Opdensteinen, P., Clodt, J. I., Müschen, C. R., Filiz, V., & Buyel, J. F. (2019). A combined ultrafiltration/diafiltration step facilitates the purification of cyanovirin-n from transgenic tobacco extracts. *Frontiers in Bioengineering and Biotechnology*, *6*(JAN). https://doi.org/10.3389/FBIOE.2018.00206

Ozogul, F., Cagalj, M., Šimat, V., Ozogul, Y., Tkaczewska, J., Hassoun, A., Kaddour, A. A., Kuley, E., Rathod, N. B., & Phadke, G. G. (2021). Recent developments in valorisation of bioactive ingredients in discard/seafood processing by-products. *Trends in Food Science & Technology*, *116*, 559–582. https://doi.org/10.1016/J.TIFS.2021.08.007

Park, P. W., Hayashida, K., Aquino, R. S., & Jinno, A. (2016). Proteoglycans. *Encyclopedia of Cell Biology*, *1*, 271–278. https://doi.org/10.1016/B978-0-12-394447-4.10032-X

Peng, Y., Yu, Y., Lin, L., Liu, X., Zhang, X., Wang, P., Hoffman, P., Kim, S. Y., Zhang, F., & Linhardt, R. J. (2018). Glycosaminoglycans from bovine eye vitreous humour and interaction with collagen type II. *Glycoconjugate Journal*, *35*(1), 119–128. https://doi.org/10.1007/S10719-017-9808-1/FIGURES/5

Pomin, V. H. (2016). Dual and antagonic therapeutic effects of sulfated glycans. *Bioorganic & Medicinal Chemistry*, *24*(18), 3965–3971. https://doi.org/10.1016/J.BMC.2016.07.037

Prescott, A. (2003). *US6660853B2 - Method for purifying high molecular weight hyaluronic acid - Google Patents*. United States Patent 6660853 B2. https://patents.google.com/patent/US6660853B2/en

Pudełko, A., Wisowski, G., Olczyk, K., & Koźma, E. M. (2019). The dual role of the glycosaminoglycan chondroitin-6-sulfate in the development, progression and metastasis of cancer. *The FEBS Journal*, *286*(10), 1815–1837. https://doi.org/10.1111/FEBS.14748

Rameshthangam, P., Solairaj, D., Arunachalam, G., & Ramasamy, P. (2020). Chitin and Chitinases: Biomedical And Environmental Applications of Chitin and its Derivatives. *Journal of Enzymes*, *1*(1), 20–43. https://doi.org/10.14302/ISSN.2690-4829.JEN-18-2043

Restaino, O. F., Finamore, R., Stellavato, A., Diana, P., Bedini, E., Trifuoggi, M., de Rosa, M., & Schiraldi, C. (2019). European chondroitin sulfate and glucosamine food supplements: A systematic quality and quantity assessment compared to pharmaceuticals. *Carbohydrate Polymers*, *222*. https://doi.org/10.1016/J.CARBPOL.2019.114984

Rodriguez-Merchan, E. C. (2018). Topical therapies for knee osteoarthritis. *Postgraduate Medicine*, *130*(7), 607–612. https://doi.org/10.1080/00325481.2018.1505182

Rudd, T. R., & Yates, E. A. (2010). Conformational degeneracy restricts the effective information content of heparan sulfate. *Molecular BioSystems*, *6*(5), 902–908. https://doi.org/10.1039/B923519A

Sadhasivam, G., Muthuvel, A., Pachaiyappan, A., & Thangavel, B. (2013). Isolation and characterization of hyaluronic acid from the liver of marine stingray Aetobatus narinari.

International Journal of Biological Macromolecules, *54*(1), 84–89. https://doi.org/10.1016/J.IJBIOMAC.2012.11.028

Sahu, B., Sharma, D. D., Jayakumar, G. C., Madhan, B., & Zameer, F. (2023). A review on an imperative by-product: Glycosaminoglycans- A holistic approach. *Carbohydrate Polymer Technologies and Applications*, *5*, 100275. https://doi.org/10.1016/J.CARPTA.2022.100275

Save Food Initiative. (2021). *Initiative Safe Food*. https://www.save-food.org/

Schaefer, K. M. (2001). Reproductive biology of tunas. *Fish Physiology*, *19*(C), 225–270. https://doi.org/10.1016/S1546-5098(01)19007-2

Shikina, E. V., Kovalevsky, R. A., Shirkovskaya, A. I., & Toukach, Ph. V. (2022). Prospective bacterial and fungal sources of hyaluronic acid: A review. *Computational and Structural Biotechnology Journal*, *20*, 6214–6236. https://doi.org/10.1016/J.CSBJ.2022.11.013

Sila, A., Bougatef, H., Capitani, F., Krichen, F., Mantovani, V., Amor, I. Ben, Galeotti, F., Maccari, F., Nedjar, N., Volpi, N., & Bougatef, A. (2018). Studies on European eel skin sulfated glycosaminoglycans: Recovery, structural characterization and anticoagulant activity. *International Journal of Biological Macromolecules*, *115*, 891–899. https://doi.org/10.1016/J.IJBIOMAC.2018.04.125

Song, Y. O., Mijeong, K., Minji, W., Baek, J. M., Kang, K. H., Kim, S. H., Roh, S. S., Park, C. H., Jeong, K. S., & Noh, J. S. (2017). Chondroitin sulfate-rich extract of skate cartilage attenuates lipopolysaccharide-induced liver damage in mice. *Marine Drugs*, *15*(6). https://doi.org/10.3390/MD15060178

Souza, A. R. C., Kozlowski, E. O., Cerqueira, V. R., Castelo-Branco, M. T. L., Costa, M. L., & Pavão, M. S. G. (2007). Chondroitin sulfate and keratan sulfate are the major glycosaminoglycans present in the adult zebrafish Danio rerio (Chordata-Cyprinidae). *Glycoconjugate Journal*, *24*(9), 521–530. https://doi.org/10.1007/S10719-007-9046-Z/FIGURES/5

Sumogod, A., Nacional, L., Nillos, M. G., & Yap, E. E. (2020). Isolation of hyaluronic acid from yellowfin tuna thunnus albacares (Bonnaterre, 1788) eyeball. *Asian Fisheries Science*, *33*(1), 1–9. https://doi.org/10.33997/J.AFS.2020.33.1.001

Sundaresan, G., Abraham, R. J. J., Appa Rao, V., Narendra Babu, R., Govind, V., & Meti, M. F. (2018). Established method of chondroitin sulphate extraction from buffalo (Bubalus bubalis) cartilages and its identification by FTIR. *Journal of Food Science and Technology*, *55*(9), 3439–3445. https://doi.org/10.1007/S13197-018-3253-4

Tamura, J. I., Arima, K., Imazu, A., Tsutsumishita, N., Fujita, H., Yamane, M., & Matsumi, Y. (2009). Sulfation patterns and the amounts of chondroitin sulfate in the diamond squid, Thysanoteuthis rhombus. *Bioscience, Biotechnology and Biochemistry*, *73*(6), 1387–1391. https://doi.org/10.1271/BBB.90037

the UK Government. (2021). *Government to introduce world-leading ban on shark fin trade - GOV.UK*. https://www.gov.uk/government/news/government-to-introduce-world-leading-ban-on-shark-fin-trade

Thomas, Dr. L., KP, Dr. M., & Mathew, Dr. S. (2021). Fish gills of Thunnus albacares: A novel source of chondroitin sulphate glycosaminoglycans. *International Journal of Fisheries and Aquatic Studies*, *9*(1), 146–152. https://doi.org/10.22271/FISH.2021.V9.I1B.2397

Ticar, B. F., Rohmah, Z., Neri, T. A. N., Pahila, I. G., Vasconcelos, A., Archer-Hartmann, S. A., Reiter, C. E. N., Dobruchowska, J. M., Choi, B. D., Heiss, C., Azadi, P., & Pomin, V. H. (2020). Biocompatibility and structural characterization of glycosaminoglycans isolated from heads of silver-banded whiting (Sillago argentifasciata Martin & Montalban 1935). *International Journal of Biological Macromolecules*, *151*, 663–676. https://doi.org/10.1016/J.IJBIOMAC.2020.02.160

Toshihisa, S., Hideki, O., Toru, I., Masao, S., Tsuyoshi, S., Sallay, S., & Soo, P. (2000). *Method for the preparation of chondroitin sulfate compounds* (Patent No. US6342367B1). National Institute of Advanced Industrial Science and Technology AIST. https://patents.google.com/patent/US6342367B1/en

United Nations. (2015). *A/RES/70/1 Transforming our world: the 2030 agenda for sustainable development transforming our world: the 2030 agenda for sustainable development preamble United Nations Gen. Assem. Resolut*.

United Nations. (2019). *World Population Prospects*. United Nations, 2019. https://population.un.org/wpp/DataQuery/

Välimaa, A. L., Mäkinen, S., Mattila, P., Marnila, P., Pihlanto, A., Mäki, M., & Hiidenhovi, J. (2019). Fish and fish side streams are valuable sources of high-value components. *Food Quality and Safety*, *3*(4), 209–226. https://doi.org/10.1093/FQSAFE/FYZ024

Vázquez, J. A., Blanco, M., Fraguas, J., Pastrana, L., & Pérez-Martín, R. (2016). Optimisation of the extraction and purification of chondroitin sulphate from head by-products of Prionace glauca by environmental friendly processes. *Food Chemistry*, *198*, 28–35. https://doi.org/10.1016/J.FOODCHEM.2015.10.087

Vázquez, J. A., Fraguas, J., Mirón, J., Valcárcel, J., Pérez-Martín, R. I., & Antelo, L. T. (2020). Valorisation of fish discards assisted by enzymatic hydrolysis and microbial bioconversion: Lab and pilot plant studies and preliminary sustainability evaluation. *Journal of Cleaner Production*, *246*, 119027. https://doi.org/10.1016/J.JCLEPRO.2019.119027

Vázquez, J. A., Fraguas, J., Novoa-Carballal, R., Reis, R. L., Pérez-Martín, R. I., & Valcarcel, J. (2019). Optimal isolation and characterisation of chondroitin sulfate from rabbit fish (Chimaera monstrosa). *Carbohydrate Polymers*, *210*, 302–313. https://doi.org/10.1016/J.CARBPOL.2019.01.075

Vázquez, J. A., Fraguas, J., Novoa-Carvallal, R., Reis, R. L., Antelo, L. T., Pérez-Martín, R. I., & Valcarcel, J. (2018). Isolation and Chemical Characterization of Chondroitin Sulfate from Cartilage By-Products of Blackmouth Catshark (Galeus melastomus). *Marine Drugs 2018, Vol. 16, Page 344*, *16*(10), 344. https://doi.org/10.3390/MD16100344

Volpi, N. (2019). Chondroitin sulfate safety and quality. *Molecules*, *24*(8). https://doi.org/10.3390/MOLECULES24081447

Volpi, N., Galeotti, F., Maccari, F., Capitani, F., & Mantovani, V. (2021). Structural definition of terrestrial chondroitin sulfate of various origin and repeatability of the production process. *Journal of Pharmaceutical and Biomedical Analysis*, *195*. https://doi.org/10.1016/J.JPBA.2020.113826

Volpi, N., & Maccari, F. (2003). Purification and characterization of hyaluronic acid from the mollusc bivalve Mytilus galloprovincialis. *Biochimie*, *85*(6), 619–625. https://doi.org/10.1016/S0300-9084(03)00083-X

Walls, G. L., & Walls, G. L. (1942). The vertebrate eye and its adaptive radiation. In *The vertebrate eye and its adaptive radiation [by] Gordon Lynn Walls.* Cranbrook Institute of Science. https://doi.org/10.5962/bhl.title.7369

Wang, T., Zhang, S., Ren, S., Zhang, X., Yang, F., Chen, Y., & Wang, B. (2020). Structural characterization and proliferation activity of chondroitin sulfate from the sturgeon, Acipenser schrenckii. *International Journal of Biological Macromolecules*, *164*, 3005–3011. https://doi.org/10.1016/J.IJBIOMAC.2020.08.110

Yamada, S., Sugahara, K., & Özbek, S. (2011). Evolution of glycosaminoglycans: Comparative biochemical study. *Communicative and Integrative Biology*, *4*(2), 150–158. https://doi.org/10.4161/CIB.4.2.14547

Yang, L., Wang, Y., Yang, S., & Lv, Z. (2018). Separation, purification, structures and anticoagulant activities of fucosylated chondroitin sulfates from Holothuria scabra.

International Journal of Biological Macromolecules, *108*, 710–718. https://doi.org/10.1016/J.IJBIOMAC.2017.11.058

Yu, S., Wang, S., Xia, L., Hu, H., Zou, M., Jiang, Z., Chi, J., Zhang, Y., Li, H., Yang, C., Liu, W., & Han, B. (2022). Injectable self-crosslinking hydrogels based on hyaluronic acid as vitreous substitutes. *International Journal of Biological Macromolecules*, *208*, 159–171. https://doi.org/10.1016/J.IJBIOMAC.2022.03.046

Zhao, T., Zhou, Y., Mao, G., Zou, Y., Zhao, J., Bai, S., Yang, L., & Wu, X. (2013). Extraction, purification and characterisation of chondroitin sulfate in Chinese sturgeon cartilage. *Journal of the Science of Food and Agriculture*, *93*(7), 1633–1640. https://doi.org/10.1002/JSFA.5937

Zhu, Y., Li, N., Huang, M., Bartels, M., Dogné, S., Zhao, S., Chen, X., Crewe, C., Straub, L., Vishvanath, L., Zhang, Z., Shao, M., Yang, Y., Gliniak, C. M., Gordillo, R., Smith, G. I., Holland, W. L., Gupta, R. K., Dong, B., … Scherer, P. E. (2021). Adipose tissue hyaluronan production improves systemic glucose homeostasis and primes adipocytes for CL 316,243-stimulated lipolysis. *Nature Communications 2021 12:1*, *12*(1), 1–15. https://doi.org/10.1038/s41467-021-25025-4