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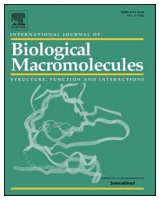

Review

Future proofing of chondroitin sulphate production: Importance of sustainability and quality for the end-applications

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ABSTRACT

Chondroitin sulphates (CSs) are the most well-known glycosaminoglycans (GAGs) found in any living organism, from microorganisms to invertebrates and vertebrates (including humans), and provide several health benefits. The applications of CSs are numerous including tissue engineering, osteoarthritis treatment, antiviral, cosmetics, and skincare applications. The current commercial production of CSs mostly uses animal, bovine, porcine, and avian tissues as well as marine organisms, marine mammals, sharks, and other fish. The production process consists of tissue hydrolysis, protein removal, and purification using various methods. Mostly, these are chemical-dependent and are complex, multi-step processes. There is a developing trend for abandonment of harsh extraction chemicals and their substitution with different green-extraction technologies, however, these are still in their infancy. The quality of CSs is the first and foremost requirement for end-applications and is dependent on the extraction and purification methodologies used. The final products will show different bio-functional properties, depending on their origin and production methodology. This is a comprehensive review of the characteristics, properties, uses, sources, and extraction methods of CSs. This review emphasises the need for extraction and purification processes to be environmentally friendly and gentle, followed by product analysis and quality control to ensure the expected bioactivity of CSs.

1. Introduction

Chondroitin sulphates (CSs) are found in the extracellular matrix of animal tissues like soft cartilage, bone, skin, and tendons. This extracellular matrix is a complex structure comprising fibrous proteins (collagen, elastin, fibronectin, and laminin), and heteropolysaccharides with unbranched chains of repeating disaccharide units [1,2]. These heteropolysaccharides are broadly called glycosaminoglycans (GAGs) because one of the two saccharide units is always an amino sugar [*N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc)]. The other saccharide unit is usually a uronic acid [glucuronic (GlcA) or iduronic (IdoA) in dermatan sulphate (DS)]. Earlier what was known as CS-B is now called DS, which is a stereoisomer of CS that contains IdoA instead of GlcA [3,4]. It is important to note from the purity as well as expected biological functions, that both CS and DS are biosynthesised from the chondroitin precursor. As a result, CS-DS hybrid chains are produced during the developmental stage in each organ like skin, cartilage, and the aorta [3,5]. The GAGs can be divided into four main groups depending on their disaccharide composition, linkage type, and the nature of sulphation. These groups are:

i) Chondroitin sulphate (CS) and dermatan sulphate (DS)
ii) Keratan sulphate (KS)
iii) Heparan sulphate (HS) and heparin
iv) Hyaluronan (hyaluronic acid, HA)

Structurally, CSs are heteropolymers comprising repeating GlcA and GalNAc disaccharide units linked with β-(1 → 3) glycosidic bonds and sulphate group(s) at different carbon positions. The positioning of the sulphate group in CS is often modified by replacing OH groups on the C-2 and C-3 positions of GlcA and the C-4 and C-6 positions of GalNAc [6,7]. Likewise, DS is also a heteropolymer with alternating disaccharide units comprising L-iduronic acid (IdoA) and *N*-acetylgalactosamine (GalNAc). These disaccharide units are esterified by a sulphate group at different carbon positions on GalNAc and IdoA resulting in different DS units. These are indicated with the notations iO [IdoA-GalNAc], iA [IdoA-GalNAc(4S)], iB [IdoA(2*S*)-GalNAc(4S)], iC [IdoA-GalNAc(6S)], iD [IdoA(2*S*)-GalNAc(6S)], and iE [IdoA-GalNAc(4S,6S)] [3]. Based on

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the positioning of the sulphate groups, the most known CS types are as follows (Fig. 1).

1) CS-0 [GlcA-GalNAc, no sulphation].
2) CS-A [GlcA-GalNAc (sulphation at C-4)].
3) CS-B [IdoA-GalNAc (sulphation at C-4)].
4) CS-C [GlcA-GalNAc (sulphation at C-6)].
5) CS-D [GlcA (sulphation at C-2)-GalNAc (sulphation at C-6)].
6) CS-E [GlcA-GalNAc (sulphation both at C-4 and C-6)].

The type of CS and their amounts in a specific tissue are dependent on the source organism and type of tissue. The molecular weight of the CS chain with repeating disaccharide units could vary between 20 and 25 kDa in tracheal biomass and 50-80 kDa in shark cartilage [8]. While the molecular weight of the DS chain also varies in different biomass samples, such as 11-25 kDa in porcine skin, 18 kDa in hagfish notochord, and 70 kDa in shark skin [3].

2. Potential applications

CS is a biopolymer that is found in the extracellular cartilage of humans and animals. It has various reported biological functions and therefore can be used as a supplement or a medicine for different health conditions. One of the main applications of CS is to treat osteoarthritis (OA), a degenerative joint disease that causes pain and stiffnessaffecting the joints and muscles. OA is the most common type of arthritis in people over 65 years old. The disability in the elderly population is mainly because of knee pain that affects load-bearing joints and is a problem in the USA, UK, and other developed countries [9]. It was found that the highly purified CS 4S and 6S improved hand function more significantly than in the placebo group [10]. Other research also indicates that the purity of CS may influence the effectiveness of the OA treatment such that highly purified CS significantly reduces the pain in the hip (-42.6%) compared to placebo (-2%). Likewise, only a small proportion of food supplements that contain CS had an in vitro behaviour similar to that of pharmaceutical-grade products [11]. CS may help slow down the breakdown of cartilage, reduce inflammation in the joints, improve joint mobility, and enhance the overall quality of life for OA sufferers [8]. CS is often combined with other ingredients, such as glucosamine, hyaluronic acid, collagen peptides, and DS to enhance its effects.

Research showed various bioactivities associated with specific structures of CS, such as antioxidant, anti-inflammatory, anti-apoptotic, inducer for type II collagen, and proteoglycan biosynthesis in joints reduce the production of pro-inflammatory mediators and proteases [2,8]. These biological functions make this molecule very special, particularly for OA treatment/management as Symptomatic Slow-Acting Drugs (SYSADOA). Potential applications of CS also include its use as an antiviral and anti-infective agent; an ingredient for tissue regeneration and engineering; as a biomarker in cancerous cells and tissues; for re-epithelialisation; for the stimulation of neo-vascularisation; and for supplying growth factors and cytokines [2,12–16]. CS can also be used in eye drop formulations for dry eyes. Likewise, CS has potential applications as a food supplement product for self-management and preventive care for the elderly [9].

Recent studies have suggested that CS is a versatile molecule that has multiple potential applications in medicine and biotechnology [16]. CS is involved in several physiological processes such as signalling and neuronal growth by interacting with cytokines, adhesion factors, and growth factors [17]. CS renders its anti-inflammatory effect through the downregulation of interleukin-1β (IL-1β), nuclear factor- κβ (NF-κβ), and matrix-degrading enzymes [16]. Apart from various bioactivities, CS is highly biocompatible and biodegradable and has mucoadhesive as well as hydrophilicity properties. The potential application of CS is as a tissue engineering scaffold, which can be used to create hydrogels, films, fibres, or nanoparticles that can support the growth and differentiation of various cells, such as chondrocytes, osteoblasts, fibroblasts, and stem cells.

Because of these additional characteristics, CS has now been applied in various biomedical applications including in tissue engineering and wound healing. This is because CS can provide a biomimetic

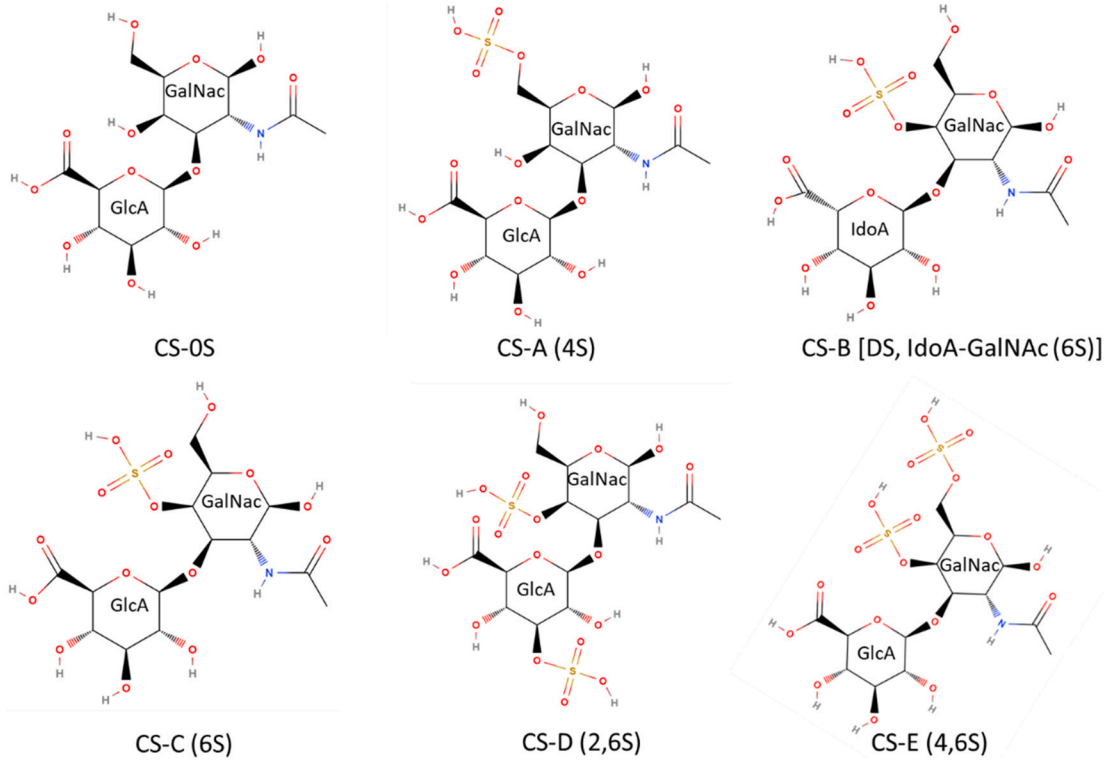

Fig. 1. Disaccharides of various CS types show the positions of sulphation within the repeating GlcA and GalNAc disaccharide units.

environment for cell growth, differentiation, and regeneration [18]. The most potential applications of CS in tissue engineering and wound healing are 1) spinal cord injury, 2) skin defects, 3) bone regeneration, and 4) drug delivery [19]. The introduction of CS can activate microglia/macrophages and thus modulate the secretion of neurotrophic factors, which can enhance the acute recovery stage after spinal cord injury [20]. Skin is the largest organ of humans that can be damaged due to several external factors like burns, physical injury, and different disease conditions. CS enhances angiogenesis, collagen synthesis, and epithelialisation and thus can promote the wound-healing process and stimulate the regeneration of skin defects [16]. Although, bone tissues are rejuvenated continuously throughout life, however, there may be a need for surgical interventions or autologous bone grafting during critical bone injuries and large-size bone defects. CS is the most potential biomaterial that can support bone regeneration by providing a scaffold for osteoblasts and osteoclasts and stimulating the secretion of bone morphogenetic proteins (BMPs) and osteogenic factors. CS can enhance the mechanical strength and biocompatibility of bone grafts and implants by forming composites with other biomaterials, such as glycoproteins, collagen, proteoglycans, hydroxyapatite, and calcium phosphate [18]. Within the bone tissue, the main roles of CS are to coordinate osteoblastic cell attachment and maintenance of bone homeostasis [16]. CS can be used as a drug delivery system as it can form complexes with various drugs, such as anticancer agents, antibiotics, anti-inflammatory drugs, and gene vectors, and enhance their stability, solubility, and bioavailability [19,21]. There is another growing application of CS that uses nanoparticles (NPs) produced by chitosan (CH)-chondroitin sulphate (CS) as a tool for delivering target gene sequences [22]. Moreover, CS can target specific tissues or organs by binding to specific receptors or enzymes on the cell surface. For example, CS can target tumour cells by binding to CD44, a receptor that is overexpressed in many cancers [21]. CS has been used as nanocarriers for tumour-targeted drug delivery by replacing the standard chemotherapy, which generally lacks selectivity of cancer cells and may allow to development of drug resistance. CS-derived nanocarriers (theranostic and therapeutic) have low toxicity, better biocompatibility, and can target both actively and passively making CS the best drug delivery vehicle (e.g., to deliver anticancer medicine doxorubicin) for cancer therapy [19,23].

The other application of CS is to prevent or treat coronary atherosclerotic heart disease, a condition where plaque builds up in the arteries and reduces blood flow to the heart. CS may help lower cholesterol levels, prevent blood clots, and protect the blood vessels from damage. It may also improve cardiac function and reduce the risk of heart attack or stroke [24]. Some studies have shown that taking CS supplements may improve joint function and slow down cartilage degradation in people with osteoarthritis, especially in combination with glucosamine [25]. However, other studies have found no significant benefit of CS over either a placebo or conventional medications [26]. Therefore, more research is needed to confirm the effectiveness and safety of CS for osteoarthritis and other joint conditions.

The quality and purity of CS supplements may vary depending on the biomass source and the extraction and purification processes. Therefore, it is important to choose products that are certified by third-party testing agencies and follow the recommended dosage and instructions. CS is considered safe when taken by mouth for up to six years, but it may cause some mild side effects, such as stomach upset, nausea, bloating, diarrhoea, or constipation [27]. People with certain medical conditions, such as asthma or prostate cancer, should consult their doctor before taking CS supplements. CS may also interact with some medications, such as warfarin (Coumadin) [28]. CS can also be used as a biomarker for disease diagnosis and prognosis [2]. This is because CS can be detected in various biological fluids (blood, urine, cerebrospinal fluid, and synovial fluid), and reflect the pathological conditions of the tissues or organs. For instance, CS can be used as a biomarker for osteoarthritis by measuring its concentration and molecular weight distribution in the synovial fluid. CS can also be used as a biomarker for other diseases, such as cancer, cardiovascular disease, neurological disease, and inflammatory disease [29,30]. However, further research is needed to explore its mechanisms of action, optimise its formulations and delivery methods, and evaluate its safety and efficacy in clinical trials.

3. Sources of chondroitin sulphates and their biological functions

Based on the current literature, the sources of CS could be marine origin (shark, fish, squid); land animals (bovine, porcine, avian); plants; or microbial fermentations [31,32], etc. (Table 1). Commercially CS, and other GAGs, have been produced from animal tissue such as the trachea and nasal cartilage of bovine, porcine, ovine, and other mammals [33–35] as well as chicken keel [36], and marine organisms including shark and fish [37–42]. Currently, the major commercial source for the production of CS is the cartilage of marine organisms. CS-C (6S), for example, is extracted from shark cartilage, and CS-A (4S) from whale cartilage [40,42].

The previous research findings have shown that the characteristic structures of CS exhibit various biological functions by interacting with other molecules present in the cells and tissues (Table 1). Their reported biological activities include, but are not limited to, antioxidant activities [43], pre-biotic [44]; anti-cancer [44,45]; anti-inflammatory [46]; fibronectin interactions [47,48]; regulation of retinal neuronal patterning [49]; Neuritogenic activity [50,51]; plasminogen activation [52,53] and monocyte and B-cell activation [54,55].

3.1. Animal chondroitin sulphate

Commercially available forms of CS are sourced from animal cartilage like bovine, porcine, chicken, and crocodile [41,56,57]. However, animal-derived CS has a negative impact on therapeutics use because of the risk of viral and/or prionic contaminations that may cause Bovine Spongiform Encephalopathy (BSE) and Epizootic Aphtha [2].

Safety and quality concerns of animal-derived CS are also likely due to mixtures of sources that may result in a CS product with mixed characteristics and properties. For example, the use of un-segregated combined raw animal materials such as tissues, bones, soft organs, and cartilage will result in a final product with unreproducible mixed structures, a variable grade of purity, and variable biological effects [58,59]. It was well-researched that the complex structure of CS strictly depends on the tissue, organ, and source of the organism, and even the age of the animals [58].

Animal-derived CS is a concern for vegetarians and people with dietary restrictions due to religious beliefs. The introduction of animal-derived CS supplements has been prohibited in Middle Eastern and Asian markets due to religious beliefs and/or the dietary practice of not using animal products [60].

3.2. Fish chondroitin sulphate

There are several fish and their by-products (such as fins, scales, skeleton, bone, cartilage, eyeballs, viscera, etc.) from the fish-processing industries that can serve as low-cost, easy-to-use sources of CS [61,62]. Cartilaginous fish [salmon, ray, and skate [43,51,63–65] fish bones and by-products [flat, goose, tilapia, rabbit] [64,66,67], and sharks [43,45,46,63,64] are the sources of various commercial CS preparations. CS obtained from tilapia (*Oreochromis niloticus*) possesses a sulphate group at the carbon position four of galactosamine (GalNAc), and was found to have a non-cytotoxic concentration of 200 μg/mL. This CS was also reported to show antioxidant activities [66]. However, enzyme chondroitinase treated total CS of tilapia showed CS-A (C—4S) disaccharide (59%) followed by CS-C (C—6S) disaccharide (36.6%) and non-sulphated (C—0S) disaccharide (3.4%) units [66]. The CS from shark cartilage may contain various disaccharide units such as C—0S, C—6S, C—4S, C-2,6diS, C-4,6diS, and C-2,4diS [64].

Table 1
All natural sources of chondroitin sulphates (CSs), methods used for extraction/production, types, biological activities, and reported yield.

Natural sources	Special tissue/ body parts	Specifics associated to extraction and purification	CS type (Di-)	CS yield (%)	Biological activity/ applications	References
Bovine	Trachea cartilage	Hazard chemicals	0S, 6S, 4S	12.6	Repair of central nervous system	[6,76,120]
	Nasal cartilage	Papain digestion		7.8		[83]
	Nasal septa	Hazard chemicals		19-23		[76]
Chicken	Keel cartilage	$MgCl_2$ + dialysis + papain digestion + ethanol precipitation		16.8	Prebiotic, anti-cancer	[44,57]
	Trachea		0S, 6S, 4S			[6]
Shark	Fin cartilage	Boiling + papain digestion + hazard chemicals		15.05	Anti-inflammatory, osteoarthritis	[46,63]
	Cartilage	Lyophilised + powder + alcalase digestion + hazard chemical + ethanol	0S, 6S, 4S, 2,6diS, 4,6diS, 2,4diS	9.7	Anti-cancer, antioxidant activities	[43,45,64]
Fish	Ray cartilage	Boiling + papain digestion + hazard chemicals		7.49	Neuritogenic activity	[51,63]
	Salmon cartilage	Lyophilised + powder + alcalase digestion + hazard chemical + ethanol	0S, 6S, 4S, 2,6diS	3.5	Antioxidant activities	[43,64]
	Tilapia viscera	Proteolysis, ion exchange and acetone fractionation	4S		Antioxidant activities	[66]
	Skate cartilage	Lyophilised + powder + alcalase digestion + hazard chemical + ethanol	0S, 6S, 4S, 2,6diS, 4,6diS, 2,4diS	12.5	Anti-inflammation, hepatic dyslipidemia	[64,65]
	Flatfish bone	Lyophilised + powder. + alcalase digestion + hazard chemical + ethanol	0S, 6S, 4S, 2,6diS, 4,6diS, 2,4diS	1.3		[64]
	Flatfish head	Lyophilised + powder. + alcalase digestion + hazard chemical + ethanol	0S, 6S, 4S, 2,6diS, 4,6diS, 2,4diS	1.4		[64]
	Goosefish Bone	Lyophilised + powder. + alcalase digestion + hazard chemical + ethanol	0S, 6S, 4S, 2,6diS, 2,4diS	2.6		[64]
Crocodile	Sternum cartilage	Boiling + papain digestion + hazard chemicals		20.09		[63]
	Trachea	Boiling + papain digestion + hazard chemicals		14.72		[63]
	Hyoid	Boiling + papain digestion + hazard chemicals		27.37		[63]
	Rib	Boiling + papain digestion + hazard chemicals		9.05		[63]
Fermentation	*Escherichia coli* O5:K4:H4	Defructosilation and selective sulphation	4S, 6S, 4,6diS			[68,69]
	Escherichia coli O5:K4:H4	Precipitation with cetavlon followed by repeated precipitations with ethanol	K4 CPS	0.08-0.09 g/L		[70]
	E. coli O10:K4:H4	Ultrafiltration with 10 kDa membranes followed by repeated precipitations with ethanol	K4 EPS, K4 CPS	332 mg/L		[71]
	Bacillus subtilis natto	Growth in shake flask	CS	237.7 mg/L		[2,121]

3.3. Vegan chondroitin-like polymers

CS has established nutraceutical and pharmaceutical applications globally. However, due to its high demand, source, and potential limitations in existing extraction/production methods, there is increasing consumer interest in an alternative production route. However, it has to be used with caution that alternative CS or CS-mimic polymers don't have sufficient research evidence for their health benefits. Therefore, animal- or shark-origin CS may still be considered as sustainable source, whose by-products are used for CS production.

Phytodroitin is a plant-derived alternative to chondroitin. It is a natural complex polysaccharide resultant of a fermentation process and comprises glucuronic acid and *N*-acetylglucosamine along with mucopolysaccharide-rich extracts of algae (https://protecnutra.com/phytodroitin/ last accessed on 15/03/23; https://www.vegetology.com/blog/phytodroitin-vegan-alternative-to-chondroitin last accessed on 15/03/23). The mucopolysaccharide structure of phytodroitin was found 'essentially similar' to CS of Avian and shark origin, but slightly different to bovine origin as per Fourier transform infrared (FTIR) spectroscopy analysis. MythoChondro is another fermentation-derived vegan chondroitin sulphate-mimic available in the market by Gnosis. It was found in a clinical trial that Mythocondro has higher bioavailability over animal-derived CS and was included as a novel food according to the Novel Food Regulation (EU) 2015/2283. (https://gnosisbylesaffre.com/ingredient/mythocondro/ last accessed on 15/03/23). Mythochondro contains shark-like CS that is produced by fermentation and chemical sulphation for food supplement applications [32]. There is another biotechnologically produced non-sulphated chondroitin such as Sinogel was recommended for biomedical applications through intraarticular injection [32]. Several prokaryotic bacteria such as *E. coli* O5:K4:H4, *E. coli* O10:K4:H4, *E. coli* K-12 MG1655, *Bacillus subtilis natto*, *Pichia pastoris*, etc. were known to produce chondroitin or chondroitin-like polysaccharides (Table 1, [2,68–71]. One of the enzymes related to CS biosynthesis was reported from two pathogenic bacteria such as *Pseudomonas aeruginosa* serotype O6 and *Yersinia enterocolitica* serotype O8 [72,73]. A functional enzyme chondroitin synthase was also recently discovered in the non-pathogenic green sulphur bacterium *Chlorobium phaeobacteroides* [74]. These known and currently undiscovered bacterial strains represent alternative ways to produce vegan chondroitin or CS-mimic through the fermentation and chemical or chemoenzymatic synthesis process.

There is increasing demand for alternative CS sources due to impurities of animal origin as well as dietary preferences. Fish processing industries produce by-products that could be a suitable source of CS. However, there are no standard extraction and characterisation methods that make CS production from fish by-products challenging, especially the quality and purity of the end-products with expected medicinal/pharmaceutical benefits [75]. Likewise, animal-derived CS production faces challenges such as variable molecular weight and sulfonation patterns, which are the key features of CS that determine their biological activities [17]. Nevertheless, as per the experimental evidence available in this field, animal and marine by-products can be considered sustainable sources of CS, as long as their green extraction and quality analysis are implemented.

4. Conventional and green extraction methods

There are different methods of extraction depending on the type of raw material and the desired purity as well as the yield of CS [42]. Some of the common methods can broadly be divided into 1) Chemical extraction method, 2) Enzymatic extraction, and 3) physical process extraction. The last two methods can be considered green extraction

methods, which may go together in steps for the best extraction and purification of high-purity CSs. The most common methods for CS extraction from all biomass types include steps such as (1) hydrolysis of biomass by chemicals; (2) breakdown of proteoglycan core; (3) chemical precipitation and removal of proteins; and 4) recovery of partially purified CS [8,34,42,63,76–78].

4.1. Chemical extraction processes

Chemical extraction processes are conventional methods of CS extraction that can be based on acid and/or alkaline extraction. In the first type of acid extraction, the cartilage tissues are treated with dilute acids, such as hydrochloric acid (HCl) or sulfuric acid (H_2SO_4), to dissolve the CS and other components. Later, the CS is precipitated with solvents like ethanol (C_2H_6O) or acetone (C_3H_6O) and purified by repeated dissolution and precipitation process. In the alkaline extraction method, the cartilage tissues are treated with dilute alkalis, such as sodium hydroxide (NaOH) or potassium hydroxide (KOH), to dissolve the CS and other components. Later, the CS is precipitated with solvents like ethanol or acetone, and the CS is then purified by repeated dissolution and precipitation. Although the process of the alkali method is simple, the use of high concentrations of alkali may cause degradation of CS, affecting its biological function [79].

The above conventional CS extractions, used in industrial production, have been carried out by alkaline hydrolysis with NaOH, urea [$CO(NH_2)_2$], or guanidine HCl (CH_5N_3·HCl), and then the GAGs are selectively precipitated by alcohols, cetylpyridinium chloride (CPC, $C_{21}H_{38}ClN$) and potassium thiocyanate (KSCN), both harmful chemicals or non-ionic detergents [76]. The final steps in these protocols are deproteinization by trichloroacetic acid (TCA, $C_2HCl_3O_2$), precipitation and purification by gel filtration, ion exchange, and/or size-exclusion chromatography [34,63]. So, in these chemical-based methods, the use of several chemicals such as acetone, chloroform, methanol, TCA, ethanol, CPC, sodium acetate, and sodium hydroxide have been reported. However, most of the chemical-based methods are not completely efficient in producing highly pure CS. Moreover, these methods are time-intensive, need high costs for scaling up, and are not environmentally friendly.

4.2. Green extraction processes

Current extraction of CS methods consists of tissue hydrolysis, protein removal, and purification. To break down the complex structure and remove CS from the other GAGs, enzymes such as alcalase, papain, trypsin, etc. have been researched; then various solvents and detergents are used. In some methods, chemical hydrolysis (after washing of biomass and heat-treatment) was undertaken to ensure a complete breakdown of the complex GAG core structure.

The conventional extraction and purification methods of CS are often time-consuming, energy-intensive, and environmentally unfriendly. Therefore, there is a need for a green and efficient process to extract and purify CS from various biomass sources. Although certain reports on CS extraction used enzymatic digestion and physical processes (ultrasonication, microwave digestion) as green extraction or environmentally friendly methods [41,78,80–82], which to our best understanding are not completely green extraction methods as these methods possess steps where chemicals or solvents are still used.

4.2.1. Enzymatic extraction of CS

This is a part of the green extraction process, which involves treating the cartilage tissues with enzymes such as alcalase, chymotrypsin, papain, pepsin, Protin NY100, or trypsin to digest the proteins and release the CS [78,81,83–85]. Proteolytic digestion of any biomass using either chemicals or enzymes is the most critical step in terms of extraction yield and subsequent purification [86]. The released CS is then dialysed against water/buffer, small peptides are precipitated with TCA or CPC, and finally, the clear supernatant containing CS is lyophilised to obtain dry CS powder. The first step for purified CS production is thus the extraction of crude GAGs either chemically or enzymatically. The removal of lipids using chloroform and acetone was undertaken for fish samples, these were dried before the extraction process [87]. In the next step, proteins are removed by TCA to recover specific GAGs from the resulting extracts, which is the most frequently used approach for various biomass sources (Fig. 2). Not that all enzymatic extraction methods are equally efficient, because: 1) type of chosen enzyme acts on differently on the polypeptides and may need higher temperature and time, 2) post-enzyme digestion steps may still have chemical-based processing, 3) ultrafiltration-diafiltration steps, alternative to chemical-based processing after enzymatic digestion, requires specific pore size membrane filters, a pump and a pressure sensor as the membranes may be blocked easily if the enzymatic digestion is incomplete [42].

4.2.2. Physical process of extraction of CS

4.2.2.1. Ultrasound-assisted extraction. Ultrasound-assisted extraction (UAE) is an efficient extraction-assistance protocol that recently became popular for use with various plant metabolites processing as well as CS extractions [82,88–90]. UAE can be considered a green and economically viable alternative to conventional solvent-based techniques for food and natural product extraction. The UAE reduces the extraction and processing time and thus reduces energy requirements, unit operations cost, and CO_2 emissions. In this UAE method, biomolecules are extracted based on single or combined mechanisms, when ultrasound acts directly on the biomass to release the metabolites. UAE enhances extraction yield, and economy and has green impacts [88]. Ultrasound helps in extracting the target compound through the phenomenon of cavitation (mechanical action) in the specific solvent medium. This cavitation phenomenon is a transient process due to the combined characteristics of mechanical and thermal mechanisms when a bubble violently collapses in the solvent medium, which aids in extraction efficiency [8]. The mechanical ultrasonic effect promotes the release of soluble compounds from the biomass through cell wall disruption, enhancing mass transfer, and increasing solvent access to cellular content [88].

CS from porcine cartilage was extracted using an ultrasound-assisted alkaline method, where the crude biomass solution was ultrasonicated to remove residual proteins. Then CS was purified with a stepwise process by using kaolin clay, activated charcoal, ethanol precipitation, and drying [90]. A recent study used ultrasound-assisted enzymatic extraction (UAEE) using various enzymes such as alcalase, papain, and Protin NY100 to extract CS from squid cartilage (*Dosidicus gigas*) [81]. The results of this study found that ultrasound-assisted alcalase had the best extraction efficiency. The response surface methodology (RSM) was used to understand the relationship between extraction conditions and the yield of CS. The ridge max analysis helped determine the maximum extraction yield, optimum extraction temperature (59.4 °C) and time (24 min), optimum pH (8.25), and optimum enzyme concentration (alcalase, 3.6%). The study suggested a green and efficient process for CS extraction and purification from squid cartilage [81]. There was another report on ultrasound-assisted extraction of CS from jumbo squid cartilage where the optimum extraction conditions identified were: extraction temperature (42 °C), extraction time (46 min), and NaOH concentration (4.15%). The resultant crude extract was ethanol precipitated to obtain CS with only 23.7% yield and 82.3% purity [82].

4.2.2.2. Microwave-assisted extraction. Microwave-assisted extraction (MAE) method is a combination of precise microwave technology and a conventional solvent extraction process. This method has been developed in recent years for various biomolecule extraction, particularly from plants and nowadays CS from fishes [80,88,89]. MAE is a technique that uses microwave radiation to accelerate the hydrolysis of

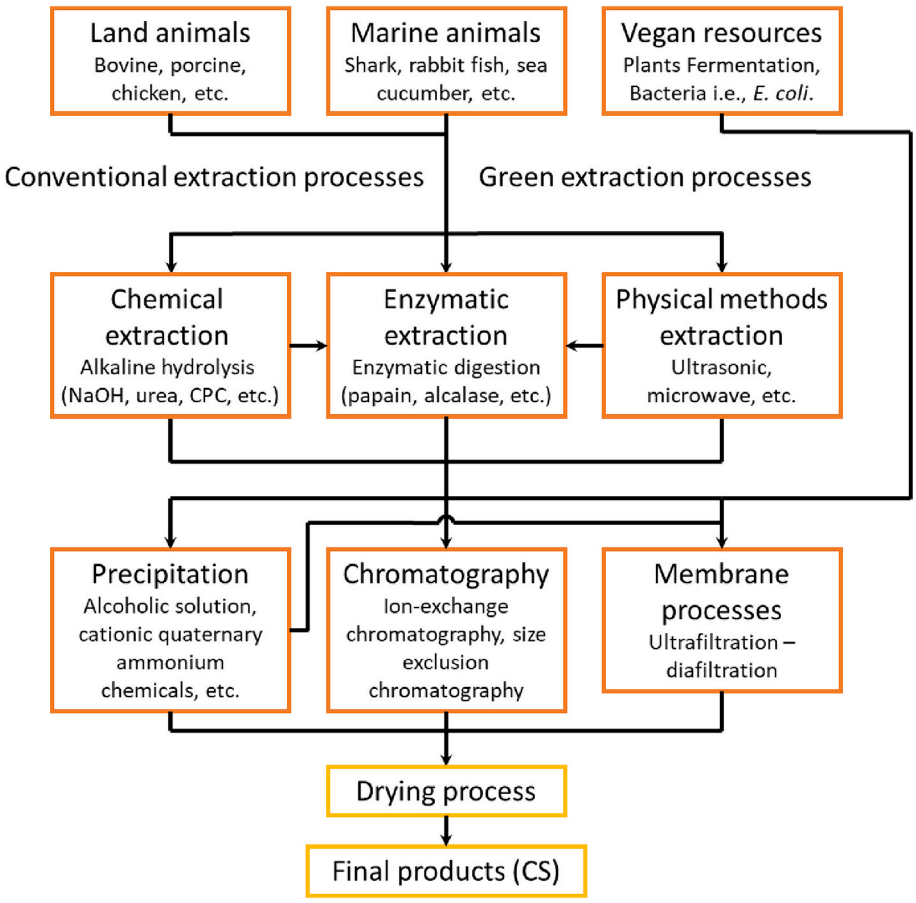

Fig. 2. Schematic showing general steps of extraction and purification of chondroitin sulphates (CSs) from various sources.

cartilage tissue and isolate CS from the complex matrix. MAE technique has several advantages over conventional methods, such as shorter extraction time, lower alkali concentration, higher yield and purity, and reduced energy consumption [80]. In this study, Cheng et al. optimised the MAE of CS from tilapia by-product by using RSM. The optimum CS extraction conditions identified were microwave power of 252 W, microwave time of 5.64 min, ratio of solid to liquid of 1:29, and NaOH concentration of 7%. Using the above optimum conditions, the mean extraction rate of CS was in good agreement with the predicted model value, and hence, this study suggested that microwave-assisted extraction of CS from tilapia by-product is a feasible and efficient method for industrial production [80]. Further, the method for CS extraction from tilapia was improved by using ultrasonic-microwave synergistic extraction [89].

4.2.2.3. Pulsed electric field (PEF) assisted extraction. The pulsed electric field (PEF) is another promising process for the extraction of CS [79]. PEF is a non-thermal technique that can enhance the extraction of bioactive compounds from plant and animal biomasses. Extraction of CS using PEF involves applying high-voltage pulses to disrupt the cartilage tissue and release the CS into the solvent. NaOH solution with a concentration of >3% is required as the electrolyte, further development is needed to reduce the consumption of chemicals. However, this extraction method has several advantages over conventional methods, such as higher yield and purity of CS, shorter extraction time and lower energy consumption, reduced use of chemicals and enzymes, retention of the biological activity, and structural conformation of CS. The study of He et al. [79] with fish bone concluded that PEF can widely be used to extract CS with non-thermal performance, high speed, and lower environmental pollution. Another study by He et al. [91] combined the semi-bionic extraction (SBE) method and PEF to rapidly extract the maximum contents of calcium, CS, and collagen from the fishbone.

4.2.2.4. High pressure assisted extraction. This method involves treating the cartilage with high-pressure water or steam to disrupt the tissue structure and release the CS [92]. The CS is then separated by filtration, centrifugation, or ultrafiltration and purified by chromatography or electrophoresis. In this study, high hydrostatic pressure (HHP) was combined with enzymatic hydrolysis (with papain) which is a new extraction process tested for isolating CS from antlers cartilaginous tissues. This work aimed to determine the effect of high pressure, temperature, and time of incubation on the effectiveness of enzyme activity. The results were promising that high pressure (100 MPa) yielded 95.1% CS extractability, while low extractability (19%) of CS was obtained in ambient pressure (0.1 MPa) [92].

4.2.2.5. Solvent-free mechanochemical extraction. This is one of the innovative solvent-free mechanochemical extraction (or solid-state mechanochemical extraction) methods used for CS extraction from shark cartilage by Wang and Tang [41]. This method is considered a superior approach to substitute the conventional heating-based extraction methods. A solvent-free mechanochemical extraction (SFMCE) method can significantly reduce the energy cost due to the reduced extraction time from 3 h to 3 min. This SFMCE method consists of two steps: (1) mechanical pre-treatment (or mechanical pre-activation) of the raw biomass; and (2) reactive mechanochemical treatment of the pre-activated biomass with solid reagent and abrasive under high-intensity mechanical stress in AGO-2 centrifugal-planetary mill. Therefore, in SFMCE both the physical change of raw biomass and the chemical transformation of the target compound (e.g., CS) are expected.

In this method of extraction, the mechanically pre-treated biomass powder was co-grounded for 3 min at room temperature to obtain mechanochemical composites (MCs). The resultant MCs were rapidly diffused in a 3% NaCl solution. The insoluble components were then removed after acidification of the NaCl solution, and centrifugation. The water-soluble content containing CS was precipitated with two volumes of ethanol. The CS pellets were dehydrated with two ethanol washes and the CS powder (sodium salt) was obtained after drying in an oven at 80 °C. The yield and purity of CS achieved using the SFMCE method were increased respectively by 9.7% and 10.07% compared to the conventional alkaline extraction method [41]. This SFMCE extraction method was also effectively used for the extraction of polysaccharides from bamboo leaves [93]; extraction of carboxymethyl cellulose from rice husks [94], polysaccharides from the fungus *Ganoderma lucidum* [95], and other biomolecules.

Table 2 summarizes different methods for the recovery and purification of CS from various biomass sources. The methods include precipitation, column chromatography, pulsed electric field (PEF) extraction, and membrane processes such as ultrafiltration-diafiltration. Organic solvent precipitation involves the use of solvents such as ethanol, sodium acetate, isopropanol, and TCA to precipitate CS from a pre-treated biomass solution. The purity and recovery rates vary depending on the solvent and biomass source used. The addition of salts could enhance the precipitation of proteins and peptides and help in upgrading CS purity. For example, using 1.4 volumes of ethanol and 3.8% sodium acetate resulted in a purity of 97.5% and a recovery rate of 96% [96]. Another common method used in the industrial production of CSs is precipitation by the organic solvent and/or inorganic salts. However, the drawback is that the use of organic solvents and/or other chemicals is not an environmentally friendly process, hence a more sustainable process is required.

Column chromatography involves the use of anion-exchange resins

Table 2
Conventional and green extraction methods used for the extraction of CS from various biomass sources.

Methods	Sources of biomass	Chemicals and/or materials	Specifics associated with extraction and/or purification	Purity	Recovery	References
Precipitation (organic solvent, salts)	–	Ethanol and sodium acetate	Sodium acetate (3.8%) and 1.4 volumes of ethanol	97.5%	96%	[96]
	–		One volume of ethanol and 0.2 M of NaOH	>96%	>96%	[96]
	Shortfin mako shark (*Isurus oxyrinchus*)	Isopropanol	Isopropyl alcohol (40%, v/v) + sodium chloride (2%, w/v)	–	57%	[122]
	Buffalo (*Bubalus bubalis*) cartilages	Trichloroacetic acid (TCA)	TCA solution (10%) at 4 °C for 12–18 h	–	–	[34]
		Alkaline-hydroalcoholic-saline solution precipitation	NaOH (0.48 M), 1.07 volumes of ethanol and 2.5 g/L of sodium chloride	–	–	[123]
	Raja porosa cartilage	Ethanol precipitation	Three volumes of anhydrous ethanol	94%	36.51%	[124]
	Chicken leg bone	Precipitation	Trichloroacetic acid (7%, w/v), then 70% (v/v) ethanol precipitation	–	Total yield of 0.14% and the recovery rate of 67.35%	[125]
Column chromatography		Ion exchange chromatography	Column (2 × 6 cm) packed with DEAE-cellulose anion-exchange resin, eluted by 50 mM sodium chloride for 150 min at 1 mL/min	–	–	[97]
	–	Ion exchange chromatography	DEAE-cellulose anion-exchange resin, eluted by 100 mM sodium chloride	–	–	[40]
	Chicken keel cartilage	Ion exchange chromatography + molecular sieve chromatography	Chromatography using 732 cation exchange resin column and Sephacryl-300 HR gel column	99.01%	28.05%	[36]
	Lumpsucker fish, *C. lumpus*	Preparative chromatography	Column with anion exchange resin (Lewatit VPOC1074/S6328 A 1:1 Lanxess), washed with 1 M or 5 M sodium chloride	–	–	[126]
Pulsed electric fields	Fish bone	High intensity pulsed electric fields	Material–liquid ratio of 1:15 g/mL, electric field intensity of 16.88 kV/cm, pulse number of 9, and NaOH (3.24%).	Highly purified CS as standard	Maximum yield of 6.92 g/L	[79]
Membrane processes	Rabbit Fish (*Chimaera monstrosa*)	Ultrafiltration-diafiltration	Ultrafiltration membranes of 100 and 30 kDa (spiral polyethersulfone, 0.56 m^2, Prep/Scale-TFF, Millipore Corporation, USA)	99%	–	[67]
	Shortfin mako shark (*Isurus oxyrinchus*)	Ultrafiltration membrane (3 kDa)		–	–	[122]
	chicken breast cartilage	Ultrafiltration membrane (30 kDa)	Polyethersulfone-based UF30 membrane with the MWCO of 30 kDa, at permeation flux of 5.0 $L{\cdot}m^{-2}{\cdot}h^{-1}$	–	–	[127]
	Central Skeleton Wastes of Blue Shark	Ultrafiltration-diafiltration (30 kDa)		97%	2.8% (w/w of skeleton)	[123]
	Blackmouth Catshark (*Galeus melastomus*)	Alkaline treatment, hydroalcoholic alkaline precipitation, then 30-kDa membrane separation	Ultrafiltration (UF) and diafiltration (DF)	81.2%, 82.3% and 97.4% respectively	–	[128]
	Head by-products of blue shark (*Prionace glauca*)	Enzyme digestion + precipitation + UF-DF	Alcalase hydrolysis + alkaline-hydroalcoholic saline solutions (NaOH: 0.54 M, ethanol: 1.17 volume, sodium chloride (2.5%) + ultrafiltration – diafiltration (sequential cascade of 100 to 30 kDa membrane)	98.5%	12.08 ± 0.72% (w/w of dry cartilage)	[78]

such as DEAE-cellulose to separate CS from other components in a solution [97]. The advantage of chromatography separation is that it can produce CS at high purity. However, the capacity of chromatography does not meet the requirements for large-scale CS production.

Membrane processes such as ultrafiltration-diafiltration involve the use of membranes with specific pore sizes to separate CS from other components in a GAG-rich solution [67]. For the membrane processes, current reports indicate that a precipitation pre-treatment is needed before filtration. Therefore, even though membrane processes are environmentally friendly, the introduction of chemicals weakens their environmental friendliness. To form a greener process, precipitation should be avoided. Current reports suggest that a precipitation step is necessary before the ultrafiltration-diafiltration (UF-DF) process. However, if the goal is to make the process entirely green, then the precipitation step could be replaced with a microfiltration process. In this continuous extraction/purification process, ground cartilage or any biomass of choice is enzymatically digested in a bioreactor and the fermentation broth is then filtered through a microfiltration membrane. CS and its by-products such as peptides and fatty acids pass through the permeate. The permeate is then filtered through an ultrafiltration membrane and small peptides and fatty acids are removed via diafiltration (Fig. 3). Recently, Tsai et al. [81] have reported a laboratory success on green extraction and purification of CS by using hollow fibre dialysis. In this study, the ethanol precipitated CS was dissolved in distilled water and lyophilised. An automatic tangential flow filtration system with a 10 kDa modified polyethersulfone (mPES) hollow fibre filter module was used to flush out the low molecular weight molecules and impurities from the crude extracts. The retentate was recovered and freeze-dried to obtain pure CS. The yield and purity of CS using a hollow fibre dialyser were higher compared to ethanol precipitation [81].

5. Quality control

Both the content and purity analysis of CS during any type of extraction and purification methods are the key requirements for CS production, not only for pharmaceutical but also for nutraceutical applications. CS, as described previously, is generally produced employing appropriate extraction followed by further purification from any selected biomass. Based on the selected methods of production, the end CS content, purity (presence of co-products), and types of CS (variously sulphated CS depending on source biomass) may be different. There is a lack of standard and common extraction and quality control techniques for CS production which is a challenge for this sector [75]. Commercial CS production may include various sources and types of raw materials, which pose a serious threat due to potential contamination with animal-derived pathogens. The heterogeneous structure and physicochemical profile of CS vary with the source organisms and types of tissues used in the extraction of CS.

The biological functions of CS are due to its interaction ability with a wide variety of macromolecules such as growth factors, protease inhibitors, adhesion molecules, matrix molecules (collagens, elastin, and microfibrillar proteins, proteoglycans including hyaluronan, and non-collagenous glycoproteins), cytokines, chemokines, and pathogen virulence factors through its unique sulphated saccharide domains [5,98]. Biological functions such as antioxidant and anti-inflammatory activities of CS are associated with its molecular weight [99,100]. For various medical applications, sulphation of CS is an essential requirement. It was reported that the origin and extraction method could influence the quality of CS [58] and that desulphation could happen during the extraction process of heating CS in dimethyl sulfoxide containing 10% of water or in methanol at 80 °C [101]. Acidic or alkaline conditions used for extractions and the subsequent exposure to certain solvents could also accelerate the de-sulphation process [102].

Current industrial production of CS usually involves alkaline hydrolysis [8], which may result in the production of poor-quality CS products. In a recent study, commercially available sixteen pharmaceutical-grade CS samples were tested and found that eleven out of the sixteen samples contained <15% of CS [103]. The remaining five samples were found to possess varied structures and had different sizes and degrees of sulphation [103]. A variety of production protocols utilise oxidising or reduction chemicals in their extraction and purification, this may alter the CS quality through their structural modifications like desulphation or over sulphation. Therefore, strict quality control is required including the traceability of CS origin before it becomes a commercial product. So, the overall commercial CS production process should include: i) process monitoring, ii) CS quantitation, and iii) safety testing for impurities.

5.1. Process monitoring

One of the main challenges in CS production is the process monitoring to check the product quality in real-time. Currently, there are several highly sophisticated molecular diagnostic methods are available that can be used (in combinations) for the quality control analysis. However, all methods are not suitable for realistic and robust monitoring of the process, as most of these sophisticated methods for CS analysis require chromatography, electrophoresis, or spectrophotometry, and are based on specific sample preparation, costly instruments, and skilled operator [60]. To monitor the process of extraction and steps during purification, the crude extracts and purified fractions can be

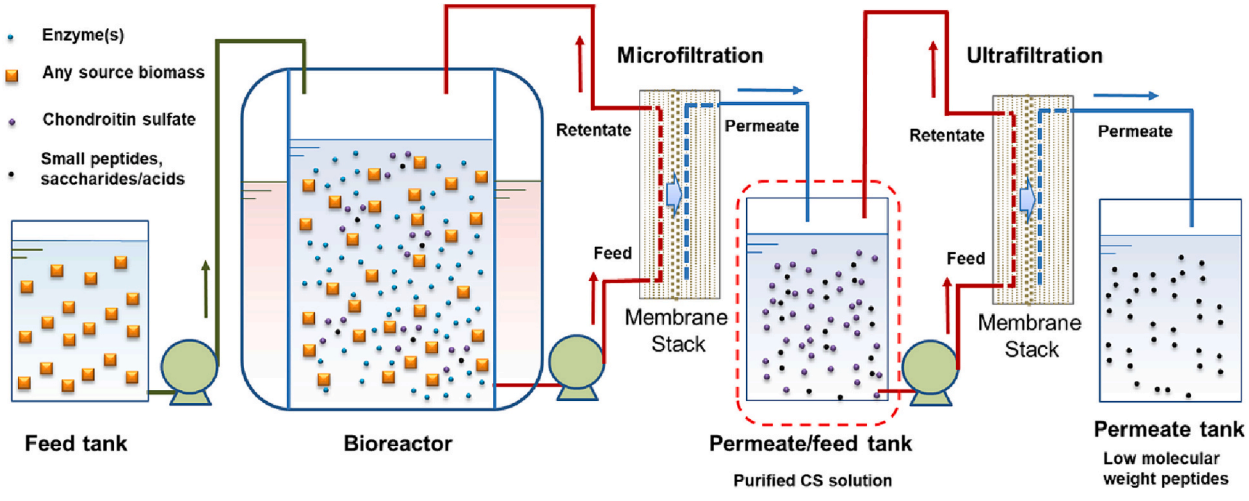

Fig. 3. Schematic diagram of green extraction and purification protocol for CS production.

tested for total CS content by a rapid Dimethyl-methylene blue (DMMB) assay [34,104]. While to estimate the quantity and identification of each type of CS, simple SAX-HPLC (strong-anion exchange HPLC) analysis [64,105], also called enzymatic HPLC (eHPLC) [58,106] is used as one of the best analytical methods of choice. In the above HPLC method, the purified CS sample to be tested is first digested with the enzyme chondroitinase ABC (lyase) to obtain the disaccharide backbone. High-performance size-exclusion chromatography (HPSEC) is another powerful process monitoring technique that can be used for sizing, quantification, and molecular weight determination of CS fragments [105]. The size range of HPSEC is defined by the pore size of the column and the operational parameters (e.g., column dimension, mobile phase, and flow settings).

Infrared spectroscopy is another powerful analytical methodology used in academic laboratories and industries for the analysis of molecules relevant to the pharmaceutical, chemical, and polymer industries including CS analysis [34,99,107–109]. Infrared spectroscopy that uses mid-infrared (MIR) wavelengths (between 20 and 2.5 μm) of light is called FTIR spectroscopy, while Infrared spectroscopy that uses near-infrared wavelengths (between 2.5 and 0.7 μm) of light is called NIR spectroscopy. These infrared spectroscopy methods can provide information about the chemical composition and structure of materials without destroying them but were not suitable for online or in-line process monitoring until recently. Nowadays, thankfully both NIR and FTIR portable devices are available that will be suitable for CS production process monitoring [108,110].

Nuclear Magnetic Resonance (NMR) spectroscopy is another powerful analytical method used to obtain detailed information about the structure, composition, and dynamics of various substances in academic institutions including in CS studies [99,105,111]. For real-time monitoring of various production processes like chemical reactions, bioprocesses, pharmaceutical manufacturing, and CS production, a portable NMR can be used. The use of an NMR device for the production process monitoring is an advantage in that it is non-invasive and non-destructive for samples during the on-going incubation conditions and the process can be a continuous flow system.

Certain laboratory-scale extraction and purification steps of CS production have been successfully monitored through gel electrophoresis [64,103,112], which is a visually compelling analytical method that can suitably be used for quality control for CSs. For example, simple agarose-gel electrophoresis was suitable for detecting the presence of other glycosaminoglycans, such as heparin, HS, DS, and hyaluronic acid in the CS preparation [106]. To confirm the absence of DS (CS-B) in the CS preparation, chondroitinase-treated samples were analysed by 0.5% agarose gel-electrophoresis in 0.04 M of barium acetate buffer (pH 5.8) [64]. Cellulose acetate electrophoresis can be carried out for standard CS or any GAG preparations from different stages of purification in 0.1 M potassium phosphate buffer (pH 7.0). It also may be possible to study the degree of sulphation by electrophoresis in 0.1 M HCl, which will allow ionisation only in sulphate group [113]. After electrophoresis, each resolved band can be stained with 0.5% (*w/v*) Alcian blue in aqueous acetic acid (5%, *v*/v) and de-stain thereafter with deionized water [112]. Da Cunha et al. [103] also used agarose gel electrophoresis to resolve CS, DS, and HS in agarose gel using 1,3-diaminopropane-acetate buffer at alkaline pH. After the electrophoresis, the gels were dried after in-gel precipitation of GAGs by cetyltrimethylammonium bromide (cetavlon). Then the dried gels were stained with toluidine blue for the development of metachromatic bands for the GAGs.

5.2. Quantitation of chondroitin sulphates

Quantitative estimation of CS content from any crude extracts and/or dietary supplements possessing CS would be extremely challenging because of their wide molecular weight variations of heteropolymers, poor UV absorbance of CS, and CS being strongly ionic. Additionally, in the presence of other types of GAGs as impurities or because of adulteration, the analytical method to be used for the quantification of CS must be precise for CS while ignoring the presence of other contaminating GAGs. The quantification of CS from the crude extracts or during purification stages can be carried out by sulphate GAGs assay [also known as DMMB assay] using a calibration curve made of standard CS [34,63]. In this assay, 1,9-dimethylmethylene blue is used as a metachromatic dye to react with sulphate glycosaminoglycan and provides an absorption peak at 525 nm. DMMB assay requires at least a tetra-saccharide to show the colour change of the assay solution. However, some researchers found interference of DNA in this assay, which could be minimised by decreasing the pH to 3 and increasing salt concentrations in the assay solution [114].

For proper purity check and quantification of CS types, enzymatic hydrolysis of CS before HPLC methods is routinely used both academically and industrially [58,64,105,106]. For HPLC quantification, first, the CS samples are depolymerised with the chondroitinase ABC (lyase) or chondroitinase AC enzyme to obtain different types of disaccharide backbones (e.g., CS-0S, CS-4S, CS-6S, etc.). Chondroitinase ABC can depolymerase both CS and DS, while chondroitinase AC can specifically depolymerase CS [115]. Then these disaccharides are resolved using the most popular SAX column, and the resolved peaks can be compared with the retention time for the identification of CS-types, and quantification based on the calibration curves of the standard CSs [64,105,106,58]. There are reports for the use of other HPLC methods including one where both amido and amino columns were used to separate disaccharides under acidic conditions [116]. Likewise, there was a report on a rapid HPLC method using a C18 column and octane sulfonic acid as a mobile phase for the determination of CS from finely powdered raw material dissolved in water. This rapid HPLC quantitation method is based on the ability of CS to absorb light at 195 nm [117].

5.3. Safety testing for impurities

To investigate the safety of animal-derived CS products, these must be screened for microbiological safety like bacterial, fungal, and yeast profiles apart from pathogenic prion protein (PrP). Bovine spongiform encephalopathy (BSE) is a fatal neurodegenerative disease affecting humans and animals caused by the abnormal form of PrP protein. In the EU, BSE and other animal transmissible spongiform encephalopathy (TSEs) are subject to EU (EC 999/2001) and national (S.I. No 156 of 2018) legislation (https://www.efsa.europa.eu/en/topics/topic/bovine-spongiform-encephalopathy-bse last accessed on 26/05/23). Standard techniques for protein detection such as Western blotting and enzyme-linked immunosorbent assays (ELISAs) are commonly employed for PrP^{TSE} evaluation. The use of tryptic cleavage of PrP^{TSE} may also allow for LC-MS determination within biological samples and may be investigated. Total bacterial and fungal counts should be estimated using standard spread plate techniques, and isolates identified, where appropriate, by their culture, morphological and biochemical properties, and appropriate agar type. Incubation periods and temperatures will vary depending on the isolation techniques employed.

6. Current and future market

The global market for CS is anticipated to be driven by the increasing demand for nutraceutical and pharmaceutical applications. Sodium CS has a global market demand for application in the nutraceutical, pharmaceutical, animal feed, personal care, and cosmetics sectors. In North America and Europe in particular, the demand for pharmaceutical-grade CS has been driven by the prevalence of arthritis among the obese and geriatric populations. The largest market for CS in 2020 was the USA, where this increased growth was due to increased awareness among consumers, and thus increased consumption as joint health supplements. Globally, the use of CS in the nutraceutical sector was 6082.2 tons in 2020 and is estimated to reach 7293.5 tons by 2028. The current commercial sources of CS include bovine, swine, poultry, shark, and

synthetic. In 2022, the global market size was $1.25 billion, which may grow from 2023 to 2030 at a compound annual growth rate (CAGR) of 3.5% [118]. Of the various by-products' sources, bovine appeared as the major source of CS with a volume share of 69% in 2020 in the market [118].

Market research for CS by *Straits Research* found that the global CS market size will be USD 1709 million in 2030, while it was valued at USD 1211 million in 2021. This means that the CS sector is expected to grow at a CAGR of 3.9% during the forecast period of 2022-2030 [9]. In another market research, it was found that the global CS market in 2018 was 1210 million US$ which is expected to increase in 2025 to 3960 million US$ with a growing CAGR of 16.0% [119]. This study also found that China, where approximately 200 manufacturers were located, is the largest producer of CS, with 79% of the global market share. China exports most of its food-grade CS to the US and the pharmaceutical-grade CS to Europe [119].

7. Overall discussion and conclusion

CS is a natural polymer that is found in the extracellular matrix and tissues of animals and has various biological functions, such as providing structural support, preventing water loss, and modulating cell growth and differentiation. CS is nowadays used as a dietary supplement for the prevention and treatment of osteoarthritis, a degenerative joint disease that causes pain, stiffness, and limited mobility. The evidence for its efficacy and safety is not conclusive, and more high-quality studies are needed to confirm its role in osteoarthritis management. CS can be obtained from various sources, such as animal cartilage, microbial fermentation, or chemical synthesis. The quality and purity of CS may vary depending on their sources and the method of extraction used [75]. Therefore, it is important to ensure that the CS supplement meets the standards of quality control and regulation.

The current demand for CS due to their known health benefits may exceed the supply, especially from animal sources, which raises ethical and environmental concerns. The demand for CS for clinical applications (tissue engineering and wound healing) needs highly pure and concentrated CS compared to their applications as food supplements and cosmetics. Therefore, alternative sources are needed to meet the growing market for CS. However, current natural sources (animals and marine organisms) as by-products of various industries can support a "no-waste economy" and act as sustainable CS sources in terms of their proven functionality. There is a need for the development of green extraction and highly repeatable purification methods of CSs from these natural sources. Finally, appropriate quality analysis of the purified products is essential to ensure the expected benefits during their end-applications.

To conclude a SWOT analysis is presented below for CS based on the information on their sources, method of extraction, and demand as described in this review article (Table 3).

Table 3
SWOT analysis of chondroitin sulphate (CS) based on their sources, method of extraction, and commercial potential.

Strengths	Weaknesses
➢ CS has a high demand as a dietary supplement and pharmaceutical ingredient for joint health. ➢ CS has proven anti-inflammatory, analgesic, and cartilage-protective properties. ➢ CS can be extracted from various animal sources, like bovine trachea, porcine intestinal mucosa, shark cartilage, and fish skin as natural sources and to support the "No-Waste Economy". ➢ CS can also be biosynthesised by engineered microorganisms and bacterial fermentation.	➢ CS production currently is dependent on animal by-product sources, which are unsustainable and prone to contamination. ➢ CS extraction from animal tissues is a complex, mostly chemical-based, and costly process that requires multiple stages and purification steps. ➢ CS quality and purity are difficult to verify by simple instrumentation due to the heterogeneity and variability of its structure and sulphation. ➢ CS may have adverse side effects like gastrointestinal discomfort, bleeding risk, and allergic reactions.
Opportunities	**Threats**
➢ CS production from specific by-products sources can be developed using complete green extraction and purification technologies. ➢ CS can be produced by developing animal-free improved methods that are more efficient, scalable, and environmentally friendly. ➢ CS quality analysis can be enhanced by developing more sensitive, simple, and reliable methods for detecting its composition and contaminants. ➢ CS applications can be expanded through scientific validation of its potential benefits for other diseases and health conditions, such as cardiovascular health, Tissue engineering and wound healing, and skin care.	➢ CS faces regulatory challenges due to the lack of standardized methods and criteria for its quality control and safety evaluation. ➢ CS commercial extraction methods are mostly chemical based. ➢ CS may be subject to adulteration or substitution by cheaper or synthetic alternatives that may compromise its efficacy or safety. ➢ CS may lose its market share if consumers become more aware of its animal origin and ethical issues.

CRediT authorship contribution statement

Sushanta Kumar Saha: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Yin Zhu:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Patrick Murray:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Lena Madden:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work presented in this review article.

Data availability

Data will be made available on request.

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