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By-products of *Scyliorhinus canicula*, *Prionace glauca* and *Raja clavata*: a valuable source of predominantly 6S sulfated chondroitin sulfate

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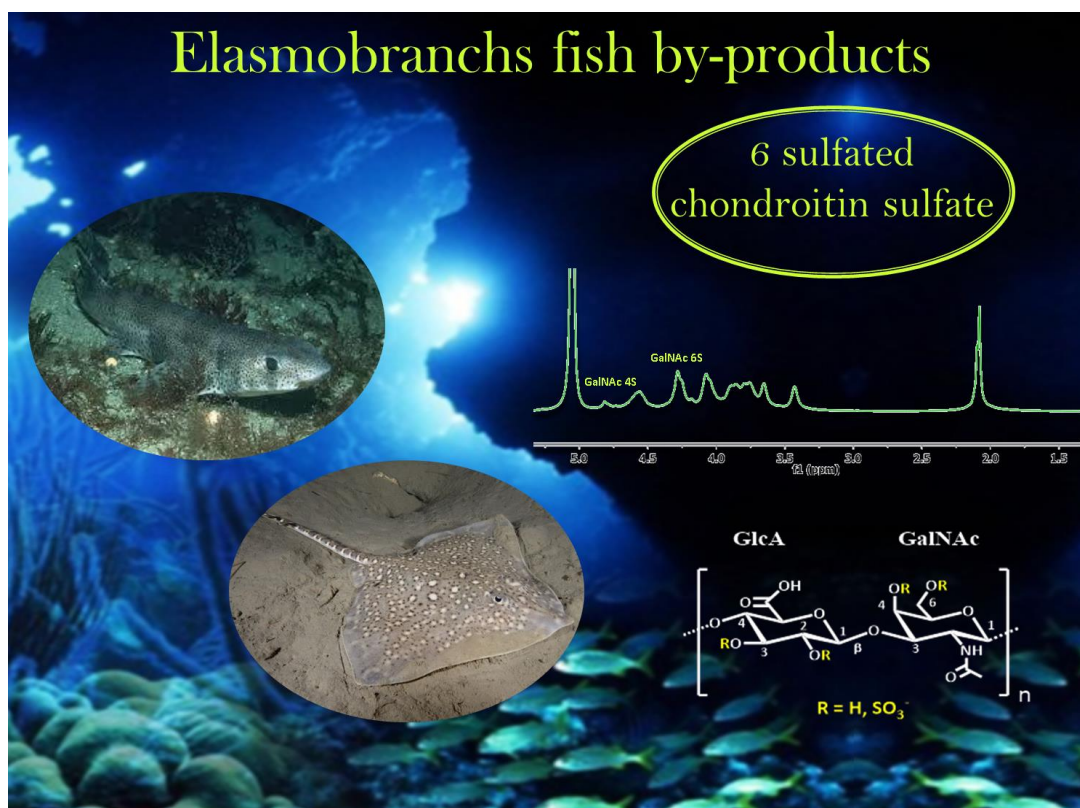
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Graphical Abstract



Highlights

- Chondroitin sulfate was isolated from fish processing industry by-products
- CS Mw was determined by GPC and sulfation position by NMR and SAX-HPLC
- CS was isolated from *Scyliorhinus canicula*, *Prionace glauca*, and *Raja clavata*
- 4S/6S ratio below 0.20 was observed for *P. glauca* and *R. clavata*

Abstract

Chondroitin sulfate (CS) was isolated from *Scyliorhinus canicula*, (fin, head and skeleton) *Prionace glauca* (head), and *Raja clavata* (skeleton) by-products from fish processing industry using environmentally friendly processes. The molecular weight was determined by gel permeation chromatography and the sugar composition and sulfation position by NMR and SAX-HPLC after enzymatic digestion. The CSs showed a prevalent 6S GalNAc sulfation for the 3 species (4S/6S ratio lower than 1). A higher 6S sulfation was observed for *P. glauca* head and *R. clavata* skeleton (4S/6S ratio below 0.20) than for *S. canicula* (4S/6S ratio ca. 0.6). The existence of CS samples with such low 4S/6S ratio has only been observed before in a rare species of shark (*Mitsukurina owstoni*, globin shark). The good extraction yields achieved make *S. canicula*, *P. glauca* and *R. clavata* fish industry by-products a useful source of 6-sulfated chondroitin sulfate.

Keywords

Chondroitin sulfate, elasmobranch, *Scyliorhinus canicula*, *Prionace glauca*, *Raja clavata*, sulfation position.

1. Introduction

Sulfated glycosaminoglycans (GAGs) are linear negatively charged polysaccharides found in high abundance in mammals and invertebrates at the cell surface or in the extracellular matrix (Yamada, Sugahara & Özbek., 2011). They give consistency to the tissues (due to the high hydration conferred by the sulfate groups)

and simultaneously mediate in many other biological processes such as the signaling pathways of cell differentiation (Fuster & Esko, 2005; Gandhi & Mancera, 2008).

A consequence of a broad range of biological activities of CS is the wide nutraceutical, therapeutic and pharmacological applications described. The most widely spread application of CS is the treatment of osteoarthritis symptoms. However, other potential medical applications are the reduction of inflammatory bowel disease, reduction of allergenic response or the use in urinary pathologies, (Lauder, 2009). The sulfation position plays a crucial role in CS biological activity and hence on derived applications. For example, the reduction of inflammatory bowel disease occurs by the inhibition of TNF- α that is stronger for over sulfated 4,6S CS (Tully, Rawat & Hsieh-Wilson, 2006). This highlights the importance of studying all potential sources of CS in a search for sulfation characteristics that might be useful in pharmacological applications.

As CS is found in mammals and invertebrates, many different sources have been reported. Commercial CS is mainly derived from cartilage of terrestrial animal sources such as bovine, porcine and avian, or from marine organisms such as shark, skate and sturgeon (Lauder, 2009; Maccari, Galeotti & Volpi, 2015; Volpi, 2007). However, mammalian cartilage represents scarcely 0.6% compared to chondrichthyes, which represent 6-8% (Lee & Langer, 1983).

CS sulfation varies with organism source, tissue, location within a tissue, and age, but a predominance of 4 sulfation is observed in animal CS (Volpi, 2007). Although comprehensive data in marine species are missing, it has been observed that the CS from marine origin are more varied (Sugahara et al., 1996; Vieira, Mulloy & Mourão, 1991, Habuchi, Moroi & Ohtake, 2002). Fishbone species, namely monkfish, codfish, spiny dogfish, salmon, and tuna (Maccari, Galeotti & Volpi, 2015), as well as elasmobranch (Higashi et al., 2015), contain GlcA 2S-GalNAc 6S as a common

characteristic (in a percentage lower than 30 %). All these fish CS contain GalNAc 4S and GalNAc 6S but the 4S/6S ratio is very variable, ranging from 2 in tuna and monkfish, 1.0-1.4 in skate, and less than 0.5 generally in sharks. According to these data, shark seems to be a unique source of mostly 6 sulfated CS but, unfortunately, there is a strong limitation of raw material for its production and strong concerns when considering endangered shark species. In this way, by-products from fish processing industries and some by-catches accessible under the landing obligation imposed by the European Union Regulation N° 1380/2013 should be the best source to obtain 6 sulfated CS.

Recently we have described two by-products from fish processing industry, heads from *Prionace glauca*, and skeleton from *Raja clavata* and a common fish by-catch small-spotted catshark (*Scyliorhinus canicula*) as alternative source of CS (Murado et al., 2010; Vázquez et al., 2016, Blanco, Fraguas, Sotelo, Pérez-Martín & Vázquez, 2015). The two by-products are very attractive sources of CS because of the extraordinary yields of production, ca 10% (w/w wet cartilage), and large volumes available in the fish processing industry. On its side, *S. canicula* has a wide geographical distribution along the Eastern and Northern Atlantic Ocean. Commercial landings are made for human consumption but they have a low commercial value and are taken as a by-catch, having high discard rates, reaching 100% in some European fisheries. The lack of use of CS obtained from these three species is mainly caused by the reduced amount of information published about their chemical composition. Only one study exists reporting the composition of *S. canicula* cartilage (Gargiulo et al., 2009) and another on *P. glauca* fin (Higashi et al., 2015), while *R. clavata* CS composition is still unknown.

Herein, we performed the chemical characterization of CS from these 3 species of fish. *S. canicula* specimens heads, fins, and central cartilage were separated and CS was isolated and analyzed independently, as well as heads from blue shark (*P. glauca*) and skeletons from ray (*R. clavata*) have been used for the production of CS. Nuclear magnetic resonance (NMR, ^1H and HSQC) and SAX-HPLC after enzymatic digestion and gel permeation chromatography (GPC) with triple detection allowed to determine the sugar composition, sulfation position and the molecular weight of purified CS, evaluating their suitability as sources to obtain 6 sulfated CS.

2. Materials and Methods

2.1 Isolation and purification of CS

The fish by-products and by-catches were obtained approximately 12 h after capture from Vigo market (Spain). Specimens were kept in ice to be transported to the laboratory where heads, fins and central cartilage were separated manually. Cartilages from head, skeleton and fin were obtained by thermal treatment (80 °C / 45 min) of individuals and manual cleaning of muscle residues. After the heating treatment (80 °C / 1 h) of these substrates, cartilages were cleaned, milled at ~1 mm size with a grinder (Thermomix TM31, Vorwerk) and stored at -20°C. The extraction and purification of the CS from each cartilaginous material was based on the processes previously reported (Blanco et al., 2015; Vázquez et al., 2016). Initially, cartilages were hydrolyzed by 0.1% (v/w) alcalase 2.4L (Novozymes S.A.) (2.4 AU/kg of cartilage) at 58 °C, agitation at 200 rpm, pH 8.5, at a solid:liquid ratio (1:3) and 10 h of hydrolysis for *S. canicula* cartilages; 55.7 °C, pH 8.2 (using Tris-HCl buffer 0.1 M), 1% (v/w) of alcalase (24 AU/kg of cartilage), agitation at 200 rpm, hydrolysis time of 8 h and a solid:liquid ratio (1:3) for *P. glauca* and *R. clavata*. The supernatants obtained by centrifugation of the

157 previous hydrolysates (6000 g for 20 min) were mixed with an alkaline hydroalcoholic saline solution: a) 1.17 volumes of EtOH (per volume of hydrolysate), 0.54 M of NaOH and 2.5% w/v of NaCl for *P. glauca* and *R. clavata*; b) 1.14 volumes of EtOH (per volume of hydrolysate), 0.64 M of NaOH, and 2.5% w/v of NaCl for *S. canicula*; for 2 h under agitation and finally centrifuged (6000 g for 20 min). The precipitates were redissolved with distilled water and neutralized (6 M HCl). These solutions rich in CS were subsequently ultrafiltrated and dialyzed through a combination of 100 and 30 kDa membranes (Prep/Scale-TFF: Spiral polyethersulfone membrane of 0.56 m², Millipore Corporation, Bedford, MA, USA) working under total recirculation of retentate. Initially, the solutions of CS were processed by means of 100 kDa membrane in order to remove high molecular weight hyaluronic acid. The permeates were recovered and ultrafiltrated by 30 kDa membrane. The operation conditions in this last performance were: filtrate average flows of 578±57 mL/min (*P. glauca*), 668±51 166 mL/min (*R. clavata*) and 520±48 mL/min (*S. canicula*); inlet pressure <1 bar and solutions temperature of 40 °C. Diafiltration of retentates generated in the previous ultrafiltration-concentration step was performed with a continuous distilled water flow (maintaining permeates flow=retentates flow) until the 5 diavolumes of water intake.

2.2 Analytical determinations

Total soluble proteins (P) were measured by the method of Lowry, Rosebrough, Farr & Randall (1951). CS quantified as glucuronic acid was determined by m-hydroxydiphenyl reaction according to the protocol of Van den Hoogen et al. (1998)

and the mathematical modifications described by Murado, Vázquez, Montemayor, Cabo & González (2005). CS purity index (I_p) was defined as $I_p (\%) = CS \times 100 / (P + CS)$. The total concentration of chloride was determined by ion chromatography (IC). Total sodium and sulfur were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES).

2.3 Enzymatic digestion of CS

The enzymatic digestion of CS was performed with chondroitin ABC lyase obtained from *Proteus vulgaris* (CASE, 18.3 U mg^{-1} , EC 4.2.2.4, Sigma Aldrich) using 0.02 U per mg of chondroitin sulfate in Tris-HCl 0.05 M, 0.15 M NaAc at 37 °C for 48 h. The reaction was stopped by heating at 70 °C for 25 min followed by centrifugation at 10000 rpm for 30 min to remove the enzymes. Aliquots were taken at different time points of the enzymatic digestion lyophilized and analyzed by GPC, gel electrophoresis, and (SAX)-HPLC.

2.4 Gel Permeation Chromatography (GPC)

GPC measurements were performed on a set of 4 columns connected inside a Malvern Viscotek TDA 305 with refractometer, right angle light scattering and viscometer detectors. Samples were measured with a Suprema Precolumn (5 μm , 8×50 mm), Suprema 30 Å (5 μm , 8×300 mm), Suprema 1000 Å (5 μm , 8×300 mm) and PLaquagel-OH-Mixed (8 μm , 7.5×300 mm). The system was kept at 30°C and 0.1 M NaN_3 in 0.01 M NaH_2PO_4 , at pH 6.7 was used as eluent at rate of 1 mL/min. Pullulan 47 kDa and polydispersity index (PDI) 1.07 (PSS standard services) was used to obtain a multidetector calibration (used to obtain the absolute M_w of the CS) and a set of 10 pullulan (180 Da to 355 kDa) was used to obtain a calibration with the refractive index detector based on the retention time (used to estimate the M_w in the digested fractions

that present a too weak LS signal). The dn/dc was measured on-line assuming that the input sample concentration is correct as recommended by Malvern.

2.5 (SAX)-HPLC

The samples were analyzed by strong anion-exchange (SAX)-HPLC separation and detection at 232 nm, as reported by Volpi (2000). HPLC equipment was equipped with a 150x4.6 mm stainless-steel column Spherisorb 5-SAX (5 mm, trimethylammoniopropyl groups $\text{Si-CH}_2\text{-CH}_2\text{-CH}_2\text{-N}^+(\text{CH}_3)_3$ in Cl^- form, Waters). Isocratic separation was performed from 0 to 5 min using 50 mM NaCl pH 4 and a linear gradient separation was used from 5 to 90 min with 100% 50 mM NaCl, pH 4 to 100% 1.2 M NaCl, pH 4 at a flow rate of 1.5 mL/min. Each disaccharide was identified using purified standards.

2.6 NMR spectroscopy

NMR experiments were acquired on a Varian Inova 750 spectrometer. Chemical shifts (D_2O) are reported in ppm (δ) and downfield from 3-(trimethylsilyl)-propionic acid- d_4 . Mnova Software 9.0 (Mestrelab Research) was used for spectral processing. The monosaccharide composition was determined from the ^1H NMR (spectra with 10 s delay to assure complete relaxation) at 750 MHz were performed at 10 or 6 °C to avoid overlapping with residual HOD (Mucci, Schenetti & Volpi, 2000; Cai et al., 2012). Sulfation position(s) was confirmed by CRISIS-HSQC. The CRISIS-HSQC pulse sequence is aimed to compensate the inefficiencies of refocusing of the $^1\text{J}_{\text{CH}}$ couplings that cause loss of quantitative information in the standard HSQC experiment (Boyer, Johnson & Krishnamurthy, 2003).

3. Results and discussion

3.1 CS isolation and purification

In the present work, we have applied a set of optimized processes in terms of reducing production costs, to avoid solvents and pollutant technologies and to be easily scaled up (Vázquez et al., 2013; Vázquez et al., 2015).

The yields obtained (weight of CS in relation to weight of dry cartilage, as %) revealed that head (6%) was the best substrate followed by fin (4%) and skeleton (2%) in *S. canicula* but significantly lower than *P. glauca* (12%) and *R. clavata* (14%) sources (Table 1). These results are interesting because they are materials recovered from fishing discards and by-products that necessarily must be valued in the context of new EU policies. The values of the index- I_p calculated for the five samples also confirmed the data of yields, although in this case, the purification of blue shark head (*P. glauca*) and ray skeleton (*R. clavata*) were more effective than small catshark (*S. canicula*) cartilage. The presence of sulphur and sodium were similar in all samples but chloride levels were significantly lower in *P. glauca* and *R. clavata* samples ($p < 0.05$). In general, these percentages are in the range of those described for commercial CS formulations.

3.2 Molecular weight characterization by GPC

3.2.1. GPC

The GPC system used was equipped with a triple detector that permit the evaluation of molecular weight of polymeric samples through a combined and simultaneous action of on-line right-angle laser light scattering (RALS), refractometer (RI), and viscometer. GPC with triple detection is not commonly applied to sulfated GAGs but has been described for heparin and dermatan sulfate (Bertini, Bisio, Torri, Bensi & Terbojevich, 2005). The triple detection, including light scattering, was

selected because it does not require any chromatographic column calibration, thus overcoming also the difficulty to obtain adequate reference standards. Available commercial polysaccharide standards such as pullulan or dextran differ strongly in conformation from rigid sulfated GAGs. The use of the light scattering provides the absolute Mw, e.g. the Mw is not related to the conformation of the polymer used for the calibration.

The GPC chromatograms of CS extracted from *S. canicula* skeleton are shown in Figure 1. A polymeric peak is observed at 20 mL in the refractive index (RI) with only slight difference in retention time in the viscosity and light scattering detectors, revealing a low polydispersity. Similar eluograms were obtained for the fin and head CS from *S. canicula* as well as for *R. clavata* skeleton and *P. glauca* head (Figures S1-S3). The Mw obtained from the multidetector calibration (combination of the 3 detector signals) is included in Table 2.

In the viscosity and RALS eluograms of *S. canicula* (Figure 1) and *P. glauca* (Figure S2), an additional and much smaller peak is also observed at lower retention times. This second peak corresponds to a very low concentration (not found in RI) of a very high Mw species. It might be ascribed to a small fraction of a high Mw GAGs but also to aggregates. The presence of a small concentration of aggregated polymer chains in solution has been described for chitosan (Ottøy et al., 1996; Lamarque et al., 2005) and has been also observed in heparin (Bertini et al., 2005).

The Mw calculated with the triple detection is close to 40 kDa in barely all samples, except for *P. glauca*, which is slightly higher (60 kDa). The low PDI obtained is probably due to the ultrafiltration step that isolates molecules between 30 and 100 kDa. This step also separates CS from other GAGs that might be present in the crude extracts but have a different Mw such as hyaluronic acid (HA) and keratan sulfate (KS).

HA is known to present Mw higher than 100 kDa and keratan sulfate is around 10-15 kDa (Tai et al., 1996, Weyers et al. 2013). The Mw of dermatan sulfate (DS) is more similar to CS Mw (Bertini 2005) but a complete overlap in the GPC will occur only with minute amounts of DS. From this fact together with the NMR (see below) we can conclude that if DS is present, it will be in minute amounts.

There are few data in the literature about the Mw of CS from elasmobranchs by GPC. Mw of CS reported for shark CS was ca. 50-60 kDa (Volpi, 2007; Mucci, Schenetti & Volpi, 2000). In these works, CS of known molecular weight, determined by analytical centrifugation, were used to construct a calibration curve based only in one detector sensitive to concentration (Volpi, 1993), not describing the polymer polydispersity, probably because analytical centrifugation cannot give this information. Additionally, the calibration is made with samples of different sulfation degrees and this influences the conformation of the polysaccharide in the GPC eluent and hence the precision in the determined Mw. In this way, the method that we selected is simpler (no need of preparing a set of standards that are not commercially available and validate them with analytical ultracentrifugation) and more adequate because the polydispersity is known. From these, and our data, it is clear that the Mw of CS from elasmobranchs is always higher than bovine, chicken or porcine (close to 20 kDa, Volpi, 2007). Interestingly, the samples from *S. canicula* fin, head and central cartilage showed very similar properties, making the whole animal an adequate source of CS with similar Mw.

3.3 Sugar composition by NMR and SAX-HPLC

¹H NMR spectra of the three elasmobranchs (Figure 2) shows the high purity in respect to proteins as observed by the lack of signals in the region 0.5-1.5 ppm (aliphatic amino acids) and 7.0-8.5 (aromatic amino acids, see full spectra in the

supplementary material Figure S5). This confirms the high purity of the obtained GAG extracts, as suggested by the index-Ip and gel electrophoresis results.

The ^1H NMR and HSQC (Figure 3 and S7-S8) spectra correspond with the detailed previous assignment of CS signals and therefore quantitative information on the monosaccharide composition can be obtained by an integration of the ^1H NMR spectra (Cai et al., 2012; Mucci, Schenetti & Volpi, 2000). The samples from *S. canicula* fin, head and skeleton showed almost identical ^1H NMR (Figure S6), allowing to conclude that using by-products from specific parts of the animal does not affect the properties of the final CS. As highlighted for the Mw obtained by GPC, the samples from fin, head and central cartilage showed very similar properties making the whole animal an adequate source of CS with the described sulfation and Mw.

The spectra show a predominance of the signals corresponding to 6 sulfation in the GalNAc (singlets of H4 and H6 at 4.26 and 4.22 ppm in the ^1H NMR, Figure 3) as compared to 4 sulfation (Mucci, Schenetti & Volpi, 2000). It is also patent that the characteristic peaks of dermatan sulfate (H1 at 4.87 ppm and H2 of 3.52 ppm of the IdoA residue) were not observed in the spectra, *i.e.* no dermatan content was observed in agreement with the single polysaccharide and narrow polydispersity found in the GPC. In this aspect, the CS is similar to the one observed by Gargiulo et al. (2009) in *S. canicula* fin and to the one observed by Higashi et al. (2015) in *P. glauca* raw fin, but different to *P. glauca* fin without skin and cartilage that shows more 4 sulfation and presence of iduronic acid (Higashi et al., 2015). The absence of keratan sulfate (already observed by GPC) is confirmed by the lack of signals corresponding to the H1 of galactose close to 5.1 ppm (Tai et al. 1996).

To determine the percentage of sulfation in position 4, the signal at 2.09-2.08 ppm, that correspond to the acetyl group (Ac) of GalNAc (*i.e.* its intensity is independent of the sulfation position), was used and compared with singlet at 4.80 ppm, assigned to H4 of 4-sulfated GalNAc (GalNAc 4S) (Mucci, Schenetti & Volpi, 2000). Apparently, no 4,6 sulfation occurs in the sample since only one singlet appears in the region of 4.8 ppm. The presence of sulfation in position 2 is also evidenced in the spectra by the characteristic peak of H2 in sulfated GlcA (4.16 ppm). However, this is just an initial indication since this signal is strongly overlapped and appears together with the H4 of the non-sulfated disaccharides.

Although NMR is the fastest method to achieve a general picture of the CS composition, it does not allow obtaining the complete map of the disaccharide composition. This is due to the high overlap of the signals both in the ^1H and HSQC NMR analysis and because of the relatively low sensitivity of NMR that difficult to determine disaccharides appearing in low percentages. To obtain more detailed information about the sugar composition of the samples, the CS was enzymatically digested, and analyzed by (SAX)-HPLC.

The samples were digested with chondroitinase ABC and analyzed following the GPC method recently described by Silva et al. (2015), to assure that 99% of the material was converted into oligosaccharides. A shift of the refractive index signal to elution times corresponding to pullulan disaccharides after 48 h of digestion is observed in the eluograms (Figure 4 and S4). The digested samples were analyzed by SAX-HPLC, chromatograms of the SAX-HPLC are included in the supplementary material (Figures S9 and 10), and the results summarized and compared to NMR in Table 3.

(SAX)-HPLC confirmed the prevalence of sulfation at position 6 over the sulfation at position 4 observed by NMR, with 4S/6S ratios much lower for *P. glauca*

and *R. clavata* (0.15 and 0.20 respectively) than for *S. canicula* (0.6). The existence of CS samples with low 4S/6S ratios in common fish by-products is a very interesting outcome because such low ratios have only been observed in a rare species of shark (*Mitsukurina owstoni*, globin shark). No 4,6 sulfated disaccharides were observed in the (SAX)-HPLC, which agrees also with the appearance of just one singlet in the region of 4.80 ppm of ^1H NMR spectra. A relatively high percentage of non-sulfated disaccharides were found by HPLC (10-20%) in the 3 species of fish. These results are in a similar range to those described for elasmobranchs (Higashi 2015). Higashi studied several elasmobranchs, including *P. glauca*, observing values of non-sulfated disaccharides ranging from 3 to 21 %. The value described for *P. glauca* fin (without skin) is 18.4, a very similar value to the 16.4 found here for *P. glauca* head. Also, the previous value found for *S. canicula* 8% (Gargiulo 2009) is comparable to the 14% that found here for *S. canicula* head.

There is only one small peak that could not be identified in the SAX-HPLC. The similar retention time to the non-sulfated CS disaccharide (Figure S9-10) suggests that it can be non-sulfated GlcA-GlcNAc. Actually, the chondroitinase ABC used for the digestion is known to slowly degrade HA (Yamagata, 1968). It could be the result of the degradation of some HA with Mw between 30 and 100 kDa remaining after ultrafiltration. In fact, HA is a high Mw polydisperse polysaccharide where a small amount of molecules in this Mw range may be present. In any case, this peak corresponds to less than 1.5 % in *P. glauca* and 4 and 6% in *R. clavata* and *S. canicula* respectively, showing that only small amounts of other GAGs are present in the CS extracts, in agreement with the GPC and NMR results.

Pondering NMR and HPLC data, we can describe for *S. canicula* whole body (fin head, and skeleton) a percentage of GlcA-GalNAc 4S of ca. 35%, GlcA 2S-GalNAc 6S of ca. 20% and GlcA-GalNAc 6S of ca 30% that agrees well with the sulfation pattern described previously for *S. canicula* (Gargiulo, Lanzetta, Parrilli & De Castro, 2009). The CS obtained from *P. glauca* head and *R. clavata* skeleton present a quite similar composition with GlcA-GalNAc 4S between 10-15%, GlcA 2S-GalNAc 6S close to 10% and GlcA-GalNAc 6S of ca 60%. The differences of the composition observed between the present and previous analysis of *P. glauca* (GlcA-GalNAc 4S of 25% and presence of iduronic described previously for *P. glauca* fin, Higashi et al., 2015) may reflect differences in the analytical determinations but also in the isolation procedure or actually differences in the specimens collected. For instance, Gargiulo, Lanzetta, Parrilli & De Castro (2009) have used papain for the enzymatic hydrolysis of cartilage instead of alcalase. Moreover, isolation and purification procedures were performed by repeating ethanol precipitations and ion-exchange gel chromatography (Gargiulo, Lanzetta, Parrilli & De Castro, 2009) whereas in the present work only one step of precipitation, using alkaline alcoholic solution and further ultrafiltration was required. Even though, this is only the second study that analyzed samples of *S. canicula* and *P. glauca* and the first for *R. clavata* and so more information should be collected to confirm the observed differences.

4. Conclusions

Small-spotted catshark (*S. canicula*, whole body), blue shark (*P. glauca*, head) and ray (*R. clavata*, skeleton) by-products from fish processing industry are useful sources of predominantly 6S sulfated chondroitin sulfate. The CSs characterized by gel permeation chromatography nuclear magnetic resonance (^1H and HSQC) and (SAX)-

HPLC after enzymatic digestion showed Mw *ca.* 43, 60, and 44 kDa for *S. canicula*, *P. glauca* and *R. clavata* respectively. The ^1H NMR and (SAX)-HPLC showed a prevalent composition of the chondroitin CS 6 sulfation for the 3 species (4S/6S ratio lower than 1). A higher 6S sulfation was observed for *P. glauca* head and *R. clavata* skeleton: 4S/6S ratio 0.15 for *P. glauca* head and 0.20 for ray skeleton vs. the 0.63 observed for *S. canicula*. The specific 4S/6S ratio (uncommon in sustainable sources) together with the good yield obtained makes these materials a useful source of 6-sulfated chondroitin sulfate with a great number of potential applications in different sectors as nutraceutical and pharmaceutical.

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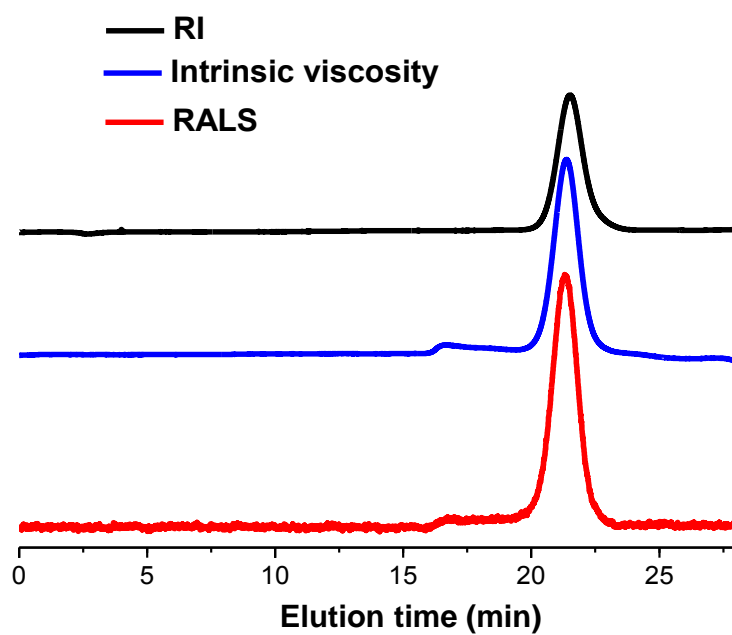


Figure 1. GPC eluograms of CS from *S. canicula* skeleton in 0.1 M NaNO₃, 0.01 M NaH₂PO₄, pH 6.7. The refractive index, intrinsic viscosity and right angle light scattering detector signals are signed in colors.

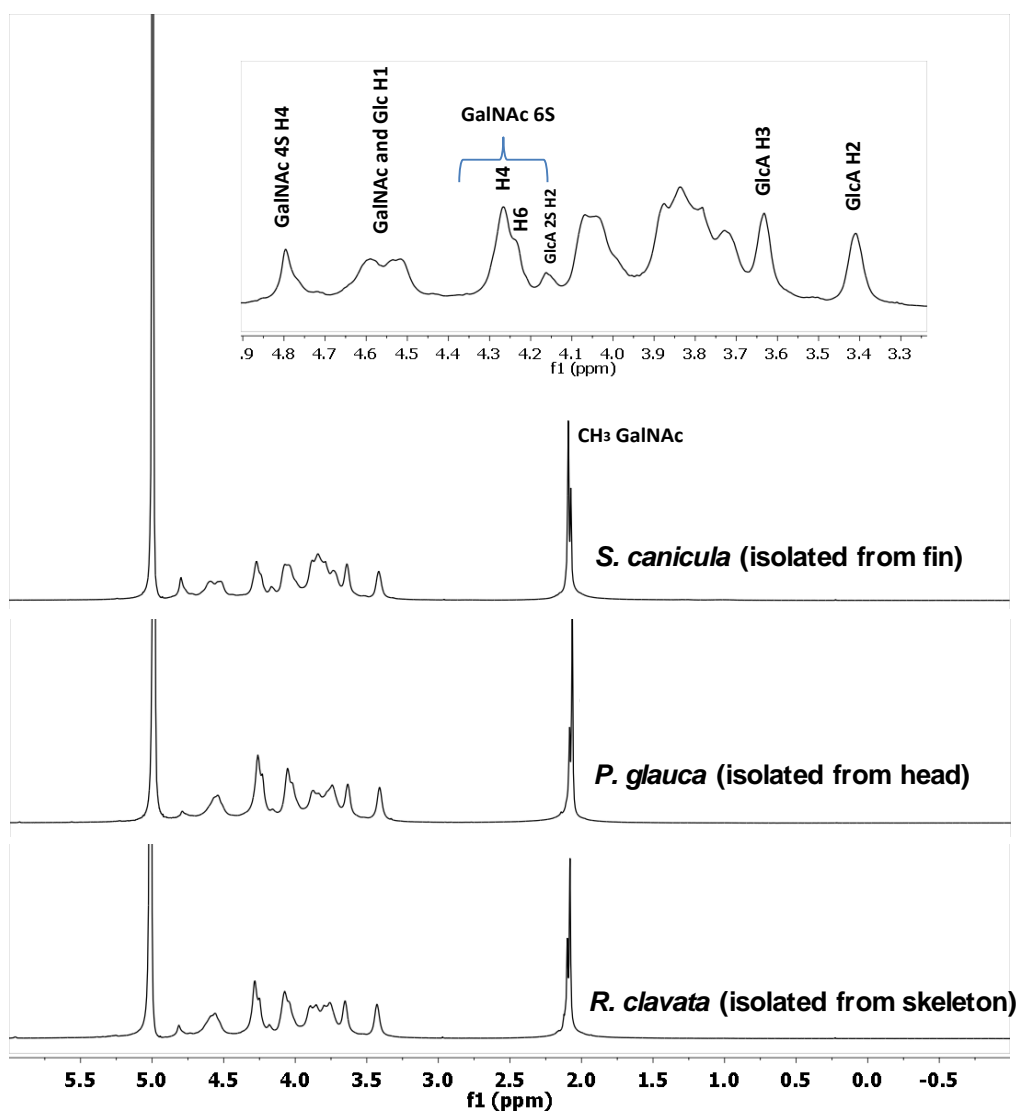


Figure 2. ^1H NMR (D_2O , 750 MHz) spectra of the CS from *S. canicula* isolated from fin (top, 10 °C), *P. glauca* isolated from head (middle, 10 °C) and *R. clavata* isolated from skeleton (bottom, 6 °C).

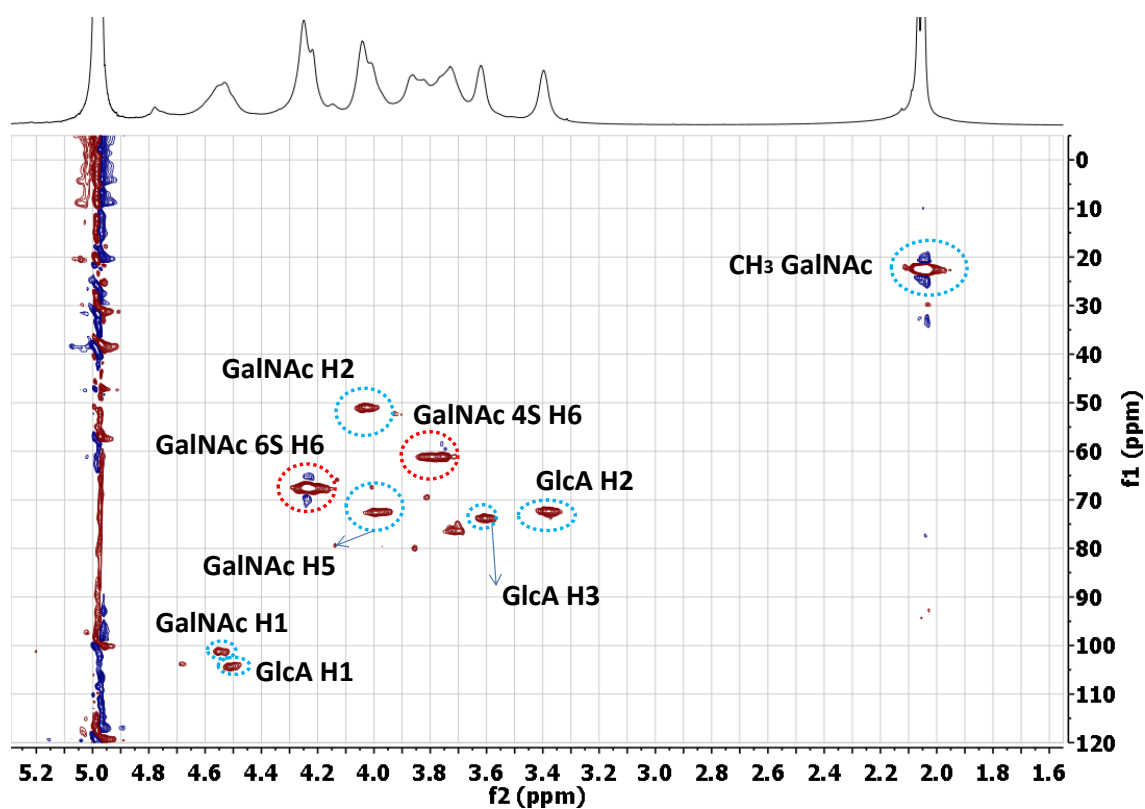


Figure 3. HSQC spectra (D₂O, 25°C, 750 MHz) of the CS from *R. clavata* isolated skeleton.

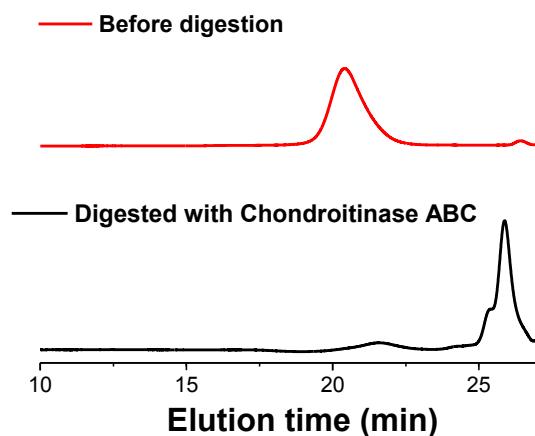


Figure 4. GPC eluograms of CS from *S. canicula* head in 0.1 M NaNO₃, 0.01 M NaH₂PO₄, pH 6.7. The refractive index signal (that shows low and high Mw species) is shown before (red line) and after digestion (black line).

Table 1. Chemical composition of the purified CS from fishing by-products and discards. Error data are the confidence intervals for $\alpha=0.05$ and $n=2$.

Sample	Y (w/w dry cartilage)	S (%)	Cl (%)	Na (%)	GAG (%)*
<i>S. canicula</i> fin	3.9±0.3	7.41±0.37	0.35±0.04	7.37±0.23	96.2±0.4
<i>S. canicula</i> head	5.8±0.3	7.59±0.42	0.37±0.06	7.50±0.32	98.0±0.2
<i>S. canicula</i> skeleton	1.9±0.2	7.39±0.28	0.34±0.05	7.45±0.36	97.4±0.3
<i>P. glauca</i> head	12.1±0.3	6.74±0.23	0.02±0.0	6.93±0.15	98.5±0.2
<i>R. clavata</i> skeleton	13.7±0.5	7.31±0.34	0.02±0.0	7.56±0.15	98.2±0.3

*GAG was determined in relation to proteins as index-I_p.

Table 2. Mw and PDI and dn/dc of the CS.

Sample	<i>S. canicula</i> fin	<i>S. canicula</i> head	<i>S. canicula</i> skeleton	<i>P. glauca</i>	<i>R. clavata</i>
Mw	43 kDa	45 kDa	43 kDa	60 kDa	44 kDa
PDI	1.49	1.33	1.21	1.18	1.30
dn/dC	0.122	0.124	0.129	0.130	0.133

Table 3. Composition of the CS from by-products of *S. canicula*, *P. glauca* and *R. clavata* determined by NMR and SAX-HPLC.

Sample	<i>S. canicula</i> fin	<i>S. canicula</i> head	<i>S. canicula</i> skeleton	<i>P. glauca</i> head	<i>R. clavata</i> skeleton
GalNAc-4S¹	39%	38%	36%	11%	16%
GlcA-GalNAc-4S²	31.5 ± 0.62	35.5 ± 0.12	39.5 ± 0.22	10.1 ± 0.1%	16.0 ± 0.1 ²
GlcA-GalNAc 6S²	32.4 ± 0.1	28.7 ± 0.2%	29.7 ± 0.6	64.2 ± 0.4	55.9 ± 0.1
GlcA-GalNAc 0S²	22.5 ± 1.4 ²	14.1 ± 0.0% ²	15.1 ± 0.1 ²	16.3 ± 0.6 ²	19.5 ± 0.1
GlcA 2S-Gal NAc 6S²	16.3 ± 0.2	18.1 ± 0.1 % ²	16.7 ± 0.5 ²	9.3 ± 0.2%	8.6 ± 0.12
GlcA 4S-Gal NAc 6S²	n.o.	n.o.	n.o.	n.o.	n.o.
4S/6S ratio²	0.63 ¹ 0.64 ²	0.61 ¹ 0.76 ²	0.59 ¹ 0.82 ²	0.17 ¹ 0.15	0.26 ¹ 0.20

n.o. not observed ¹ by NMR, ² by SAX-HPLC,