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RESEARCH ARTICLE

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Quantitative evaluation of sulfation position prevalence in chondroitin sulphate by Raman spectroscopy

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Abstract

Chondroitin sulphate (CS) as a major component of the extracellular matrix of numerous connective tissues is responsible for biomechanical properties such as resistance and elasticity. It occurs in different isomeric forms with different sites and degrees of sulfation. The characterization of crude CS and quantitative isomeric identification by high-performance liquid chromatography (HPLC) requires specific enzymes, expensive reagents, and a complicated analytical process. Poor reproducibility and imprecise quantification were found with other methodologies. Raman spectroscopy allows rapid and reproducible identification of CS isomers offering specificity and avoids the need for sample pretreatment or external markers. In the present work, a quantitative identification of major contribution of isomeric chondroitin 4 and 6-sulphate in crude CS by Raman spectroscopy has been performed. Two quantitative indices have been proposed based on areas of specific bands of interest related to main skeletal modes, an aromatic ring with axial orientation in the case of the major contribution of 4-sulphate and C-O-(S) vibration, which is heavily detected in the case of the major contribution of 6-sulphate. Both mammalian and fish sources of CS were used to validate the ability of mentioned indices to discriminate between prevalence of 4-sulphate, 6-sulphate, or none of them. Correspondence of given results with HPLC was demonstrated.

KEYWORDS

chondroitin sulphate, quantitative indices, Raman spectroscopy, sulfation position

1 | INTRODUCTION

Chondroitin sulphate (CS) is a sulphated glycosaminoglycan (GAG) consisting of a long unbranched polysaccharide chain with a repeating disaccharide structure of N-acetylgalactosamine and glucuronic acid. As a major component of the extracellular matrix of numerous connective tissues, such as cartilage, bone, skin, ligaments, and tendons, it is responsible for many important biomechanical properties such as resistance, elasticity, tissue stiffness, and resilience.[1,2] The consistency given to tissues by sulphated GAGs is due to their high capacity to retain interstitial water[2] conferred by sulphate groups, which promote the formation of large negatively-charged molecules that attract cations and water molecules, leading to the obtaining of a hydrated gel.[3] Sulphated GAGs simultaneously mediate in many other biological processes ranging from signalling pathways of cell differentiation, anticoagulation, and growth pathways to cartilage's ability to sustain stress during compression. They also

contribute to providing support while permitting cell migration and the diffusion of nutrients and soluble signalling molecules within the extracellular matrix.[3–5]

The structural characteristics of chondroitin sulphate include heterogeneity of molecular mass and charge density due to different sites and degrees of sulfation, since CS occurs in various isomeric forms such as CS-A (4-sulfation in N-acetyl-D-galactosamine) and CS-C (6-sulfation in N-acetyl-D-galactosamine).[6,7] These isomeric forms exhibit functional selectivity, CS-C (6-sulfation) being related, for instance, with wound repair, central nervous system development, and countering infection.[5] Moreover, different isomers contribute to the same tissue. In the case of the meniscus, which consists of fibril-forming collagen (60–70% of tissue dry weight), proteoglycans, matrix glycoproteins, and elastin, chondroitin 6-sulphate (C6S) represents 40% of proteoglycan content, whereas chondroitin 4-sulphate (C4S) accounts for around 10–20%, dermatan sulphate between 20% and 30% and keratan sulphate for the remaining 15%. The healthy stage of the meniscus is correlated to pure collagen type II or the coexistence of collagen types I and II along with CS.[8] A significant negative correlation between levels of C6S and age was detected together with a significant inverse correlation between the ratio of C6S to C4S and age.[9] Changes in the structure of CS and a diminished ratio of C6S/C4S have been found in synovial fluid and the cartilage of patients affected by osteoarthritis, a pathological condition, which leads to cartilage degeneration and subchondral bone damage, in advanced and terminal stages compared with early ones.[1,8,9]

The sulfation position therefore plays a crucial role in CS biological activity and hence in derived applications, the most widespread application of CS being the treatment of osteoarthritis symptoms.[10] Other applications are focused on the interactions of sulphated GAGs with a wide range of proteins. In fact, the variety of possible structure sequences and their biological significance is why GAGs remain an important and essential source of drug development studies.[5,11] Purified GAGs have a broad scale of pharmacological and cosmetic applications such as artificial skin and compress production in the case of CS.[2]

The interest of studying all potential sources of CS is then justified. It has been found in different species of mammalians and invertebrates where the CS extracted varied, in sulfation degree and isomer contribution, with organism source, tissue, location within the tissue, and age. However, mammalian CS is observed to be predominantly be 4 sulfation position, which is the main source of CS, derived from the cartilage of terrestrial animal sources such as bovine, porcine, and avian ones. Other utilized CS sources are marine organisms, mainly sharks; these show differences with terrestrial CS in terms of molecular weight and sulfation.[12] The existence of CS sources with low C4S/C6S ratios (prevalence of six sulfation position) in common and sustainable fish by-products (from the fish processing industry) such as small-spotted catshark (*Scyliorhinus canicula*, whole body), blue shark (*Prionace glauca*, head), and ray (*Raja clavata*, skeleton) was recently published.[4]

Spectrophotometry, chromatography, and electrochemical methods are common techniques used to quantify crude CS. They present certain disadvantages. In spectrophotometry, the complex operation can be affected by a number of factors, leading to poor reproducibility. Chromatography and capillary electrophoresis have strong baseline noise due to the end absorption at 200 nm with a tailing or a wide peak, resulting in imprecise quantification. Quantification with strong anion exchange (SAX)-HPLC requires the enzymatic depolymerisation of the chain with specific and expensive bacterial enzymes followed by complicated disaccharide analysis.[2] Although offering high sensitivity, electrochemical methods demand strict conditions, present some difficulties in operation, and their specificity is not good. There is therefore a need for an appropriate and precise method for the quantitative analysis of CS.[7]

Raman spectroscopy allows for rapid, simple, reproducible, and nondestructive analysis and has the advantage that sample pretreatment, external markers, and preseparation are not required.[2,7] It measures inelastic scattered light following the excitation of a target compound, tissue, or molecule by a monochromatic laser. The collection of Raman spectra from biological samples produces a fingerprint representing the molecular vibrations specific to chemical bonds. It is highly sensitive to structure, allowing one to identify chemical and structural changes.[13] The main disadvantages of this technique are its low intensity and the occurrence of autofluorescence, which interferes with the interpretation of the spectrum. To avoid this problem, it is common to use near infrared lasers or ones at a higher wavelength to avoid the effect of fluorescence.[13]

Several studies have evaluated sulphated GAGs by Raman spectroscopy. Bansil et al. (1978) performed the first Raman study of C4S and C6S, revealing significant differences in their spectra.[14] Certain differences were related to differences in chemical composition whereas others were interpretable in terms of the geometry of various substituents. They proposed assignments for vibrational frequencies of several groups for both isomers and concluded that Raman is sensitive to the orientation of the sulphate group, related to the pyranose ring. Ishwar et al. (2009) investigated molecular bonds and chemical groups present in certain peptide-GAG

relationships by using Raman spectroscopy and affinity capillary electrophoresis to potentially identify differences in how the different GAGs interact with peptides.[15] These authors proposed Raman spectroscopy as a useful tool for screening peptide-GAG binding data in real time and under hydrated conditions. Recently, Orkoula and Kontoyannis (2014) applied micro-Raman spectroscopy to human menisci samples to evaluate the presence of GAGs in healthy and osteoarthritic areas.[8] The main Raman spectroscopy peaks of C6S and C4S were both detected and identified. Liu et al. (2014) determined the CS content of tablets using Raman and near-infrared (NIR) spectroscopy methods and validated the Raman approach in quantitative analysis by comparing the results obtained with Raman and NIR.[7] The development of a calibration and validation model to predict CS content of unknown samples was the objective. Quantitative identification of different CS isomers was not performed.

In the present work, we present a quantitative evaluation of a major contribution of isomeric C4S and C6S by Raman spectroscopy. Two quantitative indices have been established based on specific bands of interest related to main skeletal modes, orientation of the aromatic ring, and C-O-(S) vibration. HPLC characterization and two CS groups (mammalian and fish sources) were used to validate the proposed indices.

2 | MATERIALS AND METHODS

2.1 | CS production

Isolated and purified samples of CS from fish sources (6-sulfation predominance), particularly *P. glauca* (head), *R. clavata* (skeleton), *Scyliorhinus canicula* (fin), *Chimaera monstrosa* (head, skeleton, and fin), and *Galeus melastomus* (head, skeleton, and fin) were provided by IIM-CSIC (Vigo, Spain). Moreover, CS from mammalian sources (4-sulfation predominance), particularly bovine and porcine trachea were also isolated and purified by the same institution. Briefly, the procedure of CS extraction was based on the following steps[16–18]: (a) enzymatic hydrolysis of cartilaginous material by Alcalase 2.4 L (Novozymes A/S, Nordisk, Bagsvaerd, Denmark), (b) selective precipitation of CS from hydrolysates by alkaline-hydroalcoholic-saline solutions, (c) purification of redissolved aqueous CS by membranes of ultrafiltration-UF (30 kDa) with a first stage of UF-concentration and subsequent protein elimination and desalination operating in diafiltration-DF mode. Commercial bovine CS from Bioibérica S.A.U. (Barcelona, Spain) was also analysed.

2.2 | Raman equipment and methodology

Raman spectra were collected using a FT-Raman Bruker RFS 100 equipped with an Nd:YAG (neodymium-doped yttrium aluminium garnet) laser (1,064 nm) with incident laser radiation up to 500 mW, 64 scans, and 4 cm^{-1} resolution. The Raman peaks were assigned to different biochemical variations, based on the literature. Band areas were calculated by using a standard software program (MagicPlot) limiting the selected wavenumber intervals, related with biochemical vibrations of interest, by a linear baseline. An example of the area measurement in one of the CS Raman spectrum is presented as supplementary material (Figure S1). Ratios between areas of two wavenumber regions of interest for C4S or C6S were performed to obtain corresponding indices.

2.3 | Disaccharide composition by SAX-HPLC

Disaccharide composition of CS was determined by SAX chromatography after enzymatic digestion with chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4., 1.66 U mg^{-1}, Prod. No. C2905, Sigma-Aldrich) at 0.2 U mg^{-1} of CS. The reaction was carried out in a buffer of 0.05 M Tris–HCl and 0.15 M sodium acetate at pH 8 and 37°C. After 24 hr, the enzyme was inactivated by heating it at 70°C for 25 min, followed by centrifugation at 12,857 g. Supernatants were collected and filtered through 0.2 μm polyethersulfone (PES) syringe filters. Unsaturated disaccharide standards were purchased from Grampenz (Aberdeen, United Kingdom) and dissolved in water. Samples and standards were manually injected into an HPLC system (Agilent 1200) consisting of a binary pump (G1312A), column oven (G1316A), and UV-visible detector (G1314B). Separation was carried out with a Waters Spherisorb SAX column (5 μm, 4.6 × 250 mm, Prod. No. PSS832715) fitted with a guard cartridge (Waters Spherisorb, 5 μm, 4.6 × 10 mm) based on a previously reported method (Volpi 2000). Elution was performed in isocratic mode from 0 to 5 min with 50 mM NaCl at pH 4. Linear gradient was applied from 5 to 20 min starting with 50 mM NaCl at pH 4 and ending with 76% 50 mM NaCl at pH 4 and 24% 1.2 M NaCl at pH 4. A sample volume of 20 μl was injected into the system with a flow rate of 1.5 ml min^{-1}. Detection was made at 232 nm. An external calibration curve was built with each standard to calculate the amount of disaccharide units in the sample and reported as a percentage of the weight.

2.4 | Statistical analysis

Two independent measurements of 64 scans/each of the different chondroitin sulphate sources were performed by Raman spectroscopy to obtain their corresponding mean spectra. Three area measurements were carried out per each analysed wavenumber range with MagicPlot software to obtain a mean and standard deviation, incorporating the potential random error attributed to the software or the experimenter. Statistical differences $p < 0.05$ (95% cases) were confirmed by the nonparametric Mann-Whitney U test with IBM SPSS Statistics, version 23. Intervals of confidence at 95% for mean values were estimated by the formula: mean value $\pm$ Z^* (σ/n1/2), where Z is the critical value corresponding to the accumulative area of 0.975, and its value is 1.96, σ is the standard deviation, and n is the number of samples analysed.

3 | RESULTS AND DISCUSSION

3.1 | Raman qualitative evaluation

Raman spectra of representative CS from fish sources: head from *P. glauca* and from mammalian source: commercial bovine are shown in Figure 1a in the wavenumber range of 1,800–350 cm^{-1}. Both spectra were analysed, and attributions assigned. As a first approach the spectra were coincident with an enrichment in C6S isomer for fish source and in C4S isomer for mammalian source, as expected.[2,4,14] This fact implies the detection of certain Raman peaks at a specific intensity or their absence, depending on the major isomer in the composition of the analysed chondroitin.

Going into detail with attributions, a broad band with the main peak at 1,664 cm^{-1} in C6S (*P. glauca*) and at 1,658 cm^{-1} in C4S (commercial bovine), with shoulders at 1,637 cm^{-1} and 1,639 cm^{-1}, respectively are attributed to amide I vibration.[2,8,13,14,19,20] Weak peaks at 1,454 cm^{-1} and 1,452 cm^{-1} respectively, correspond to CH_2 deformation. Peaks at 1,408 cm^{-1} and 1,409 cm^{-1} respectively are assigned to COO— symmetric vibration, whereas strong peaks at 1,375 cm^{-1} for both spectra are attributed to CH_3 symmetric deformation. Finally, amide III is assigned at 1,340 cm^{-1} with medium intensity peaks[19] and at 1,269 cm^{-1} with broad peaks for both spectra (as referenced by Parker (1983)[20] at 1,260–1,266 cm^{-1}; Bjarnason and Tu (1978)[21] attributed the position at 1,269 cm^{-1} due to a *p* reverse turn conformation). The vibration of SO_3— asymmetric stretching appeared as a shoulder at 1,237 in C6S and at 1,238 cm^{-1} in 4-sulphate. A peak at

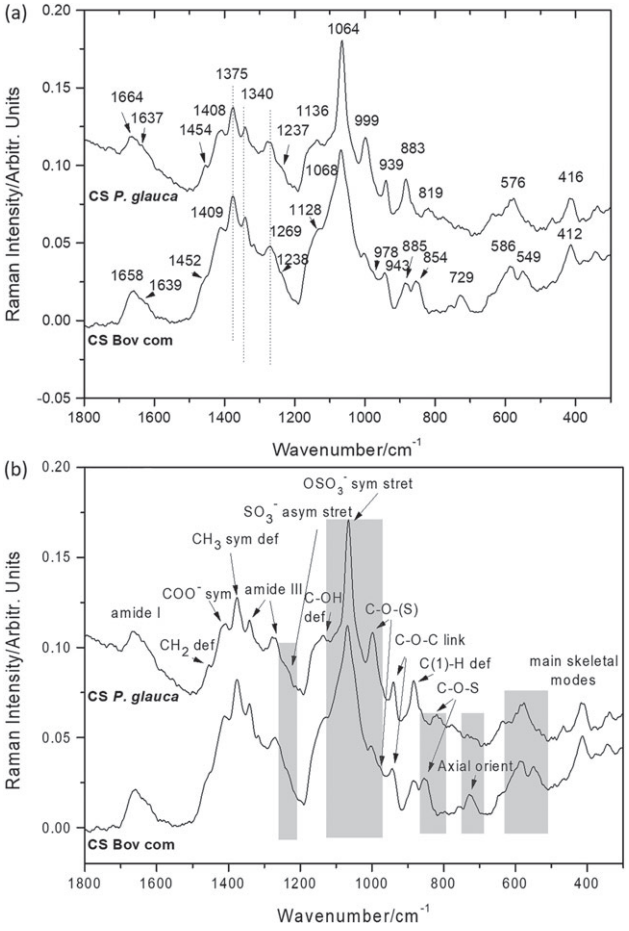

FIGURE 1 Raman spectra of representative chondroitin sulphate (CS) *Prionace glauca* from fish source and CS Bov com (commercial bovine CS) from mammalian source in the wavenumber range of 1,800–350 cm^{-1} (a). Corresponding attributions with bands directly related with sulphate group vibrations or specific orientation of the isomers highlighted in grey (b)

1,136 cm^{-1} and shoulder at 1,128 cm^{-1} corresponding to C—OH deformation.[14] A strong peak at 1,064 cm^{-1} in C6S (*P. glauca*) and at 1,068 cm^{-1} in C4S (Bov com) were attributed to OSO_3— symmetric stretching. Peaks at 999 cm^{-1} and shoulder in 978 cm^{-1} are ascribed to C—O—S) vibration. Finally, a medium intense peak at 939 cm^{-1} in C6S and a less intense one in 4-sulphate at 943 cm^{-1} were attributed to skeletal C—O—C linkage vibrations. Medium intense peaks at 883 cm^{-1} and 885 cm^{-1} respectively correspond to $C_{(1)}$—H deformation for β anomers. A low intense peak at 819 cm^{-1} for C6S and a medium strong one for C4S at 854 cm^{-1} are attributed to asymmetric vibration of C—O—S linkages. A medium strong band near 725 cm^{-1} has been attributed to the axial orientation of the aromatic ring,[20] which appeared in our commercial bovine spectrum as a medium strong broad band at 729 cm^{-1}. Finally, peaks at 576 cm^{-1} in C6S and at 586 cm^{-1}

and 549 cm^{-1} in C4S were also detected, together with 416 cm^{-1} and 412 cm^{-1}, respectively, which correspond mainly to skeletal modes.

In Figure 1b, both spectra are presented with the attributions, already referred to above. The bands, which are directly related to the sulphate group vibrations or specific orientation of the isomer have been highlighted in grey. Differences can be observed in the position and amplitude of the OSO_3— *sym stret* band, which appears sharper and in lower wavenumbers (1,064 cm^{-1}) in the C6S band (CS *P. glauca*) than in C4S, where it appears at 1,068 cm^{-1} in a wider band (assigned at 1,069 cm^{-1} by Orkoula and Kontoyannis 2014[8] and at 1,079 cm^{-1} by Bansil et al. 1978[14]). On the other hand, vibration C—O—(S) is referenced with a strong band in 995 cm^{-1} for C6S and medium at 978 cm^{-1} for 4-sulphate.[14] In our case, a strong band appeared at 999 cm^{-1} in 6-sulphate and a medium-weak one in C4S, with a weak shoulder at 978 cm^{-1} at this latter. These variations in the commercial bovine CS spectra may indicate the presence of other isomers apart from the prevalence of C4S at this mammal source. Bansil et al. (1978) established a medium band in 820 cm^{-1} in C6S and medium strong in 853 cm^{-1} in 4-sulphate attributed to asymmetric vibration of C—O—S linkages.[14] The difference in wavenumber of 33 cm^{-1} has been interpreted to reflect the equatorial configuration of OSO_3— in C6S and axial configuration in C4S. A broad band at 729 cm^{-1} in commercial bovine spectrum is referred in literature as a medium strong band near 725 cm^{-1}, which may arise from an aromatic ring of axial orientation.[2,20] In fact, Mainreck et al. (2010) established that C4S and 6-sulphate differ for the bands at 730 cm^{-1} and 853 cm^{-1} of axial orientation and at 822 cm^{-1} and 1,000 cm^{-1} of equatorial orientation.[2] Again the presence of both bands, the one at 729 cm^{-1} and another weak band at 999 cm^{-1} in commercial bovine CS spectra confirmed C4S composition (with axial orientation) influenced by other isomers with equatorial orientation.

The Raman spectra in the wavenumber range of interest (1,300–350 cm^{-1}) were obtained for the whole series of chondroitin sulphates analysed and are presented in Figure 2. Thus, the spectra of the four CS of fish source (6-sulphate prevalence) are presented in Figure 2a and the three mammalian sources (4-sulphate prevalent) in Figure 2b. Clear similarities in chemical composition within each group, the four tested fish origin CS, or the three tested mammalian origin CS have been detected by Raman spectroscopy. At the same time, clear differences between both types of sources in relation with the Raman vibrational modes have been already detailed.

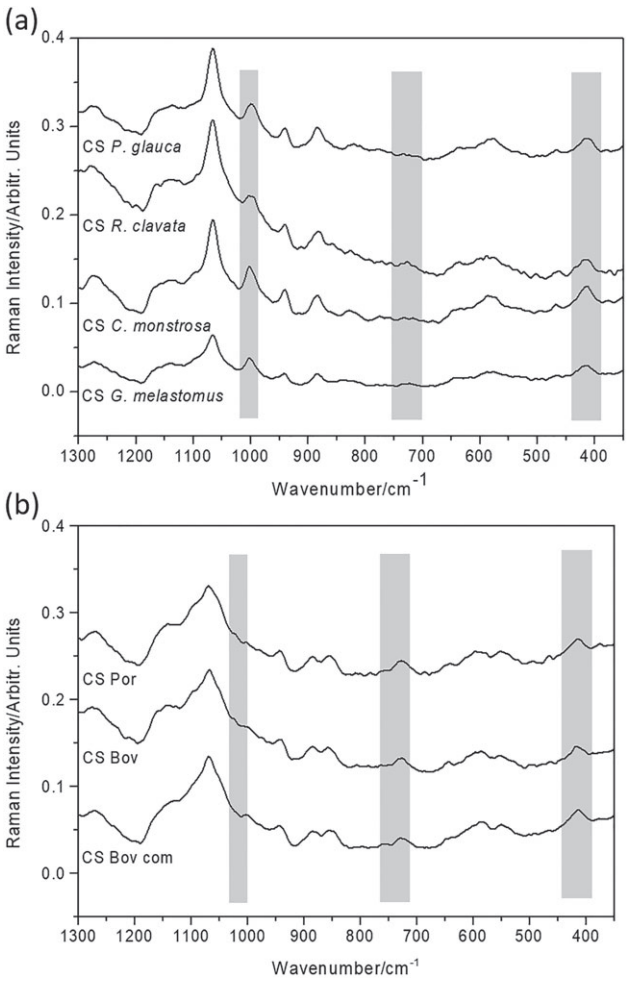

FIGURE 2 Raman spectra in the wavenumber range of interest (1,300–350 cm^{-1}) obtained for the whole series of chondroitin sulphate of fish sources (a) and of mammalian sources (b)

3.2 | Raman quantitative evaluation

3.2.1 | Proposed indices and validation

According to all this, the determination of certain indices will allow for the rapid discrimination by Raman spectroscopy of the major contribution of C6S or C4S in a tested sample of crude CS. Ratios between mean of band areas in the following three ranges of wavenumbers: 390–440 cm^{-1}, 700–750 cm^{-1}, and 980–1,015 cm^{-1} were established for the four fish and the three mammalian sources. The range of 390–440 cm^{-1} contains vibrations of the main skeletal modes, which appear with the same intensity in the seven CS evaluated. The range of 700–750 cm^{-1} contains the medium strong band near a 725 cm^{-1} aromatic ring with an axial orientation, only present in C4S spectra. Finally, the range of 980–1015 cm^{-1} corresponds with the vibration C—O—(S) with a strong band in 999 cm^{-1} for C6S. Thus, the following ratios are suggested:

- Ratio 1 = 700–750 cm^{-1}/390–440 cm^{-1} (area of the Raman bands)
- Ratio 2 = 980–1,015 cm^{-1}/390–440 cm^{-1} (area of the Raman bands)

Area values for the range 390–440 cm^{-1}, used as a constant for both ratios, measured for each of the three mammalian sources, the four fish sources, and for both groups: Mammalian and fish sources are shown in Table 1, where statistically significant differences were not found. At the same time, values obtained for Ratio 1 and Ratio 2 are also presented. Mean values of Ratio 1 obtained for mammalian ones tend to 1, whereas mean values from fish CS tend to 0. These obtained data confirmed what was expected, as the band of 700–750 cm^{-1} is only present in C4S. The values of Ratio 2, using a band specific for C6S (980–1,015) revealed that data of mammalian CS tend to 0 (as expected), whereas the ones of fish sources tend in general to 1.

The group means of the ratios obtained for the three CS of mammalian sources tested and for the four of fish sources, already shown in Table 1, have been represented in Figure 3. The statistical analysis revealed significant difference for both ratios between both groups for 95% of cases ($p < 0.05$).

These Raman quantitative ratios (Table 1) have then established as a major contribution of 4-sulphate for the three tested mammalian sources and of 6-sulphate for the four of the fish sources evaluated. These results were confirmed with an analysis of the disaccharide compositions by SAX-HPLC of CS from three of the mammalian sources and four of the fish ones, shown in Table 2. A major contribution of C4S (GlcA-GalNAc 4S), over 50% was measured for the commercial bovine source, bovine, and porcine CS, whereas a major contribution of C6S (GlcA-GalNAc 6S) of over 50% was measured for *C. monstrosa*, *G. melastomus*, *P. glauca*, and *R. clavata*. Lower contributions of GlcA-GalNAc 0S and GlcA 2S-

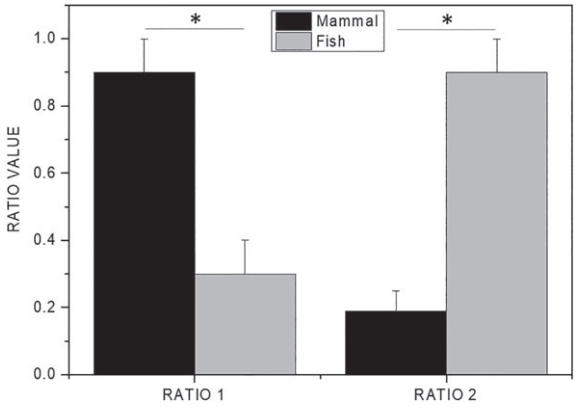

FIGURE 3 Group mean of Ratio 1 and Ratio 2 obtained for mammalian sources and fish sources. Significant statistical difference ($p < 0.05$) indicated as *

GalNAc 6S were detected (<20%) together with a very low presence of GlcA-GalNAc, 4,6S, and GlcA 2S-GalNAc 4S (<2.5%) for both source types, being these four variations higher for the fish sources. These data are reflected in the 4S/6S ratios with a prevalence of sulfation at position 6 over the sulfation at position 4 for CS from fish sources in comparison with mammalian ones, with values close to 0 for those coming from fish and higher than 1 in mammalian CS. The quotients between isomer percentages C4S/C6S obtained by these HPLC results are also presented in Table 2, ranging from 1.47 to 2.53 in mammalian and from 0.16 to 0.43 in fish sources.

Finally, intervals of confidence at 95% for both group mean values were estimated at each Raman ratio to establish Raman sulfation indices. These indices, named as Index For Prevalence of 4S Sulfation and Index For Prevalence of 6S Sulfation are shown in Table 3 and will allow one to rapidly distinguish dominant C4S from C6S contribution for 95% of cases. The indices have been established under the requirement to meet two conditions. Thus, Ratio 1 with a value between 0.8 and 1.0

TABLE 1 Area values for the range 390–440 cm-1, used as a constant for both ratios, established for each of the three mammalian sources, the four fish sources, and for both groups: Mammalian and fish sources (statistically not significantly different) are shown. Values for Ratio 1 and Ratio 2 are also presented. All data are indicated as mean ± standard deviation

	Bov com	Bovine	Porcine	*Prionace glauca*	*Raja clavata*	*Chimaera monstrosa*	*Galeus melastomus*
390–440 cm^{-1}	0.45 ± 0.07	0.30 ± 0.03	0.33 ± 0.02	0.42 ± 0.04	0.41 ± 0.02	0.48 ± 0.02	0.28 ± 0.01
Group mean ± *SD*	0.36 ± 0.08	0.39 ± 0.08					
Ratio 1	0.8 ± 0.2	1.0 ± 0.1	1.03 ± 0.02	0.27 ± 0.07	0.46 ± 0.09	0.23 ± 0.07	0.37 ± 0.01
Group mean ± *SD*	0.9 ± 0.1	0.3 ± 0.1					
Ratio 2	0.22 ± 0.01	0.24 ± 0.04	0.12 ± 0.01	0.96 ± 0.09	0.67 ± 0.03	0.95 ± 0.04	0.94 ± 0.03
Group mean ± *SD*	0.19 ± 0.06	0.9 ± 0.1					

TABLE 2 Disaccharide composition of chondroitin sulphate from terrestrial and fish sources determined by strong anion exchange-high-performance liquid chromatography

	Bov com	Bovine	Porcine	*Chimaera monstrosa*	*Galeus melastomus*	*Prionace glauca**	*Raja clavata**
C4S (GlcA-GalNAc 4S)	55.2 ± 0.4	56.8 ± 0.1	68.6 ± 0.1	22.1 ± 0.3	23.8 ± 0.1	10.1 ± 0.1	16.0 ± 0.1
C6S (GlcA-GalNAc 6S)	37.5 ± 0.2	36.9 ± 0.1	27.1 ± 0.2	52.7 ± 0.1	54.9 ± 0.4	64.2 ± 0.4	55.9 ± 0.1
GlcA-GalNAc 0S	5.7 ± 0.5	5.9 ± 0.3	3.7 ± 0.1	4.4 ± 0.2	4.2 ± 0.5	16.3 ± 0.6	19.5 ± 0.1
GlcA 2S-GalNAc 6S	0.7 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	17.4 ± 0.1	15.0 ± 0.1	9.3 ± 0.2	8.6 ± 0.12
GlcA-GalNAc 4,6S	0.6 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	2.4 ± 0.1	1.5 ± 0.0	n.o	n.o
GlcA 2S-GalNAc 4S	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	1.0 ± 0.0	0.6 ± 0.0	n.o	n.o
C4S/C6S	1.47	1.54	2.53	0.42	0.43	0.16	0.29

Results expressed as mean % ± standard deviation ($n = 2$). C4S/C6S = (% GlcA-GalNAc 4S/ %GlcA-GalNAc 6S).

*Novoa-Carballal et al. (2017).[4] n.o: not observed.

TABLE 3 Raman quantitative sulfation indices (of double condition) for confidence interval of 95% of cases established to distinguish a major contribution of 4-sulphate or 6-sulphate isomer of the chondroitin sulphate evaluated

	Ratio 1	Ratio 2
Index for prevalence of 4s sulfation	(0.8–1.0) &	(0.12–0.26)
Index for prevalence of 6s sulfation	(0.2–0.4) &	(0.8–1.0)

and Ratio 2 between 0.12 and 0.26 constitute an indicator of prevalence of C4S at the CS source. In the same way, Ratio 1 with a value of between 0.2 and 0.4 and Ratio 2 with between 0.8 and 1.0 signify a prevalence of C6S.

3.2.2 | Case study: No-prevalent CS6 or CS4 source

Finally, CS from a fish source, *S. canicula* fin, with an equal contribution of C4S and C6S isomers was evaluated by both methodologies, Raman spectroscopy, and HPLC. Raman spectra of CS from *P. glauca* (6-sulphate prevalence), from Bov com (4-sulphate prevalence), and of *S. canicula* are presented in Figure 4, where band areas of interest, to obtain the mentioned indices have been highlighted in grey (390–440 cm^{-1}, 700–750 cm^{-1}, and with 980–1,015 cm^{-1}). Bands of C—O—S linkage at 819 cm^{-1} in CS with C6S predominance and at 854 cm^{-1} in CS with C4S predominance are highlighted in circles with dashed lines, together with main skeletal modes at 576 cm^{-1} for C6S and at 586 cm^{-1} and 549 cm^{-1} for C4S. The Raman spectrum obtained for *S. canicula* did not fit with any of the other two presented. A Raman band of low intensity was observed at 980–1,015 cm^{-1} region in *S. canicula* spectrum as well as in Bov com spectrum, however the characteristic band at 700–750 cm^{-1} of axial

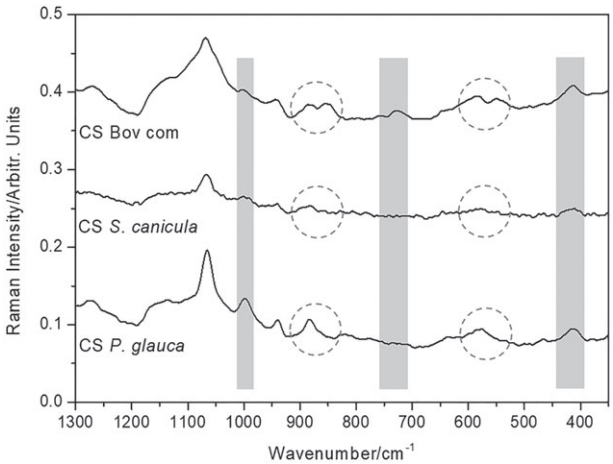

FIGURE 4 Raman spectra in the wavenumber range of interest (1,300–350 cm^{-1}) obtained for chondroitin sulphate from *Scyliorhinus canicula* in comparison with chondroitin sulphate (CS) Bov com (4-sulphate prevalence) and CS *Prionace glauca* (6-sulphate prevalence)

orientation of the aromatic ring observed at C4S (Bov com) was not present at *S. canicula* spectrum. In relation with the bands of C—O—S linkage and main skeletal modes (circles with dashed lines), the spectrum of *S. canicula* did not present a clear tendency with respect to any of the other two, as one clear peak appeared at CS of *P. glauca* or a double-peak band at Bov com spectrum.

Raman Ratio 1 obtained for *S. canicula* was 0.20 ± 0.07, and Ratio 2 was 0.40 ± 0.03. These values do not fit both requirements for any of the proposed indices for the prevalence of 4 sulfation position or to that of 6 sulfation position (Table 3). This means that there is no major contribution of isomer C4S or C6S for this chondroitin and that other isomers could represent a significant contribution. SAX-HPLC data for this *S. canicula* CS (Table 4) confirmed the equal contribution of around 30%

TABLE 4 Disaccharide composition of chondroitin sulphate from *Scyliorhinus canicula* determined by strong anion exchange-high-performance liquid chromatography

	CS *Scyliorhinus canicula**
C4S (GlcA-GalNAc 4S)	31.5 ± 0.6
C6S (GlcA-GalNAc 6S)	32.4 ± 0.1
GlcA-GalNAc 0S	22.5 ± 1.4
GlcA 2S-GalNAc 6S	16.3 ± 0.2
GlcA -GalNAc 4,6S	n.o
GlcA 2S-GalNAc 4S	n.o
C4S/C6S	0.97

Results expressed as mean % ± standard deviation (n = 2). C4S/C6S = (% GlcA-GalNAc 4S/ %GlcA-GalNAc 6S).

*Novoa-Carballal et al. (2017).[4] n.o: not observed.

for C4S (GlcA-GalNAc 4S) and for C6S (GlcA-GalNAc 6S) and significant contributions of GlcA-GalNAc 0S and GlcA 2S-GalNAc 6S of around 20%. The quotient C4S/C6S of 0.97 situates this chondroitin in an intermediate position between mammalian (ranging from 1.47 to 2.53) and the other previously tested four fish sources (ranging from 0.16 to 0.43). These results reinforce the validity of the proposed indices.

Therefore, the use of the proposed indices allowed us to distinguish the major contribution of C4S or C6S in a given chondroitin in an objective, quantitative, and rapid manner by means of Raman spectroscopy. Given the results obtained, the sources of the CS analyzed have been divided into two groups: mammalian and fish by-products sources, as the first ones presented a clear prevalence of 4-sulphate chondroitin whereas in fish-derived CS, 6-sulphate was prevalent for most cases. In fact, CS obtained from whale cartilage, a marine mammal, has been commercialized as a 4-sulphate prevalent CS,[2] and the ones currently commercialized from shark cartilage present a predominance of C6S isomer, which seems to follow the same trend. These sources are of enormous interest with regards to treating neuralgia, arthritis, tinnitus, cancer, hyperlipaemia, and other conditions.[22,23] Treatment outcome is directly related to the quality of the CS preparation, however the quantification of intact CS with no UV or fluorescence chromophores is difficult by normal spectral and chromatographic methods.[7] Reliable and accurate methods for the determination of CS content are therefore important for the quality control of CS preparations. A quantitative determination of CS by Raman spectroscopy was recently obtained with relatively high accuracy.[7] The quantitative identification of a major contribution of isomeric C4S or C6S in the present work adds one more tool with high sensitivity and specificity to facilitate the characterization of crude CS. In fact, the design of specific software where proposed indices are incorporated would enable a reliable, accurate quantitative characterization in seconds.

4 | CONCLUSIONS

Raman spectroscopy has been demonstrated to be a reliable technique for quantitative identification of the major contribution of isomeric C4S or C6S. Two quantitative indices have been proposed based on areas of specific bands of interest related to main skeletal modes at 390–440 cm^{-1}, the aromatic ring with axial orientation in the case of the major contribution of C4S at 700–750 cm^{-1} and C—O—(S) vibration, which is detected at high levels at 980–1,015 cm^{-1} in the case of the major contribution of C6S. The indices were defined as being linked to a double condition as follows: Index for the Prevalence of 4S Sulfation (95% confidence interval) meaning values between 0.8 and 1.0 for 700–750/390–440 (Ratio 1) and between 0.12 and 0.26 for 980–1,015/390–440 (Ratio 2); Index for Prevalence of 6S Sulfation (95% confidence interval) meaning (0.2–0.4) for Ratio 1 and (0.8–1.0) for Ratio 2. Three mammalian and five fish by-products sources validated the ability of the mentioned indices to differentiate between the prevalence of C4S, C6S, or neither of them. Correspondence between the results obtained by the Raman indices proposed and HPLC characterization of the different CS evaluated was also demonstrated.

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