**Optimal isolation and characterisation of chondroitin sulfate from
Rabbit fish (*Chimaera monstrosa*)**

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Abstract

Chondroitin sulfate (CS) is a glycosaminoglycan widely explored for cartilage regeneration. Its bioactivity is influenced by sulfation degree and pattern, and distinct sulfation in marine CS may open new therapeutic possibilities. In this context, we studied for the first time the isolation and characterisation of CS from Rabbit Fish (*Chimaera monstrosa*). We propose an efficient process starting with enzymatic hydrolysis, followed by chemical treatments and ending in membrane purification. All steps were optimised by response surface methodology. Chemical treatment by alkaline-hydroalcoholic precipitation led to 99% purity CS suitable for biomedical and pharmaceutical applications, and treatment by alkaline hydrolysis yielded CS adequate for nutraceutical formulations (89% purity). Molecular weight and sulfation profiles were similar for both materials. Gel permeation chromatography analyses resulted in molecular weights (M_n) of 51-55 kDa. NMR and SAX-HPLC revealed dominant 6S-GalNAc sulfation (4S/6S ratio of 0.4), 17% of GlcA 2S-GalNAc 6S and minor quantities of other disaccharides.

Keywords: Chondroitin sulfate isolation; *C. monstrosa* cartilage; sulfation pattern; molecular weight; process optimisation; response surface methodology.

1. Introduction

Glycosaminoglycans (GAGs) are linear polysaccharides ubiquitous at the cell surface and in the extracellular matrix of most animal tissues (Yamada & Sugahara, 2008). Beyond structural functions, GAGs participate in fundamental biological processes such as cellular communication, differentiation, and growth. As a consequence, GAGs have found applications in the pharmacological and nutraceutical fields and are actively explored for regenerative medicine (Celikkin et al., 2017; Choi & Choi, 2012; Lima, Rudd, & Yates, 2017). The biological activity of GAGs depends on their capacity to interact with proteins. In sulfated GAGs, this interaction is influenced by changes in charge density based on the relative abundance of sulfated disaccharides; the presence of particular sulfated units; and, sometimes, specific saccharide sequences (Valcarcel, Novoa-Carballal, Pérez-Martín, Reis, & Vázquez, 2017)

Chondroitin sulfate (CS) is a sulfated GAG formed by disaccharide units of glucuronic acid (GlcA) and N-acetyl galactosamine (GalNAc) linked by β -(1,4)-glycosidic bonds. Sulfation can occur in both rings at different positions, leading to considerable heterogeneity in CS chains. In the marine environment, CS shows distinct characteristics compared to conventional porcine and bovine sources, mainly in terms of molecular weight and sulfation (Valcarcel et al., 2017). Abundance of particular units in CS chains seems to be related to therapeutic properties and is characteristic of particular marine organisms (Kozłowski et al., 2011). CS from different marine species display antiviral, anti-metastatic and anticoagulant properties; has shown to improve the mechanical and signalling

properties of cartilage engineering structures; and promote neurite outgrowth when hybridized with dermatan sulfate (DS) (Valcarcel et al., 2017; Yamada & Sugahara, 2008).

In this context, extraction of CS from sources not previously explored holds potential to discover novel sequences and ultimately therapeutic applications. This is the case of chimaera (*Chimaera monstrosa*), an ancient cartilaginous fish related to sharks, occasionally appearing as by-catch in deep water North Atlantic trawlers. To the best of our knowledge, only two previous reports have dealt with the characterization of CS from *Chimaera* species (Higashi et al., 2015b; Rahemtulla, Höglund, & Løvtrup, 1976), none of them describing optimal isolation processes nor the molecular weight of the polymers.

Depending on the application, CS must meet specific characteristics, which are influenced by the isolation process. Commercially, marine CS is released from shark after hydrolysis to disrupt the cartilage structure and eliminate proteins, followed by recovery and purification steps. Conventional processes include hydrolysis with high concentrations of alkali and toxic chemicals, such as guanidine hydrochloride, and final purification with chromatographic methods (Vázquez et al., 2013).

Bearing this in mind, the present work aims at isolating for the first time CS from the cartilage of *C. monstrosa*, optimizing the process from a sustainability perspective. This comprises alternative enzymatic hydrolysis to replace corrosive

alkali and comparison of selective precipitation of CS using alkaline hydroalcoholic solutions with less specific NaOH treatment. Optimal conditions are defined by response surface methodology. Finally, CS purity is increased by ultrafiltration-diafiltration techniques instead of time-consuming chromatographic separations, achieving CS of variable purity suitable for different applications. A schematic flowchart describing the steps developed to isolate CS from rabbit fish is shown in Figure A (Supplementary material).

2. Materials and Methods

2.1. Reagents

Ethanol 95-97% (Prod. No. 20905.365, VWR), sodium hydroxide (Prod. No. BDH9292, VWR), Sodium azide (Prod. No. S2002, Sigma-Aldrich), sodium dihydrogen phosphate monohydrate (Prod. No.1.06346.1000, Merck), deuterium oxide (Prod. No. 151882, Sigma-Aldrich), tris (hydroxyethyl)-aminomethane (Prod. No. KB313482, Merck), sodium acetate (Prod. No. 27653.292, VWR), sodium chloride (Prod. No. 27810.295, VWR), phenol (Prod. No. 1.00206.0250, Merck), hydrazine sulfate (Prod. No. 1.04603, Merck), sulphuric acid 95% (Prod. No. 20700.298, VWR), sodium carbonate anhydrous (Prod. No. 27771.290, VWR), copper (II) sulfate pentahydrate (Prod. No. BDH9312, VWR), potassium/sodium L(+)-tartrate tetrahydrate (Prod. No. 27068.290, VWR), Folin-Ciocalteu reagent (Prod. No. RE00180250, Scharlau), disodium tetraborate decahydrate (Prod. No. S007050500, Scharlau), sodium salicylate (Prod. No.71948, Sigma-Aldrich), sodium nitroprussiate (Prod. No. 31444, Sigma-Aldrich), sodium

dichloroisocyanurate dihydrate (Prod. No. 110887, Merck), diethyl ether (Prod. No. ET00792500, Scharlau).

2.2. Cartilage preparation

Rabbit fish (*Chimaera monstrosa*) individuals of 800-2000 g, captured as discards, were kindly provided by Opromar (Marín, Spain) and stored at -18°C until use. Specimens were eviscerated and heated in a water bath at 90°C for 30 min. Cartilaginous material was then milled and homogenized at ~1-3 mm size using a grinder and stored at -18°C. Chemical composition of cartilage was assessed by determination of water and ash content, fat concentration, total nitrogen and total protein as total nitrogen $\times$ 6.25 (AOAC, 1997). Total carbohydrate content was estimated by subtracting protein, fat, ash and moisture from total sample weight. This value of carbohydrates was confirmed by total sugar determination (Dubois et al., 1956) after exhaustive enzymatic proteolysis of cartilage.

2.3. Experimental designs for CS isolation

Initially, the joint effect of *pH*, temperature (*T*) and enzyme/substrate ratio (*E/S*) on cartilage digestion by Esperase (Esperase 8L: 8 KNovo Protease Unit/g, KNPU/g, Novozymes, Nordisk, Denmark). was studied. In this case, 20 experiments were run (with 6 replicates in the center of the experimental domain) following the combinations indicated in Table A (supplementary material), being the ranges of the independent variables: *pH* (6-10), *T* (30-80°C) and *E/S* (0.01-2%). Subsequently, two strategies were followed for the chemical processing of the previous hydrolysates: 1) alkaline treatment, evaluating the simultaneous effect of

time (ranging between 1 and 24 h) and NaOH concentration (from 0.2 to 1.5M) and 2) selective precipitation of CS with alkaline-hydroalcoholic solutions (NaOH concentration (0.1-0.8M) and EtOH volume (0.3-1.4 v) as independent variables). In both designs, 13 experiments were performed (with 5 replicates in the center of the experimental domain). In all cases, the experimental procedure was based on the performance of rotatable second order designs (Box, Hunter, & Hunter, 2005). Codified and natural values for all experimental conditions tested in the factorial designs are summarized in Tables A and B (supplementary material). In the three factorial designs, the responses (dependent variables) evaluated were the concentration of CS recovered and the CS purity index (I_p) defined below. In addition, the maximum hydrolysis (H_m) was also studied as response for enzyme hydrolysis experiments.

Orthogonal least-squares calculation on factorial design data were used to obtain empirical equations describing the different response assessed (Y) in function of the independent variables:

$$Y = b_0 + \sum_{i=1}^n b_i X_i + \sum_{i=1}^{n-1} \sum_{\substack{j=2 \\ j>i}}^n b_{ij} X_i X_j + \sum_{i=1}^n b_{ii} X_i^2 \quad [1]$$

where: Y is the dependent variable evaluated, b_0 the constant coefficient, b_i the coefficient of linear effect, b_{ij} the coefficient of combined effect, b_{ii} the coefficient of quadratic effect, n the number of variables and X_i and X_j are the independent

variables studied in each case. Student's t-test ($\alpha=0.05$) was employed to determine statistical significance of coefficients. Coefficient of determination (R^2) and adjusted coefficients of determination (R^2_{adj}) were used to establish goodness-of-fit and the following mean squares ratios from Fisher F test ($\alpha=0.05$) were calculated to define model consistency: $F1 = \text{Model} / \text{Total error}$, being the model acceptable when $F1 \geq F_{den}^{num}$; and $F2 = (\text{Model} + \text{Lack of fitting}) / \text{Model}$, being the model acceptable when $F2 \leq F_{den}^{num}$. F_{den}^{num} are the theoretical values to $\alpha=0.05$ with corresponding degrees of freedom for numerator (num) and denominator (den). All calculations were performed in an Excel-spreadsheet specifically developed for this purpose. Statistical and graphical results were confirmed by Statistica v. 8.0 software (StatSoft, Inc, Tulsa, OK, USA).

2.4. Proteolysis of *C. monstrosa* cartilage

Proteolysis was carried out in a controlled pH-Stat system with a 100 mL glass-reactor containing 25 g of fresh cartilage and 50 mL distilled water. Agitation was maintained constant at 200 rpm, while pH , T and E/S varied as defined in Table A (supplementary material). At the end of the hydrolysis (6 h), samples were centrifuged (6000 g for 20 min) and liquid fractions (hydrolysates) stored at -18°C until analysis. Degree of hydrolysis (H , as %) was quantified following the pH-Stat method (Adler-Nissen, 1986) and equations previously described (Vázquez, Blanco, Massa, Amado, & Pérez-Martín, 2017). Hydrolysis kinetics were finally modelled by Weibull equation (Vázquez et al., 2017):

$$H = H_m \left\{ 1 - \exp \left[-\ln 2 \left(\frac{t}{\tau} \right)^\beta \right] \right\} \quad [2]$$

where, H is the degree of hydrolysis (%); t hydrolysis time (min); H_m maximum degree of hydrolysis (%); β a parameter related with the maximum slope of muscle hydrolysis (dimensionless); and τ is the time required to achieve the semi-maximum degree of hydrolysis (min).

2.5. Chemical treatments of the hydrolysates

Once optimal pH, T and E/S ratio were established for proteolysis, a larger enzymatic hydrolysate (EH) of cartilage (1300 g of substrate + 3600 mL of distilled water) was produced under these conditions and split into two aliquots. The first aliquot was subjected to fractionation by selective precipitation with alkaline hydroalcoholic mixtures, while alkaline hydrolysis with NaOH was applied to the second aliquot. Both chemical treatments were optimized following the experimental design outlined in Table B (supplementary material). In the alkaline hydroalcoholic treatment, CS in the EHs was precipitated by slow addition of NaOH solutions with varying ethanol content (hydroalcoholic media, see Table B, supplementary material). Mixtures also contained 2.5 g/L NaCl to improve subsequent CS redissolution in water. After 1-2 h in agitation at medium speed (100 rpm) and room temperature, the suspensions were centrifuged (6000 g , 20 min) and the sediments redissolved in water and neutralized with 6 M HCl. In the alkaline treatment, NaOH was added to the EHs up to the required concentrations

and the mixtures maintained in continuous agitation at 200 rpm and room temperature for the different times evaluated (Table B, supplementary material). At the end of the hydrolysis, mixtures were centrifuged at 6000 g for 20 min and supernatants neutralized with 6 M HCl.

2.6. Analytical determinations

Samples from the different experiments were analysed to quantify total soluble proteins (Pr) (Lowry, Rosebrough, Farr, & Randall, 1951), and CS concentration as GlcA (van den Hoogen et al., 1998), according to the mathematical modifications reported in Murado, Vázquez, Montemayor, Cabo, & González (2005). CS purity index (I_p), defined as $I_p (\%) = \text{CS} \times 100 / (\text{CS} + \text{Pr})$, was also calculated in all purification steps.

2.7. Ultrafiltration-diafiltration (UF-DF) system for CS purification

Ultrafiltration membranes of 100 and 30 kDa (spiral polyethersulfone, 0.56 m², Prep/Scale-TFF, Millipore Corporation, USA) were used to purify the neutralized solutions rich in CS generated, under optimal conditions, in the previous chemical treatments. The membrane system was configured in a sequential cascade of 100 to 30 kDa operating in total recirculation and maintaining an inlet pressure of 1 bar (Amado, Vázquez, González, & Murado, 2013). Initially, UF-100 kDa was run for concentration of CS solutions and then DF procedure of retentates was applied. Due to CS not being retained by 100 kDa, permeates were passed through 30 kDa for concentration (UF step, factor of volumetric concentration= 4-5) and then desalination and protein removal was achieved by DF. Experimental DF data were

modelled using the following equation (Blanco, Fraguas, Sotelo, Pérez-Martín, & Vázquez, 2015):

$$R = R_f + R_0 e^{-(1-s)D} \quad [3]$$

where, R is the concentration of permeable CS or soluble protein in the retentate (% from the level at initial DF), R_f the retentate concentration of CS or protein in the asymptotic phase (%), R_0 the permeate concentration (%), D the relative diavolume (volume of added water/constant retentate volume), and s is the specific retention of CS or protein ranging from 0 (the solute is totally filtered) to 1 (the solute is completely retained). Consequently, when values are normalized as a percentage: $R_0 + R_f = 100$, where $R_0 = 0$ if all CS or protein is permeable. Percentage of CS or protein permeated by three diavolumes (R_{3D}) was also calculated. The CS purified solutions were finally dried at 60°C/48 h in a lab oven.

2.8. Molecular weight of CS

Molecular weight of CS was determined on an Agilent 1260 HPLC consisting of quaternary pump (G1311B), injector (G1329B), column oven (G1316A), refractive index (RI, G1362A) and dual-angle static light scattering (DALS, G7800A) detectors. Standard and samples were dissolved in the mobile phase (0.1M NaN₃: 0.01M NaH₂PO₄, pH 6.6) and separated with four columns (PSS, Germany): Suprema precolumn (5 µm, 8x50 mm), Suprema 30Å (5 µm, 8x300 mm), Suprema 100Å (5 µm, 8x300 mm) and Suprema ultrahigh (10 µm, 8x300 mm) at 1 mL/min

after a 100 μ L injection. Column oven and DALS were kept at 30°C, while the RID was maintained at 40°C. Detectors were calibrated with a polyethylene oxide standard (PSS, Germany) of 106 kDa (Mp) and polydispersity index 1.05. Refractive index increments (dn/dc) were calculated from the RI detector assuming accurate CS concentrations.

2.9. Sulfation of CS

Initial evaluation of the chemical composition was carried out by NMR (Nuclear Magnetic Resonance). Spectra were obtained on a Bruker DPX 600 operating at 600 Mhz and 10 °C to avoid overlapping with residual HOD. Spectra were processed with MestReNova 10.0.2 software (Mestrelab Research, Spain). Samples were dissolved in D₂O at 1 g/L for ¹H and 40 g/L for HSQC experiments. The latter were carried out to confirm sulfation positions in ¹H spectra.

Detailed disaccharide composition was determined by strong anion exchange (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 in 0.05M Tris-HCl:0.15 M sodium acetate pH 8. After 24 h at 37 °C, samples were heated at 70 °C for 25 min, centrifugated at 12857 g for 30 min and supernatants filtered (0.2 μ m polyethersulfone). Unsaturated disaccharide standards (Grampenz, UK) were dissolved in water. Samples and standards were injected (20 μ l) onto an Agilent 1200 HPLC consisting of pump (G1312A), column oven (G1316A) and UV-visible detector (G1314B) equipped with a Waters Spherisorb SAX column (5 μ m, 4.6x250 mm) fitted with a guard cartridge (Waters

Spherisorb, 5 μ m, 4.6x10mm). Elution was performed at 1.5 ml min⁻¹ in isocratic mode from 0 to 5 min with 50 mM NaCl pH 4 and then in linear gradient from 5 to 25 min, starting with 50 mM NaCl pH 4 and ending with 76% 50 mM NaCl pH 4 and 24% 1.2 M NaCl pH 4. Disaccharides were detected at 232 nm and quantified by external calibration.

2.10. Numerical and statistical analyses

Data fitting procedures and parametric estimations were carried out by minimisation of the sum of quadratic differences between observed and model-predicted values, using the non-linear least-squares (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel spreadsheet. Confidence intervals from the parametric estimates (Student's t-test) and consistence of mathematical models (Fisher's F test) were evaluated by "SolverAid" macro.

3. Results and Discussion

In the specimens tested, 8.3 $\pm$ 0.3% (fresh weight) of the whole body consists of cartilage, with a moisture content of 70.9 $\pm$ 2.8%. Proximate analysis of cartilage resulted in 52.5 $\pm$ 1.0% protein, 37.6 $\pm$ 0.9% ash, 7.7 $\pm$ 0.4% fat and 1.0 $\pm$ 0.3% carbohydrates.

3.1. Hydrolysis of C. monstrosa cartilage by Esperase

Enzymatic digestion of cartilage from different fish species has been studied in various reports (Lignot, Lahogue, & Bourseau, 2003; Murado, Fraguas, Montemayor, Vázquez, & González, 2010; Yang et al., 2015). The application of

commercial proteases (Alkalase, Papain, Actinase E, etc.) has generally led to high hydrolysis values and complete digestion of solid cartilage. In the present study the enzyme selected is Esperase, a serine-type endoprotease which has shown high proteolytic activity on fish and cephalopod waste substrates (Akagündüz et al., 2014; Vázquez et al., 2017).

Kinetic hydrolysis profiles in each of the 20 experimental conditions tested are accurately predicted by means of Weibull equation [2] (Figure 1). Parameter estimations and statistical analysis of modelling reveal the validity of the equation employed to describe and characterise cartilage proteolysis by Esperase (Table C, supplementary material). At the end of hydrolysis, samples were partially purified under suboptimal conditions (1.17v EtOH and 0.54 M NaOH) to determine CS concentration (Vázquez, Blanco, Fraguas, Pastrana, & Pérez-Martín, 2016). The simultaneous effect of pH , T and E/S on the responses evaluated (H_m , CS and I_p) are displayed in Figure 2 and polynomial equations that define theoretical 3D-surfaces describing such effects are summarised in Table 1.

Figure 1. Cartilage hydrolysis over time of *C. monstrosa* by Esperase in each one of the experimental conditions defined in Table A (supplementary material). The experimental data (symbols in red colour) were fitted to the Weibull equation [2] (continuous black line).

Figure 2. Experimental data (points) and theoretical surfaces defined by the polynomial equations defined in Table 1 for the enzymatic hydrolysis of *C. monstrosa* cartilage. Each graph represents the combined effect of the corresponding independent variables when another variable was maintained constant (at level signed in red colour).

Correlation between experimental and predicted data, based on R^2 and R_{adj}^2 values, is remarkable for H_m and CS responses and scarce for I_p . F-Fisher ratios were also validated in all cases. Quadratic coefficients of T and pH are statistically

significant and negative for H_m and CS , but $(E/S)^2$ term is positive or non significant depending on the response. Optimal values of pH , T and E/S that maximise each response, obtained by numerical derivation, are also described in Table 1. The operative optimum condition is established at 53.1°C, 2% of E/S ratio and $pH=8.38$, calculated as the average of each response.

Table 1. Second order equations describing the effect of T , E/S and pH on Esperase cartilage digestion (H_m -parameter), CS isolation and I_p -index (coded values according to criteria defined in Table A, supplementary material). The coefficient of determination and adjusted determination (R^2 and R^2_{adj}) and F-values are also shown. NS: non-significant.

Parameters	H_m	CS	I_p
b_0 (intercept)	19.0±0.83	6.33±0.20	87.96±1.46
b_1 (T)	0.72±0.55	-0.15±0.14	NS
b_2 (pH)	4.35±0.55	-0.14±0.13	NS
b_3 (E/S)	3.27±0.55	0.18±0.13	NS
b_{12} ($T \times pH$)	NS	NS	NS
b_{13} ($T \times E/S$)	2.59±0.72	NS	NS
b_{23} ($pH \times E/S$)	NS	NS	NS
b_{123} ($T \times pH \times E/S$)	-1.98±0.50	NS	NS
b_{11} (T^2)	-1.24±0.53	-0.35±0.14	-1.19±0.94
b_{22} (pH^2)	-2.58±0.54	-0.28±0.13	NS
b_{33} ($(E/S)^2$)	1.12±0.53	NS	NS
R^2	0.940	0.761	0.611
R^2_{adj}	0.896	0.676	0.578
$F1$	21.4	8.90	12.60
	$[F_{11}^8 = 2.95] \Rightarrow S$	$[F_{14}^5 = 2.96] \Rightarrow S$	$[F_{18}^1 = 4.14] \Rightarrow S$
$F2$	0.652	0.486	0.154
	$[F_8^{13} = 3.26] \Rightarrow S$	$[F_5^{13} = 4.66] \Rightarrow S$	$[F_1^{13} = 244.7] \Rightarrow S$
	H_m	CS	I_p
T_{opt} (°C)	52.5	51.9	55.0
pH_{opt}	9.07	7.69	No effect
E/S_{opt} (%)	2.0	2.0	No effect
Y_{max}	18.5%	6.37 g/L	87.9%

The present study is the first to apply Esperase for the hydrolysis of cartilaginous tissue. Our results of H_m are in agreement with those previously found for ray

(27%), small-spotted catshark (23.4%), blue shark (18%) and blackmouth catshark (22.9) cartilages treated with Papain or Alcalase (Blanco et al., 2015; Murado et al., 2010; Vázquez et al. 2016; Vázquez et al., 2018). It reveals the validity of this protease for the digestion of cartilage in substitution of the commercial enzymes more commonly employed.

3.2. Chemical treatment of Esperase hydrolysates

Based on the optimised values, EH at a larger scale (1300 g of cartilage along with 2600 mL of distilled water in a reactor of 5 L) was produced under the following hydrolysis conditions: pH=8.38, 53.1°C, t=6 h, agitation=200 rpm, E/S ratio= 2%. The hydrolysate obtained after removing (by centrifugation) the non-hydrolysable solid material (mainly of mineral nature) was aliquoted for the factorial designs of chemical treatments. Both treatments are fundamental to further hydrolyse peptides and protein material prior to membrane purification of CS. Additionally, the selective precipitation of CS in alkaline-hydroalcoholic medium should improve the final purity of CS. However, this process is significantly more expensive than using only alkaline medium.

The selection of the ranges of NaOH concentrations and EtOH volumes to study for the alkaline-hydroalcoholic process of rabbit fish EH were due to the excellent results previously found when CS was precipitated from hydrolysates of blue shark cartilage (Vázquez et al., 2016) and blackmouth shark cartilage (Vázquez et al., 2018). Figure 3 shows experimental data of the chemical treatments applied to the

hydrolysates (alkaline and alkaline-ethanolic precipitation), along with the predicted surfaces defined by fitting to second order equations [1]. Hydrolysis of cartilage hydrolysates in alkaline solution is affected by processing time and NaOH concentration (Table 2). In fact, quadratic and linear terms, for both variables and CS-response, are significant and negative. Once again, I_p theoretical surface is statistically less consistent than that for CS in terms of determination coefficient values. Optimal conditions range from 0.70-0.85 M NaOH and 11.4-12.5 h of treatment, defining the compromise option for alkaline hydrolysis in 0.8 M NaOH and 12 h.

Table 2. Second order equations describing the joint effect of *NaOH* and *time* on CS processing (coded values according to criteria defined in Table B, supplementary material). The coefficient of determination and adjusted determination (R^2 and R^2_{adj}) and F-values are also shown. NS: non-significant.

Parameters	CS	I_p
b_0 (intercept)	6.24±0.30	64.53±2.47
b_1 (N)	-0.38±0.24	NS
b_2 (t)	-0.23±0.22	NS
b_{12} (Nxt)	NS	NS
b_{11} (N ²)	-0.53±0.25	-3.18±2.10
b_{22} (t ²)	-0.82±0.26	-4.10±2.10
R^2	0.925	0.587
R^2_{adj}	0.887	0.485
$F1$	24.55 [$F_8^4 = 3.84$] ⇒ S	4.75 [$F_{10}^2 = 4.10$] ⇒ S
$F2$	0.526 [$F_4^8 = 6.04$] ⇒ S	0.489 [$F_2^8 = 19.37$] ⇒ S
	CS	I_p
$NaOH_{opt}$ (M)	0.70	0.85
t_{opt} (h)	11.4	12.5
Y_{max}	6.32 g/L	64.5%

On the other hand, the simultaneous effect of EtOH and NaOH concentrations (including NaCl in the mixtures to improve CS redissolution) was also examined by multivariable analysis (Figure 3 and Table 3).

Table 3. Second order equations describing the joint effect of *NaOH* and *Ethanol* on selective precipitation of CS (coded values according to criteria defined in Table B, supplementary material). The coefficient of determination and adjusted determination (R^2 and R^2_{adj}) and F-values are also shown. NS: non-significant.

Parameters	CS	I_p
b_0 (intercept)	5.98±0.36	88.21±0.59
b_1 (N)	1.28±0.28	12.69±0.46
b_2 (E)	2.12±0.28	8.73±0.46
b_{12} (NxE)	-0.47±0.40	-3.88±0.65
b_{11} (N ²)	-1.38±0.30	-13.79±0.50
b_{22} (E ²)	-1.49±0.30	-7.07±0.50
R^2	0.936	0.817
R^2_{adj}	0.890	0.687
F1	20.44 [$F_7^5 = 3.97$] ⇒ S	6.27 [$F_7^5 = 3.97$] ⇒ S
F2	0.665 [$F_5^8 = 4.82$] ⇒ S	0.764 [$F_5^8 = 4.82$] ⇒ S
	CS	I_p
$NaOH_{opt}$ (M)	0.54	0.55
$EtOH_{opt}$ (v)	1.10	1.05
Y_{max}	6.90 g/L	92.9 %

The values of the coefficients of determination suggest good correlation between experimental data of recovered CS and I_p and theoretical responses predicted by second order equations. In both cases, all parameters are statistically significant (t-Student test) and with equal signs for the two dependent variables quantified. For instance, terms characterizing the joint (N x E) and quadratic (N², E²) effects are always negative and define the convex 3D graphs represented in Figure 3, bottom.

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NaOH TREATMENT

NaOH-EtOH TREATMENT

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Figure 3. Experimental data (points) and theoretical surfaces defined by the polynomial equations defined in Tables 2 and 3 for the chemical treatments of the cartilage hydrolysates.

416 These surfaces are indistinguishable and optima conditions similar for both
417 responses (0.54 M and 1.10v for CS, 0.55 M and 1.05v for I_p -index). From these
418 data, optimal working values are calculated as 0.55M/1.08v. Such results are in
419 agreement with previous reports for CS obtained from blue shark head discards

(Vázquez et al., 2016). In the last decade, “*environmentally friendly*” strategies to isolate glycosaminoglycans from animal sources substituting organic solvents, such as cetylpyridinium chloride or cetyltrimethylammonium bromide (Higashi et al., 2015b; Souza et al., 2007), by alcohol have been explored, obtaining excellent outcomes (Kim et al., 2012; Murado, Montemayor, Cabo, Vázquez, & González, 2012).

In the optimal levels for the two proposals studied, CS was recovered at 6.3 g/L and 65% purity for alkaline hydrolysis and 6.9 g/L and 93% for alkaline-hydroalcoholic precipitation (Tables 2 and 3).

3.3. Purification of CS by ultrafiltration (UF) membranes

Final purification of CS solutions produced by enzymatic cartilage digestion and subsequent chemical treatments of the EHs under the optimal conditions previously defined, was carried out by combination of UF membranes with 100 and 30 kDa of molecular weight cut-off. The evolution of CS and Pr retentates recovered in both membranes are displayed in Figure 4. Experimental data of concentration factor for NaOH treated samples processed throughout 100 kDa show low correlation with the corresponding theoretical values, indicating low protein and CS retention. This was verified in the next step of DF where protein and CS were completely permeated after 3-4 diavolumes. Samples generated by NaOH-EtOH precipitation display similar behaviour (Figure 4).

Figure 4. UF-DF technology applied for the purification of CS obtained by NaOH and NaOH-EtOH treatments. Sequential operation using membranes of 100 and 30 kDa was performed. Top: concentration of retained protein (○) and CS (●) in linear relation with the factor of volumetric concentration (fc) showing experimental data (points) and theoretical profiles corresponding to a completely retained solute (discontinuous line). Bottom: progress of protein (○) and CS (●) retention with the increase of diavolume from DF process (D). Equation [3] was used to simulate the experimental data. Error bars are the confidence intervals ($\alpha = 0.05$; $n = 2$).

Permeates obtained from UF at 100 kDa were then concentrated by a 30 kDa membrane. Total correlation concordance is observed among experimental and predicted data of CS concentration factors (6-fold) and (5-fold) for samples from NaOH and NaOH-EtOH treatments, respectively. However, Prs were almost fully filtrated in both cases. The flow of filtrates in each membrane performance was 226±16 mL/min for NaOH-100 kDa, 291±18 mL/min for NaOH-30 kDa, 364±9 mL/min for NaOH-EtOH-100 kDa and 267±19 mL/min for NaOH-EtOH-30 kDa.

In all situations, experimental data of DF is accurately modelled by equation [3] (Table D, supplementary material). Coefficients of specific retention (s) and R_{3D} values obtained from fittings confirmed the commented results: 1) low values of s and high values of R_{3D} for CS and Pr retention at 100 kDa of sample from NaOH and NaOH-EtOH treatments; 2) value of s close to 1 and low value of R_{3D} for CS retention at 30 kDa from alkaline treatment with opposite results for Pr; 3) high s and low R_{3D} values for CS retention and medium s value for Pr at 30 kDa from alkaline-hydroalcoholic treatment.

The most common procedures applied to the final purification of glycosaminoglycans from animal sources are based on chromatographic separations (Souza et al., 2007; Xie, Ye, & Luo, 2014). On the contrary, UF is generally cheaper, more scalable and efficient, however, it has been rarely exploited to the purification of such polysaccharides (Blanco et al., 2015; Lignot et al., 2003; Murado et al., 2010; Murado et al., 2012; Nishigori, Takeda, & Ohori, 2000).

Summarising, the protocols developed in literature are far from optimal, time-consuming, expensive in the cost and use of reagents and were never validated at the pilot plant scale (Arima et al., 2013; Maccari, Galeotti, & Volpi, 2015; Takeda, Horai, & Tamura, 2016). The results here obtained reveal that the three optimised sequential steps (enzymatic, chemical and membrane processing) can be used as a large scale methodology to isolate CS from fish cartilage. In fact, various pilot plant trial-runs were performed (using cartilage from chondrichthyes fish) confirming the validity of the present proposal. Briefly, working with blue shark head (*Prionace glauca*) by-products in batches of 25-50 kg of cartilage under the following experimental conditions (Vázquez et al., 2016): 1) Enzyme hydrolysis with Alcalase 2.4L (E/S ratio=1%) at 60°C, pH8, 200 rpm, r(S:L)=1:4 and t=9 h; 2) Alkaline treatment with NaOH 0.5M at room temperature, 200 rpm of agitation and adding HCl 6M for neutralisation; 3) Diafiltration of CS solutions employing 6 diavolumes; 4) Final drying. The yield of recovered CS was $6.1\pm0.3\%$ (w/wet weight of cartilage) with a purity of $I_p = 85.5\pm1.8\%$. Selected pictures of those pilot plant procedures are shown in Figure B (Supplementary material).

Final purity of CS retentates (as I_p values) were $98.73\pm0.39\%$ and $88.81\pm1.59\%$ for NaOH-EtOH and NaOH treatments, respectively. Desalination was almost complete after diafiltration with final levels of chloride lower than 0.3% (w/w). Based on isolation data here reported comparing processing times between chemical treatments (1-2 h versus 12 h), it appears that hydroalcoholic precipitation in alkaline medium is the best procedure for CS isolation. However, other aspects such as alcohol cost, legal limitations to its acquisition and waste

management must be considered, as they represent disadvantages to its use when high purities are not demanded (e.g., in nutraceutical formulations). The yields of CS isolated after purification steps were: $2.7\pm0.2\%$ and $2.5\pm0.2\%$ (w/wet weight cartilage) for more and less pure CS, respectively. Although these yields were lower than those obtained for CS from blue shark (12.1% w/w of dry head cartilage), small-spotted catshark (4.8% w/w of wet head cartilage) and blackmouth catshark (3.7 % w/w of wet head cartilage), the habitual presence of rabbit fish individuals in the fraction discarded by the fishing fleets makes the alternative presented here an interesting way for its valorisation.

The CS obtained with both purity levels can be suitable for different applications. In the European Union, CS is approved as a prescription drug and is available in pharmaceutical grade for symptomatic treatment of osteoarthritis (Reginster et al., 2015). As such, the CS used must comply with strict regulations concerning quality, purity and properties (Volpi, 2009). This is illustrated by a recent study looking at sixteen pharmaceutical-grade CS raw materials, which found mean purity of $96.5\pm4.2\%$ in the top five samples (da Cunha et al., 2015). Purities of 98% achieved here fall within this range and prove the suitability of the hydroalcoholic precipitation method for applications where high purity ($I_p>98\%$) is required. In nutraceutical formulations, regulation is not so strict. In fact, several works have detected discrepancies between label claims and actual CS quantities (Adebawale, Cox, Liang, & Eddington, 2000; Martel-Pelletier, Farran, Montell, Vergés, & Pelletier, 2015). For this purpose the cheaper chemical treatment using NaOH could be sufficient (Martel-Pelletier et al., 2015).

3.4. Molecular weight of CS

Molecular weights (Mn) of CS obtained under alkaline hydrolysis and alkaline-hydroalcoholic precipitation are estimated at 55 and 51 kDa, respectively (Table 4). The similarity of Mn values indicate that harsher conditions in alkaline hydrolysis treatment do not produce depolymerization of CS, as illustrated by GPC eluograms in Figure 5.

Comparison of Mn with other works is not possible, since similar reports in Chimaera species are to the best of our knowledge nonexistent. In other cartilaginous fish, CS molecular weight ranges from 31 kDa in shark (Li et al., 2016) to 142 kDa in ray fish (Hashiguchi et al., 2011). These large differences seem very much influenced by the method of analysis, as illustrated by the latter study. The authors estimated CS molecular weights of 123 and 142 kDa in shark and ray fish respectively using GPC calibrated with dextran standards. Unlike CS, dextrans are not negatively charged and hence the conformation of both molecules in solution is different. As a consequence, the molecular weight of CS is overestimated.

Table 4. Molecular weight and disaccharide composition of CS isolated from *C. monstrosa* following alkaline hydrolysis and hydroalcoholic-alkaline precipitation. Mn: number average molecular weight, PDI: polydispersity index (Mw/Mn), dn/dc: refractive index increment; Disaccharide composition expressed as mean % $\pm$ standard deviation; ¹NMR, ²SAX-HPLC; *Higashi et al., 2015b

	Alkaline hydrolysis	Hydroalcoholic precipitation	alkaline <i>Chimaera phantasma</i> *
Mn	55 kDa	51 kDa	
PDI	1,252	1,378	
dn/dc	0,112	0,110	

GlcA-GalNAc 4S ¹	17,74	19,01	
GlcA-GalNAc 4S ²	21,51 ± 0,04	22,13 ± 0,27	27,10
GlcA-GalNAc 6S ²	53,53 ± 0,04	52,70 ± 0,08	40,70
GlcA-GalNAc 0S ²	4,12 ± 0,00	4,37 ± 0,20	5,71
GlcA 2S-GalNAc 6S ²	17,21 ± 0,02	17,38 ± 0,09	20,70
GlcA -GalNAc 4,6S ²	2,60 ± 0,02	2,39 ± 0,10	2,91
GlcA 2S-GalNAc 4S ²	1,03 ± 0,01	1,03 ± 0,01	2,91

548

549 Such accuracy problems can be circumvented by GPC with light scattering
550 detection, as no calibrating standards are necessary. Detector constants are
551 determined by injection of one low polydispersity standard, but molecular weight
552 data is absolute and do not depend on the chemical nature of the calibrant. Studies
553 taking advantage of this technique on CS are scarce, but a recent work reports 31
554 kDa in commercial CS from shark cartilage (Li et al., 2016). A more comprehensive
555 study in shark and skate found Mn of CS of 43-45 kDa in *Scyliorhinus canicula*, 60
556 kDa in *Prionace glauca*, and 44 kDa in *Raja clavata* (Novoa-Carballal et al., 2017).
557 Therefore, Mn of CS from *C. monstrosa* falls in the high end of the 31 to 60kDa
558 range reported in other cartilaginous fish.

559

Alkaline hydrolysis

Figure 5. Gel Permeation Chromatography (GPC) eluograms (left) of CS extracted from *C. monstrosa*. Red line: right angle light scattering signal (RALS); Blue line: low angle light scattering signal (RALS); Black line: refractive index (RI) signal. Right: SAX-HPLC chromatograms after enzymatic digestion of CS extracted from *C. monstrosa* with chondroitinase ABC. O: Δ UA-GalNAc; A: Δ UA-GalNAc(4S); C: Δ UA-GalNAc(6S); D: Δ UA(2S)-GalNAc(6S); E: Δ UA-GalNAc(4,6S); B: Δ UA (2S)-GalNAc(4S). Samples treated with alkaline hydrolysis (top) and hydroalcoholic alkaline precipitation (bottom).

3.5. Disaccharide composition of CS

NMR is a fast method that provides an overview of CS composition. Typical CS ^1H NMR signals are concentrated in the region from 1.5 to 5.0 ppm (Figure 6; Figure C, Supplementary Material). In CS fractionated by alkaline-hydroalcoholic precipitation, lack of signals upfield and downfield this interval and between 2 and 3 ppm is an indication of the high purity of the CS obtained, in agreement with the *Ip* index (99%). In particular, absence of signals typical of aliphatic (0.5 to 1.5 ppm) and aromatic (7.0 to 8.5) aminoacids reflects the suitability of the isolation method to remove proteins. In CS obtained by alkaline treatment, additional signals appear at 7.4 and 8.1 ppm; 1.0 and 1.5 ppm; and 2.4 to 3.2 ppm, most likely due to protein impurities. These signals are however of small intensity and reflect the purity of the sample (*Ip*=89%).

Figure 6. ^1H NMR spectra of CS in D_2O obtained at 10°C after hydroalcoholic precipitation (top) and alkaline hydrolysis (bottom).

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585 Besides CS, cartilage is composed of other GAGs such as hyaluronan (HA) and
586 keratan sulfate (KS), which could partially remain as impurities in CS. Both HA and
587 KS contain N-acetylglucosamine (GlcNAc) in place of GalNAc in CS. Signals of
588 GlcNAc anomeric carbons appear at 4.54 ppm in HA and 4.7 ppm in KS (Pomin,
589 2013) and should be absent in pure CS. Spectra in Figure 6, show no signals at
590 4.7 ppm indicating no KS contamination. Unfortunately, the HA signal at 4.54 ppm
591 overlaps with those of GalNAc and GlcA (H1), making it impossible to assess its
592 presence. However, if CS samples contain HA it is only in minute amounts, since
593 GPC eluograms show no peaks at lower elution times than CS (Figure 5). This
594 would be expected for high Mw species such as HA, typically greater than 1 MDa.

595

596 In some shark species, CS from cartilage has been found hybridized with DS
597 chains (Higashi et al., 2015a). DS and CS share GalNAc in their structure, but
598 GlcA in CS is replaced by its epimer iduronic acid (IdoA) in DS. Characteristic
599 signals in DS at 4.87 ppm (H1 of IdoA) and 3.52 ppm (H2 of IdoA), between H2
600 and H3 of GlcA in CS, are absent from the spectra (Figure 6) or with very low
601 intensity. Consequently, significant DS contamination can be ruled out, in
602 accordance with previous reports on Chimaera (Higashi et al., 2015b; Rahemtulla
603 et al., 1976).

604

605 Typical CS signals in the region of interest are almost identical for CS treated by
606 either alkaline treatment or alkaline-hydroalcoholic precipitation, as shown by ¹H

NMR (Figure 6) and HSQC (Figure D, Supplementary material) spectra. Therefore, we can anticipate that neither treatment affects disaccharide composition.

Quantification of individual disaccharide units by NMR is difficult because of the strong signal overlap that occurs in the ^1H and even in the higher resolution HSQC spectra (Figure D, Supplementary Material). Also the the sensitivity of NMR might not be enough to detect disaccharides present in small proportions especially if their chemical shift overlap with the mayor components. It is however possible to estimate the percentage of units sulfated in position 4 of GalNAc (CS-A). Comparison of signal intensities of the acetyl group in GalNAc at 2.05-2.07 ppm with the singlet at 4.78 ppm, assigned to H4 of 4 sulfated GalNAc (Mucci, Schenetti, & Volpi, 2000) (Figure 6) results in 18-19% of A units (Table 4)

This information was complemented with SAX-HPLC analysis of CS samples after digestion with ABC chondroitinase. Percentages of each unit displayed in Table 4 confirm similarity of results for CS obtained after alkaline hydrolysis or hydroalcoholic precipitation. In both cases, more than 50% of the CS units consist of CS 6S (CS-C), with CS-A content slightly above 20% and non-sulfated CS accounting for only 4%. Remarkably, the sum of disulfated disaccharides rises to 20%, equalling CS 4S proportion. The major contribution to this figure is due to GlcA 2S-GalNAc 6S (CS D) at 17%, with only minor quantities of GlcA -GalNAc 4,6S (CS-E) and GlcA 2S-GalNAc 4S (CS-B). Abundance of CS-C is typical of most cartilaginous fish (Valcarcel et al., 2017) and is relatively high in *C. monstrosa* (55%) compared to values reported in shark, ranging from 30 to 60% (Higashi et

al., 2015b; Mucci et al., 2000; Novoa-Carballal et al., 2017). In the case of CS-D, shark cartilage is the main source despite the fact that it only accounts for up to 20% of total CS (Higashi et al., 2015b). At 17%, *C. monstrosa* appears as a good source of CS D in relative terms.

Previous reports on CS composition of *Chimaera* species are limited to a recent study where CS was extracted from the fins of Silver Chimaera (*Chimaera phantasma*) (Higashi et al., 2015b). Results are also included in Table 4 for comparison purposes. The main difference is a lower proportion of CS-C (41%) in *C. phantasma* than in the present study (55%). Scarcity in CS data from Chimaera species makes impossible to ascribe this difference to a particular cause. In shark, differences in CS sulfation appear to be greater among species than among different tissues of the same species (Novoa-Carballal et al., 2017). This might be also true for Chimaera, but further data is needed to confirm this hypothesis.

4. Conclusions

In the present work, we isolate CS from *C. monstrosa* cartilage by combining sequential 1) enzymatic proteolysis, 2) chemical treatment and 3) membrane purification, with all steps mathematically optimized by response surface methodologies. The conditions to maximize the recovery of CS are established as: 1) 53.1°C, 2% of Esperase/cartilage ratio and pH=8.38 for enzyme digestion of cartilaginous material; 2) 0.8 M NaOH and 12 h for alkaline treatment of the EH and 0.55 M NaOH, 1.08 v EtOH and NaCl 2.5 g/L for alkaline hydroalcoholic precipitation of the EH; 3) UF at 30 kDa using at least 4 diavolumes of water in DF

step to obtain CS with more than 89% of purity (99% with NaOH-EtOH solutions). Molecular weight and sulfation profiles are similar for both chemical treatments. Mn are estimated at 51-55 kDa, in the high end of the typical values reported for other cartilaginous fish. Sulfation profiling reveals dominant CS 6S (4S/6S ratio of 0.4), a substantial amount of GlcA 2S-GalNAc 6S (17%) and less than 4% of other disulfated and unsulfated disaccharides.

Conflict of interest

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table A. Experimental domains and codification of independent variables in the factorial rotatable designs performed to study the cartilage hydrolysis of *C. monstrosa* by Esperase.

	T (°C) : <i>T</i>	pH	E/S (%): <i>E/S</i>
-1.68	30.0	6.0	0.01
-1	40.1	6.8	0.41
0	55.0	8.0	1.01
+1	70.0	9.2	1.60
+1.68	80.0	10.0	2.00

Codification: $V_c = (V_n - V_0) / \Delta V_n$
 Decodification: $V_n = V_0 + (\Delta V_n \times V_c)$
 V_n = natural value of the variable to codify
 V_0 = natural value in the centre of the domain
 V_c = codified value of the variable
 ΔV_n = increment of V_n per unit of V_c .

Table B. Experimental domains and codification of independent variables in the factorial rotatable designs executed to study the chemical treatments of the Esperase hydrolyzates using alkaline or alkaline-hydroalcoholic solutions.

Coded values	Natural values			
	NaOH treatment		NaOH-EtOH treatment	
	NaOH (M): <i>N</i>	Time (h): <i>t</i>	NaOH (M): <i>N</i>	Ethanol (v): <i>E</i>
-1.41	0.20	1.0	0.10	0.30
-1	0.39	4.4	0.20	0.46
0	0.85	12.5	0.45	0.85
+1	1.31	20.6	0.70	1.24
+1.41	1.50	24.0	0.80	1.40

Codification: $V_c = (V_n - V_0) / \Delta V_n$; Decodification: $V_n = V_0 + (\Delta V_n \times V_c)$
 V_n =natural value of the variable to codify
 V_0 =natural value in the centre of the domain
 V_c =codified value of the variable ; ΔV_n = increment of V_n per unit of V_c .

Table C. Parametric estimations obtained from Weibull equation [2] applied to Esperase hydrolysis kinetics under the experimental conditions studied. Independent variables are expressed in natural values in brackets. Numerical values of the parameters are shown with their confidence intervals. Determination coefficients (R^2) and p-values from F-Fisher test are also summarised.

Experimental conditions	$H_m(\%)$	$\tau(\text{min})$	β	R^2	p-value
T:-1 (40.1°C) - pH:-1 (6.8) - E/S:-1 (0.41%)	11.40±8.31	102±98	0.51±0.03	0.996	<0.001
T:1 (70.0°C) - pH:-1 (6.8) - E/S:-1 (0.41%)	4.05±0.01	7.27±0.30	1.22±0.06	0.984	<0.001
T:-1 (40.1°C) - pH:1 (9.2) - E/S:-1 (0.41%)	17.26±0.12	47.20±0.80	0.77±0.01	0.998	<0.001
T:1 (70.0°C) - pH:1 (9.2) - E/S:-1 (0.41%)	15.11±0.07	14.92±0.50	0.67±0.02	0.991	<0.001
T:-1 (40.1°C) - pH:-1 (6.8) - E/S:1 (1.60%)	10.59±0.25	69.37±3.20	0.95±0.05	0.984	<0.001
T:1 (70.0°C) - pH:-1 (6.8) - E/S:1 (1.60%)	21.51±0.27	19.71±1.20	0.66±0.04	0.972	<0.001
T:-1 (40.1°C) - pH:1 (9.2) - E/S:1 (1.60%)	21.81±0.17	29.10±0.70	0.69±0.02	0.995	<0.001
T:1 (70.0°C) - pH:1 (9.2) - E/S:1 (1.60%)	22.11±0.80	18.18±0.80	0.90±0.05	0.978	<0.001
T:-1.68 (30.0°C) - pH:0 (8.0) - E/S:0 (1.01%)	14.25±0.22	78.86±2.40	0.82±0.02	0.998	<0.001
T:1.68 (80.0°C) - pH:0 (8.0) - E/S:0 (1.01%)	19.09±0.14	13.69±1.00	1.13±0.11	0.938	<0.001
T:0 (55.0°C) - pH:-1.68 (6.0) - E/S:0 (1.01%)	3.75±0.05	75.56±1.50	213 (NS)	0.966	<0.05
T:0 (55.0°C) - pH:1.68 (10.0) - E/S:0 (1.01%)	21.97±0.23	21.72±1.00	0.68±0.03	0.982	<0.001
T:0 (55.0°C) - pH:0 (8.0) - E/S:-1.68 (0.01%)	18.47±0.08	64.51±0.60	0.82±0.01	1.000	<0.001
T:0 (55.0°C) - pH:0 (8.0) - E/S:1.68 (2.00%)	28.23±0.23	19.40±0.80	0.68±0.03	0.988	<0.001
T:0 (55.0°C) - pH:0 (8.0) - E/S:0 (1.01%)	18.92±0.01	20.37±0.10	0.78±0.00	0.995	<0.001
T:0 (55.0°C) - pH:0 (8.0) - E/S:0 (1.01%)	17.58±0.12	22.91±0.60	0.68±0.02	0.994	<0.001
T:0 (55.0°C) - pH:0 (8.0) - E/S:0 (1.01%)	19.95±0.13	21.65±0.60	0.67±0.02	0.994	<0.001
T:0 (55.0°C) - pH:0 (8.0) - E/S:0 (1.01%)	19.20±0.17	25.23±0.70	0.63±0.02	0.995	<0.001
T:0 (55.0°C) - pH:0 (8.0) - E/S:0 (1.01%)	19.27±0.15	27.55±0.60	0.66±0.02	0.996	<0.001
T:0 (55.0°C) - pH:0 (8.0) - E/S:0 (1.01%)	18.70±0.17	25.41±0.70	0.65±0.02	0.994	<0.001

Table D. Parametric estimates from UF-DF purification data of CS and proteins modelled by equation [3]. Determination coefficients (R^2) are also shown. NS: non-significant.

UF-DF sequence		CS	Proteins
NaOH TREATMENT	100 kDa	R_0	98.1 ± 10.1
		R_f	1.98 ± 1.97
		s	0.066 ± 0.065
		R^2	0.999
		R_{3D}	92.06
	100→30 kDa	R_0	101.5 ± 12.8
		R_f	-0.31 (NS)
		s	$10^{-5} \pm 10^{-5}$
		R^2	0.995
		R_{3D}	98.10
NaOH-EtOH TREATMENT	100 kDa	R_0	9.09 (NS)
		R_f	103.3 ± 12.21
		s	-1.52 (NS)
		R^2	0.125 ± 0.124
		R_{3D}	0.999
	100→30 kDa	R_0	98.37
		R_f	86.04 ± 8.62
		s	14.12 ± 1.66
		R^2	0.232 ± 0.100
		R_{3D}	0.994
	100 kDa	R_0	100.0 (NS)
		R_f	0.0 (NS)
		s	10^{-5} (NS)
		R^2	0.961
		R_{3D}	100
	100→30 kDa	R_0	116.6 ± 19.9
		R_f	0.0 (NS)
		s	0.747 ± 0.073
		R^2	0.999
		R_{3D}	62.62

Figure A. Flowchart describing the steps applied to isolate CS from rabbit fish cartilage. FPH: fish protein hydrolysate, SED: sediment, SUP: supernatant, PER: permeate, RET: retentate.

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Figure B. Pictures showing, in a summarised way, the necessary procedures for CS isolation from cartilage of blue shark heads at pilot plant scale.

Figure C. ^1H NMR spectra spectra CS obtained after hydroalcoholic precipitation (top) and alkaline hydrolysis (bottom).

Figure D. HSQC spectra CS obtained after hydroalcoholic precipitation (top) and alkaline hydrolysis (bottom).