Optimisation of the extraction and purification of chondroitin sulphate from head by-products of *Prionace glauca* by environmental friendly processes.

José Antonio Vázquez[1]*, María Blanco[2], Javier Fraguas[1,2], Lorenzo Pastrana[3] & Ricardo Pérez-Martín[1].

[1] Grupo de Reciclado y Valorización de Materiales Residuales (REVAL)
Instituto de Investigacións Mariñas (IIM-CSIC)
C/ Eduardo Cabello, 6. Vigo-36208. Galicia – Spain

[2] Grupo de Bioquímica de Alimentos
Instituto de Investigacións Mariñas (IIM-CSIC)
C/ Eduardo Cabello, 6. Vigo-36208. Galicia – Spain

[3] Laboratorio de Bioquímica, Departamento de Química Analítica y Alimentaria
Facultad de Ciencias, Universidade de Vigo
As Lagoas s/n. Ourense-32004. Galicia – Spain

* Corresponding author e-mail: jvazquez@iim.csic.es
Tel: +34 986214469; fax: +34 986292762

Abstract

The goal of the present work was to optimise the different environmental friendly processes involved in the extraction and purification of chondroitin sulphate (CS) from *Prionace glauca* head wastes. The experimental development was based on second order rotatable designs and evaluated by response surface methodology combined with a previous kinetic approach. The sequential stages optimised were: 1) the enzymatic hydrolysis of head cartilage catalysed by alcalase (55.7ºC/pH 8.2); 2) the chemical treatment of enzyme hydrolysates by means of alkaline-hydroalcoholic saline solutions (NaOH: 0.54 M, EtOH: 1.17 v, NaCl: 2.5%) to end the protein hydrolysis and to precipitate and selectively redissolve CS versus the peptidic material and 3) the selective purification and concentration of CS and the concomitant protein permeation of extracts which were obtained from previous treatment using ultrafiltration and diafiltration (UF-DF) technologies at two different cut-offs.

Keywords: Chondroitin sulphate production; cartilage *P. glauca* wastes; by-products upgrade; process optimisation; response surface methodology.

Introduction

The generation of discards and by-products from fishing activities is one of the most important problems to maintain the sustainability of the marine resources and the extractive marine industry. European Union (EU) has shown a big concern about this problematic; thus, new and more restrictive policies (zero discards) have been legislated in order to minimize the adverse ecological, environmental and pollutant impact of the biomass wasted, to attain their reduction and the development of valorisation alternatives. These measures are within the objectives of the Horizon 2020 European initiative called as *Blue technology* (EU, 2012). It is focused on how technologies can put marine resources to productive use and create sustainable growth for the exploration of bioactive compounds with potential interest in the food, feed or pharmaceutical industries.

The blue shark (*Prionace glauca*) is a pelagic elasmobranch found throughout the world's oceans, in all tropical and temperate waters. It is principally caught as by-catch on longlines and in gill nets fisheries targeting tuna and swordfish (Carvalho, Murie, Hazin, Hazin, Leite-Mourato, & Burgess, 2011). Catches of this species appears to be on the rise, due to consumer habits which are changing and the meat of this species is now appreciated. In this sense, blue shark was the fourth most important frozen fish species landed in 2013 in the largest fishing port in Europe, located in Vigo (North-West Spain). Fresh and frozen volumes of blue shark auctioned in Vigo fish auction, accounted up to 8600 Tn in 2013. Transformation of this species throughout the manufacture chain, include different

presentations as loins, fillets or fish bites, where the cartilage is removed and primarily used for low added-value processes.

One of the most valuable materials from chondrichthyes fishes, including sharks, is the cartilage. Cartilage is a tissue composed by a matrix of collagen associated with proteoglycans. They are macromolecules with a core protein linked covalently to glycosaminoglycans (GAGs) among which chondroitin sulphate (CS) is the most important (Vázquez, Rodríguez-Amado, Montemayor, Fraguas, González, & Murado, 2013). CS is a polymer constituted by alternating molecules of glucuronic acid and N-acetylgalactosamine sulphated. This material is an essential component of extracellular matrix of connective tissues being a fundamental key for the control of cell development and adhesion or the elasticity of the articular cartilage (Schiraldi, Cimini, & de Rosa, 2010). In the last decade, a numerous and varied type of CS applications has been reported mainly related to the bioengineering of tissues (Chang, Liu, Lin, Chou, & Lin, 2003; Silva et al., 2012; Leite, Sher, & Mano, 2014). But it has also demonstrated to have antiviral properties (Yamada & Sugahara, 2008), to be used as food preservative with emulsifying features (Hamano et al., 1989) and to be administered in nutraceutical formulations to prevent and reduce osteoarthritis (Michel et al., 2005; Volpi, 2009).

Bovine, porcine and chicken cartilage have been traditional sources for obtaining CS (Nakano, Dodd, & Scott, 1997), however since the bovine spongiform encephalopathy outbreak, the uses of alternative materials have received increasing attention, and so this polymer has been isolated from various tissues

93 from different vertebrate (Kinoshita-Toyoda et al. 2004; Zhao et al., 2013) and
94 invertebrate (Gu et al., 1999) marine species. Recently, much attention has been
95 paid to the isolation of this polysaccharide from elasmobranch sources (Lignot,
96 Lahogue, & Bourseau, 2003; Murado, Fraguas, Montemayor, Vázquez, &
97 González, 2010; Kim et al., 2012; Xie, Ye, & Luo, 2014), due to the benefits in
98 terms of safety, potential bioactivities and structural issues (González, Leyva, &
99 Moraes, 2001) associated with elasmobranch cartilage. According to our
100 knowledge, only one previous work studied the extraction of CS from *P. glauca*
101 cartilage (Xie, Ye, & Luo, 2014) but in this article no optimisation of conditions for
102 CS production is reported. The present work is the first time dealing with a
103 complete optimisation of the extraction conditions for the production of CS from
104 head cartilage by-product of *P.glauca*.
105
106 The main problem of CS recovery from cartilage is the digestion of this tissue and
107 the subsequent elimination of the proteins that are present in the proteoglycan
108 matrix (collagen and aggrecans). The final purity needed is dependent on the type
109 of application, higher for clinical uses and lower in nutraceutical and dietary
110 formulations. The fundamentals of many of the isolation procedures developed for
111 mammalian and marine sources (bovine and porcine trachea, shark fins, etc.) are
112 based on (Vázquez, Rodríguez-Amado, Montemayor, Fraguas, González, &
113 Murado, 2013; Shi, Meng, Li, Chen, Liu, & Bai, 2014): 1) chemical hydrolysis of
114 cartilage; 2) breakdown of proteoglycan core; 3) selective elimination of proteins
115 and CS isolation; 4) final purification of CS. Commonly, the steps of digestion,
116 hydrolysis, differential recovery of GAG and purification, are performed using

alkalis, cysteine or guanidine HCl and chemical precipitation by cetylpyridinium chloride, trichloroacetic acid or detergents combined with chromatography of gel filtration for purification (Michelacci, & Horton, 1989; Souza, Kozlowski, Cerqueira, Castelo-Branco, Costa, & Pavão, 2007). However, those processes are not fully efficient for obtaining high CS purity and also they are time-consuming, expensive to be scaled and environmentally unfriendly. Thus, alternatives based on enzyme hydrolysis and membranes technology performances have been explored in recent years (Lignot, Lahogue, & Bourseau, 2003; Murado, Fraguas, Montemayor, Vázquez, & González, 2010).

In the present study, different processes for the extraction and purification of chondroitin sulphate from *P. glauca* head cartilage by-products have been reported. In this context, a sequential combination of stages of cartilage hydrolysis mediated by alcalase, selective precipitation of CS in alkaline hydroalcoholic medium and, finally, CS purification and protein removing using ultrafiltration and diafiltration (UF-DF) procedures.

Materials and Methods

Cartilage preparation

Head wastes of blue shark (*Prionace glauca*) were kindly provided by Propegal S.L. (Vigo, Spain) and stored at -20ºC. These materials were initially heated in water bath at 80ºC/30 min and muscle was manually discarded. The cartilaginous

material were then milled and homogenized at ~1 mm size using a grinder (Thermomix TM31, Vorwerk) and stored at -20ºC until use.

Analytical determinations

Levels of ash, total carbohydrate, crude protein, moisture and fat were determined in triplicate, from subsamples taken before cartilage storage. Total nitrogen was measured according to the Kjeldahl method (AOAC, 1997) in a DigiPREP HT digestor, DigiPREP 500 fully automatic steam distillation (SCP Science, Canada) and a TitroLine easy titration unit (SCHOTT Instruments, Germany) and crude protein content calculated as total nitrogen multiplied by 6.25. Ash was quantified by calcination at 600ºC in a muffle furnace and moisture content determined after heating the sample at 105 ºC in an oven until constant weight. Fat concentration was obtained by the methodology developed in AOAC (1990). Total carbohydrate content was calculated by the difference between total weight (subtracting protein, fat and ash) and moisture content.

In CS extracts, total soluble proteins (Pr) were quantified using the method of Lowry et al. (1951). CS concentration, as glucuronic acid, was determined by the method of Van den Hoogen et al. (1998) according with the mathematical modifications reported in Murado et al. (2005). CS purity index (I_p), defined as I_p (%) = CS×100/(CS+Pr), was also calculated in all purification stages.

Alcalase hydrolysis process

Samples of 25 g of cartilage were suspended in 75 mL of distilled water and pre-incubated in the conditions of *pH* and *T* defined by the factorial design described in Table 1 (see also experimental design section). After addition of 0.3% (v/w) (7.2 AU/kg of cartilage) of alcalase 2.4L (Novozymes S.A.), the kinetics of hydrolysis were carried out in a 100 mL-thermostated reactor connected to a pH-Stat system (Metrohm, UK) with agitation (200 rpm) under the experimental conditions from Table 1. At the end of hydrolysis time (4 h), the enzymatic reactions were stopped by boiling, freezing and centrifuged (6000 g, 20 min) to recover the supernatants (hydrolysates). In these hydrolysates, the concentrations of total proteins and CS were analyzed after performing a chemical treatment with alkaline hydroalcoholic in the suboptimal conditions of 0.5 M (NaOH) and 1.1 v (EtOH).

The calculation of the degree of hydrolysis (*H*, in %) was performed according to the pH-Stat protocol reported by Adler-Nissen (1986). It was proportional to the alkali added in order to maintain the pH at the established value and determined by the following equation:

$$H = \frac{B N_b}{\alpha M_p h_{tot}} \qquad [1]$$

where, B is the volume (mL) of 0.2 M NaOH consumed during hydrolysis; N_b is the normality of NaOH; M_p is the mass (g) of initial protein (N x 6.25); h_{tot} is the total number of peptide bonds available for proteolytic hydrolysis (8.6 meq/g), and α is

184 the average degree of dissociation of the amino groups in the protein substrate and
185 it was calculated as follows:
186
187 $$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}} \quad [2]$$
188
189 The *pK* value is dependent on the temperature of hydrolysis (in K degrees),
190 therefore it can be also calculated according to the following expression:
191
192 $$pK = 2400\left(7.8 + \frac{298-T}{298\ T}\right) \quad [3]$$
193
194 Subsequently, the progress of the hydrolysis data (*H*) of *P. glauca* cartilage was
195 modelled by the Weibull equation (Vázquez, Lorenzo, Fuciños, & Franco, 2012):
196
197 $$H = H_m\left\{1-\exp\left[-\ln 2\left(\frac{t}{\tau}\right)^{\beta}\right]\right\} \quad \text{with} \quad v_m = \frac{H_m \beta \ln 2}{2\tau} \quad [4]$$
198
199 where, *H* is the degree of hydrolysis (%); *t* is the time of hydrolysis (min); H_m is the
200 maximum degree of hydrolysis (%); β is a parameter related with the maximum
201 slope of cartilage hydrolysis (dimensionless); τ is the time required to achieve the
202 semi-maximum degree of hydrolysis (min) and v_m is the maximum hydrolysis rate
203 at the τ-time (% min^{-1}).

Chemical treatment using alkaline hydroalcoholic media

Three litres of hydrolysate prepared in the best conditions for the alcalase hydrolysis of cartilage (55.7ºC/pH 8.2 using Tris-HCl buffer 0.1M/1% (v/w) of alcalase/200 rpm) were used to assess the joint influence of NaOH concentration and ethanol (EtOH) volume in the extraction of CS. Thus, the precipitations of CS were performed by adding to the hydrolysates, slowly and with middle agitation at room temperature, different hydroalcoholic solutions of NaOH in the proportions defined for the experimental design which are summarized in Table 1. In all the alkaline hydroalcoholic mixtures, 2.5% of NaCl was added for a better redissolution of CS in water. After 2 h in agitation, the suspensions were centrifuged (6000 g, 20 min) and the sediments were redissolved with water and neutralized with 6 M HCl.

Experimental designs and statistical analysis

Enzyme hydrolysis of head cartilage and selective precipitation of CS were studied using a rotatable second order designs with quintuple replication in the centre of the experimental domains (Box, Hunter, & Hunter, 2005) (Table 1). In the first case, the combined effect of *T* and *pH* on the parameters describing the hydrolysis degree of cartilages and final CS concentration and purity was evaluated. In the second step, NaOH concentration and volumes of EtOH required for the final alkaline proteolysis of proteoglycan and selective precipitation of CS were optimised.

The experimental results of the factorial designs were modelled by second-order polynomial equations as:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \qquad [5]$$

where Y represents the response to be modelled; b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of squared effect, n is the number of variables and X_i and X_j define the independent variables.

The goodness-of-fit was established as the adjusted determination coefficient (R^2_{adj}), the statistical significance of the coefficients was verified by means of the Student t-test (α=0.05) and the model consistency by the Fisher F test (α=0.05) using the following mean squares ratios:

	the model is acceptable when
F1 = Model / Total error	$F1 \geq F_{den}^{num}$
F2 = (Model + Lack of fitting) / Model	$F2 \leq F_{den}^{num}$
F3 = Total error / Experimental error	$F3 \leq F_{den}^{num}$
F4 = Lack of fitting / Experimental error	$F4 \leq F_{den}^{num}$

Ultrafiltration-diafiltration process

245 Ultrafiltration-diafiltration (UF-DF) of neutralized extracts of CS was performed
246 employing spiral polyethersulfone membranes of 0.56 m^2 (Prep/Scale-TFF,
247 Millipore Corporation, Bedford, MA, USA) with 100 and 30 kDa molecular weight
248 cut-offs (MWCO). A sequential cascade of 100 to 30 kDa membrane was
249 executed, in both cases operating with total recirculation mode for the retentates at
250 40ºC and inlet constant pressure (~1 bar). Firstly, UF obtained after 100 kDa
251 membrane was immediately followed by DF procedure. The final retentate (after
252 DF) was finally dried. Permeate obtained after UF was passed through 30 kDa
253 membrane for concentration (UF step) and subsequent desalination and protein
254 elimination by DF. Permeate of the 30 kDa-UF was discarded. The samples taken
255 from all UF-DF kinetics were analyzed in terms of CS and protein content.
256
257 For modelling the membrane process, a constant volume was fixed for the DF step
258 (filtration flow = water intake flow), where the concentration of a permeable solute
259 in the retentate was predicted by using the first-order equation (Amado, Vázquez,
260 González, & Murado, 2013):
261
262 $$R = R_f + R_0 \exp\left[-(1-s)D\right] \qquad [6]$$
263
264 where, R is the concentration of permeable protein or CS in the retentate (% from
265 the level at initial DF), R_0 is the permeate concentration (%), R_f is the asymptotic
266 and retentate concentration (%), D is the relative diavolume (volume of added
267 water/constant retentate volume) and s is the specific retention of protein or CS

with variation between 0 (the solute is filtered as the solvent) and 1 (the solute is totally retained). Thus, using normalized values (%): $R_0 + R_f = 100$, with $R_0 = 0$ if all protein or CS are permeable. In addition, the percentage of protein or CS eliminated by three diavolumes (R_{3D}) was calculated by substituting in equation [6] the value of parameter D by 3.

Numerical methods for non-linear curves modelling

Degree of hydrolysis data and DF kinetics were modelled by minimisation the sum of quadratic differences between observed and 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, freely available from de Levie's Excellaneous website: http://www.bowdoin.edu/~rdelevie/excellaneous/.

3. Results and Discussion

The percentage of fresh cartilage was 15.33 ± 2.96 % (w/w of wet head).The chemical composition of head cartilages from *P. glauca* expressed as percentage of dry weight was as follows (average ± SD, in % of dry weight): 54.44 ± 0.70, 33.55 ± 0.52, 11.75 ± 0.23 and 0.26 ± 0.01 for protein, ash, carbohydrates and fat, respectively. The content of moisture (%) was 79.01 ± 0.46 of total weight.

3.1. Optimisation of alcalase hydrolysis on P. glauca head cartilages.

A varied set of enzyme hydrolysis trends were generated for the different conditions of *pH* and *T* tested (Figure 1). In some *pH* and *T* levels the activity of alcalase to solubilise head cartilages was null and therefore no degree of hydrolysis was observed. The kinetics data were accurately modelled by Weibull equation [4]. The numerical value of the parameters and the corresponding statistical analysis of fittings are showed in Table A (supplementary material).

The parameters H_m and v_m from equation [4] as well as the CS concentration and I_p index at the end of the hydrolysis time were the dependent variables used to study the joint influence of *pH* and *T* on cartilage hydrolysis using alcalase. For the partial purification of CS in the hydrolysis samples, we have applied the suboptimal conditions of chemical treatment reported in Material and Methods (NaOH 0.5 M and EtOH 1.1 v). Table 2 shows the second order equations defining the predicted responses for the enzyme action on cartilage solubilisation affected by the aforementioned independent variables.

In general, the goodness of fitting and the correlation among data and expected values were good and superior to 70% (94% for I_p). Moreover, they were completely consistent by F-Fisher test evaluation and confirmed their adequate capacity of prediction of the effects studied. In all responses, the quadratic coefficients were always statistically significant and negatives. In Figure 2 (Top) a representation of the theoretical dome surfaces generated by the models from Table 2 are displayed. The optimal values of *T* and *pH* that maximize the

responses can be calculated as follows (H_m as example) (Wardhani, Vázquez, & Pandiella, 2010):

$$H_m = 13.0 - 3.72pHT - 3.01T^2 - 6.28pH^2$$

$$\frac{\partial H_m}{\partial T} = -3.72pH - 6.02T \text{ and } \frac{\partial H_m}{\partial pH} = -3.72T - 12.56pH$$

Equating both derivatives to zero, we obtain: T_{opt}= 0 (55ºC in natural value) and pH_{opt}= 0 (9.0 in natural value). These are the conditions that lead to the maximum degree of hydrolysis: 13%. The optima values for the rest of the responses are summarized in Table B (supplementary material). In this context, the best options for cartilage hydrolysis were defined as the average of such optimal data: T= 55.7ºC and pH= 8.2.

Based on these optimal values, a set of longer kinetics of hydrolysis were performed with increasing alcalase concentrations and the results established that 8 h of hydrolysis and 1% (v/w) of alcalase are the best conditions for both variables (data not shown). Different types of proteases have been tested, in general with good results, for cartilage hydrolysis (Lignot, Lahogue, & Bourseau, 2003; Kim et al., 2012) being papain the most commonly utilised (Garnjanagoonchorn, Wongekalak, & Engkagul, 2007; Gargiulo, Lanzetta, Parrilli, & De Castro, 2009; Murado, Fraguas, Montemayor, Vázquez, & González, 2010). Nevertheless, alcalase has been successfully applied in a great number of fish applications (Safari, Motamedzadegan, Ovissipour, Regenstein, Gildberg, & Rasco 2012;

Amado, Vázquez, Murado, & González, 2013) including, recently, the purification of GAGs (Kim et al., 2012; Xie, Ye, & Luo, 2014) showing better results for CS content than those reported by other proteases (Gu et al., 1999) Conventional digestion of cartilage was performed previously using concentrations of alcalase higher than 2% in comparison to our results, that established 1% (24 AU/kg) as the most optimal and cost-effective concentration of enzyme to be applied (Kim et al., 2012).

3.2. Optimisation of chemical treatment of alcalase hydrolysates.

In this step, hydrolysates obtained taking into account the previous optimal conditions for cartilage hydrolysis were processed with alkaline hydroalcoholic solutions, in order to optimise the joint effect of EtOH and NaOH concentrations on CS recovery. This chemical treatment helps to liberate CS from the proteoglycan matrix and produce the selective precipitation of CS. Additionally, the constant salt concentration added to the solutions improves the aqueous redissolution of GAG (Murado, Fraguas, Montemayor, Vázquez, & González, 2010; Murado, Montemayor, Cabo, Vázquez, & González, 2012).

The results for CS and I_p responses (experimental and predicted) from such treatment on *P. glauca* head hydrolysates are listed in Table C (supplementary material). The corresponding empirical models obtained by application of surface response methodology and multivariable analysis to those experimental data are summarized in Table 3. The adjusted coefficients of determination were superior to 0.83 suggesting a good correlation among experimental data and theoretical

361 responses. In all cases, the unique non significant coefficient was the linear
362 interaction among *E* and *N* ($P<0.05$). The mathematical and graphical results for
363 both responses were similar showing indistinguishable convex surfaces (Figure 3).
364 This resulted in almost equal optima conditions of alkali and alcohol: 0.53 M and
365 1.17 v for CS, 0.54 M and 1.18 v for I_D-index.
366
367 Different solvents have been checked for the selective precipitation of GAGs and
368 proteins from a digested cartilage (Gargiulo, Lanzetta, Parrilli, & De Castro, 2009;
369 Shi, Meng, Li, Chen, Liu, & Bai, 2014). Cetylpyridinium chloride,
370 cetyltrimethylammonium bromide, guanidine HCl and alcoholic solutions are the
371 most employed (Takai & Kono, 2003; Souza, Kozlowski, Cerqueira, Castelo-
372 Branco, Costa, & Pavão, 2007; Vázquez, Rodríguez-Amado, Montemayor,
373 Fraguas, González, & Murado, 2013). In general, all of them produced acceptable
374 results, but current tendencies towards the application of “green” chemicals make
375 the alcohols the most adequate and economical choice. Although methanol and
376 isopropyl alcohol solutions have been used for precipitation of various shark
377 cartilages, ethanol is the first option reported in the bibliography (Michelacci, &
378 Horton, 1989; Tadashi, 2006; Kim et al., 2012). The results here obtained for *P.*
379 *glauca* were in concordance with those previously found for *Raja clavata*;
380 nevertheless, the optimal concentration of NaOH in blue shark is 2.5-times higher
381 than in ray (Murado, Fraguas, Montemayor, Vázquez, & González, 2010).
382

383 *3.3. Membrane procedures for CS purification*

384 A sequence of UF-DF using membranes of 100 and 30 kDa was utilised for the
385 final CS purification step. In this context, batches of neutralised CS solutions
386 obtained under the conditions optimised in previous sections were firstly processed
387 at 100 kDa performing complete UF and DF stages. The most remarkable kinetics
388 of CS and protein concentration are depicted in Figure 3 (Top, left). Experimental
389 data of concentration factor showed acceptable correlation with the corresponding
390 theoretical values in the case of total proteins however, such correlation was low
391 for CS data. This result indicates a low retention of CS molecules and good
392 concentration of proteins at 100 kDa. These results were confirmed in the process
393 of diafiltration (Figure 3, bottom-left) in which most of CS was permeated after 3
394 diavolumes meanwhile proteins were mainly concentrated. The kinetics of DF were
395 perfectly described by equation [6], in both cases obtaining very high R^2 values
396 (Table D, supplementary material). As expected, the coefficient of retention (*s*) for
397 proteins was higher than for CS. Furthermore, in the case of GAG R_f parameters
398 were lower and R_{3D} parameters were higher, compared to proteins.
399
400 Permeates from UF at 100 kDa were subsequently concentrated by 30 kDa cut-off.
401 In the first step of UF, total correlation agreement between CS experimental and
402 predicted concentration factor profiles (8-fold) was found (Figure 3, top-right).
403 Proteins were not retained and the filtrate flows were maintained in an average
404 value of 578 ± 57 mL/min. Experimental data of retention obtained in DF stage
405 were accurately modelled using equation [6] (Figure 3, bottom-right and Table D
406 supplementary material). Parameter concerning CS and protein retention at 30 kDa

407 indicated null permeation (s =1, R_f =100% and R_{3D} =0%) for CS, and complete
408 permeation of proteins after 3.5 volumes of water diafiltration. In addition, the
409 desalination of CS retentate at 30 kDa was total (data not shown). The purity of CS
410 retentate (in terms of I_p-values) after drying was of 98.5%. The final yield of CS
411 was 12.08 ± 0.72 % (w/w of dry cartilage).
412
413 Although the application of membrane technologies (UF-DF) is one of the most
414 common techniques utilized for the recovery of high-value macromolecules
415 obtaining by microbial or enzymatic processes, its use in the purification of CS from
416 biological tissues has not been broadly reported. Other protocols based on
417 chromatographic separation, using different types of columns and elution solvents
418 and gradients, have been usually applied at lab-scale for CS purification (Souza,
419 Kozlowski, Cerqueira, Castelo-Branco, Costa, & Pavão, 2007; Gargiulo, Lanzetta,
420 Parrilli, & De Castro, 2009; Xie, Ye, & Luo, 2014). However, the efficiency of UF-
421 DF, its commercial cost, reproducibility and facility of equipment scaling is higher in
422 comparison with chromatography procedure (Nishigori, Takeda, & Ohori, 2000).
423 Lignot et al. (2003), using skate cartilage as substrate and ceramic membranes of
424 UF, indicated that more than 40% of salts were not dialisated at 0.1 μm pore-size
425 and predicted that 7 diavolumes of water are required for total protein and salt
426 elimination. In the present study, we have demonstrated that at least 3.5
427 diavolumes should be performed to reject both disposable solutes. On the other
428 hand, the high predictive capacity of equation [6] to simulate diafiltration kinetics
429 followed the similar good agreement among experimental and calculate data, that
430 in the assessment of DF dynamics for: total proteins from shrimp wastewaters

(Amado, Vázquez, González, Esteban-Fernández, Carrera, & Piñeiro, 2014), CS from rays (Lignot, Lahogue, & Bourseau, 2003; Murado, Fraguas, Montemayor, Vázquez, & González, 2010) and small-spotted catshark (Blanco, Fraguas, Sotelo, Pérez-Martín & Vázquez, 2015).

Conclusions

A set of biological, chemical and physical processes, including enzyme hydrolysis, selective precipitation and redissolution of polysaccharides and separation by membranes (UF-DF) have been optimised to extract and purify CS from fishing by-products of *P. glauca*. To obtain this purpose, a combination of kinetic and response surface methodologies based on rotatable factorial designs was applied. The optimal conditions for CS isolation were 55.7ºC/ pH 8.2/ 8 h/ 1% alcalase 24 AU/kg for enzyme cartilage hydrolysis and NaOH 0.54 M, EtOH 1.17 v, NaCl 2.5% for alkaline hydroalcoholic saline treatment. UF-DF performances at 30 kDa utilising at least 3 diavolumes of water in the diafiltration were needed for yielding CS of high purity (>98%). Chemical characterisation works should be further conducted in order to know the type of CS present in *P. glauca* head cartilage.

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FIGURES CAPTIONS

Figure 1. Cartilage hydrolysis over time of *P. glauca* heads by alcalase in each one of the experimental conditions defined in Table 1. The experimental data (symbols) were fitted to the Weibull equation [4] (continuous line).

Figure 2. Top: Predicted response surfaces by empirical equations summarized in Table 2 corresponding to the joint effect of *pH* and *T* on alcalase hydrolysis for the different dependent variables quantified. Bottom: Theoretical surfaces calculated by second order equations described in Table 3 to model the combined influence of *NaOH* and *EtOH* on the selective treatment of CS from hydrolysate cartilages of *P. glauca.*

Figure 3. UF-DF process for CS purification from *P. glauca* head cartilages at 100 kDa and 100➔30 kDa sequences. 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 [6] was used to fit the experimental data. Error bars are the confidence intervals (α = 0.05; n = 2).

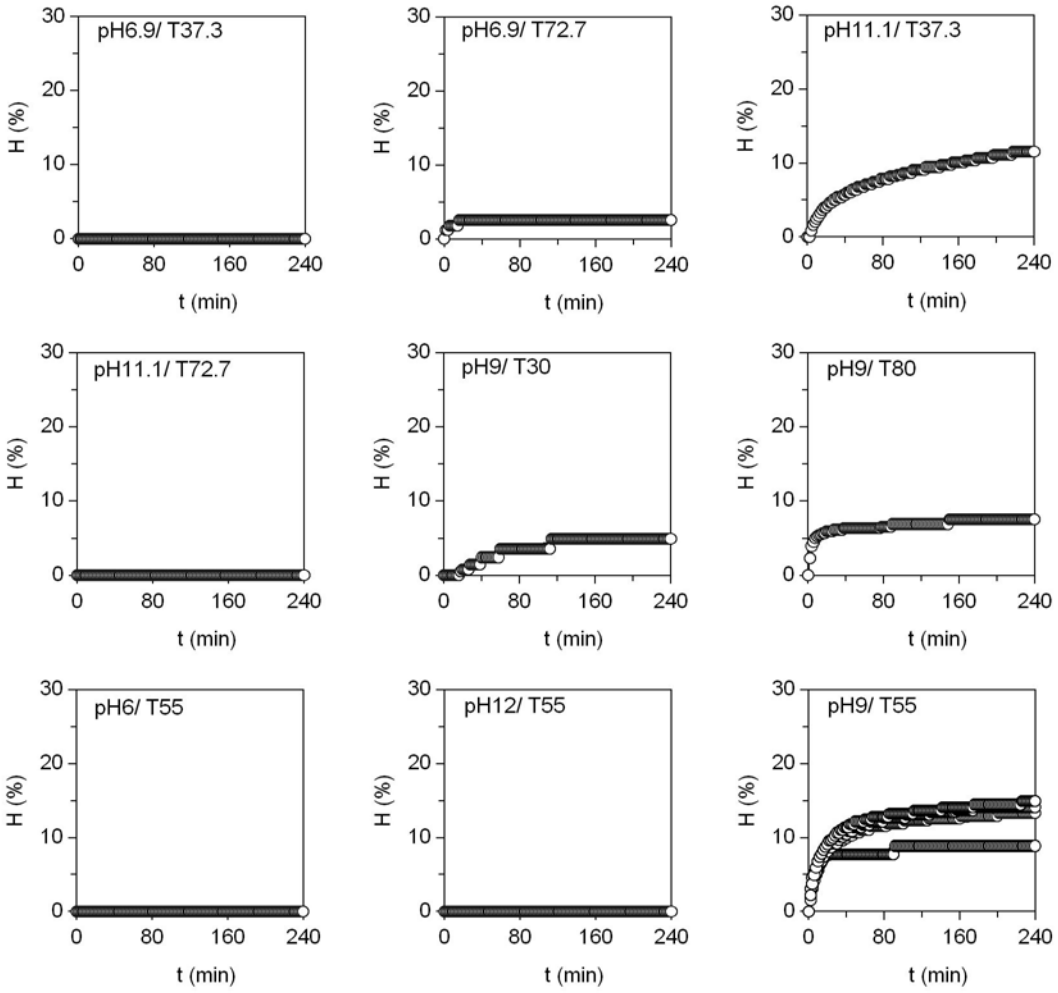
TABLES CAPTIONS

Table 1. Experimental domains and codification of independent variables in the factorial rotatable designs executed to study the cartilage hydrolysis by alcalase and the subsequent chemical treatment of the hydrolysates in alkaline hydroalcoholic solutions.

Table 2. Second order equations describing the effect of *T* and *pH* on alcalase cartilage hydrolysis, CS production and I_p-index (coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R^2_{adj}) and F-values (F_1, F_2, F_3 and F_4) is also shown. S: significant; NS: non-significant.

Table 3. Second order equations describing the effect of *N* and *E* on selective precipitation of CS (coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R^2_{adj}) and F-values (F_1 and F_2) is also shown. S: significant.

Figures



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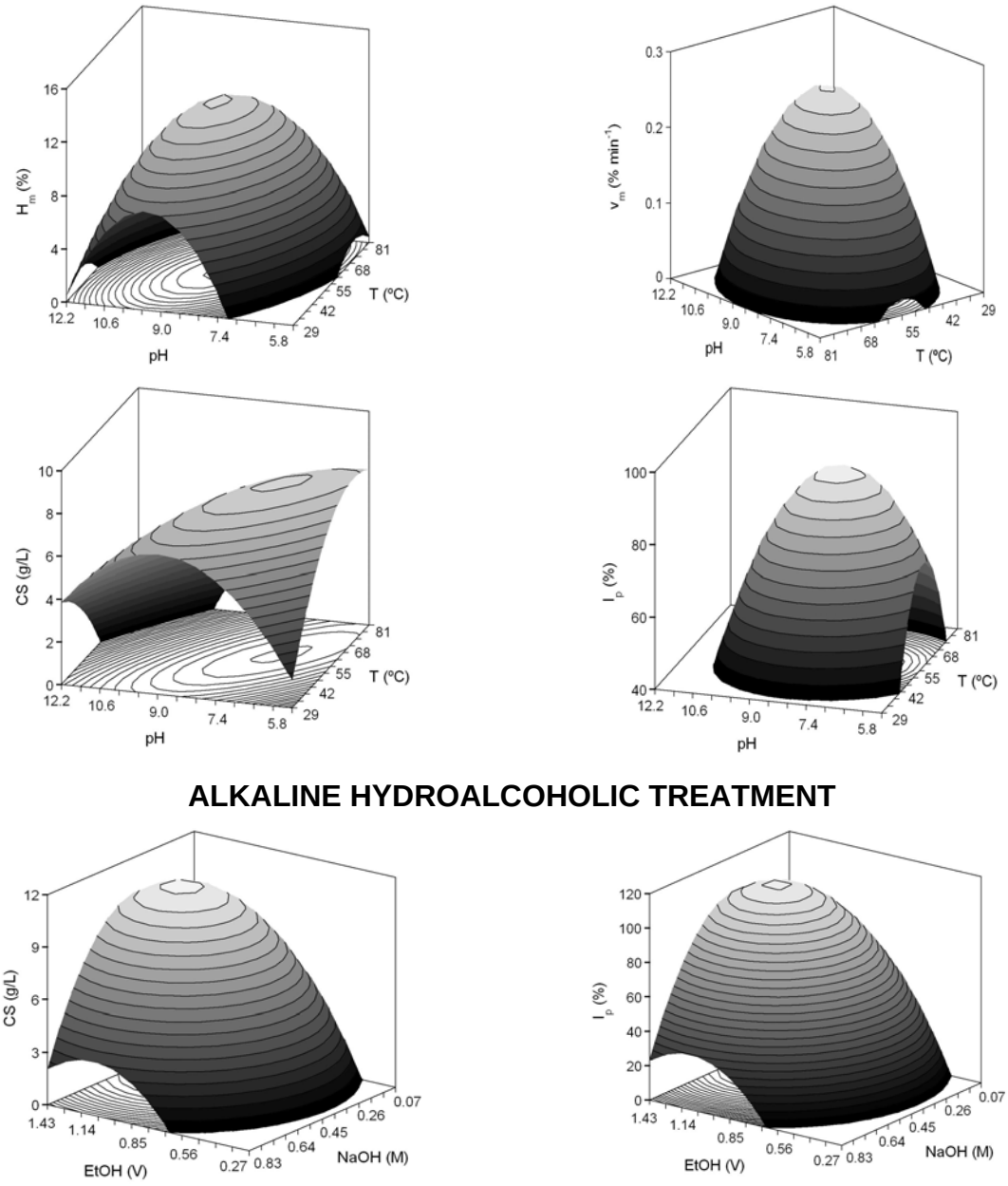

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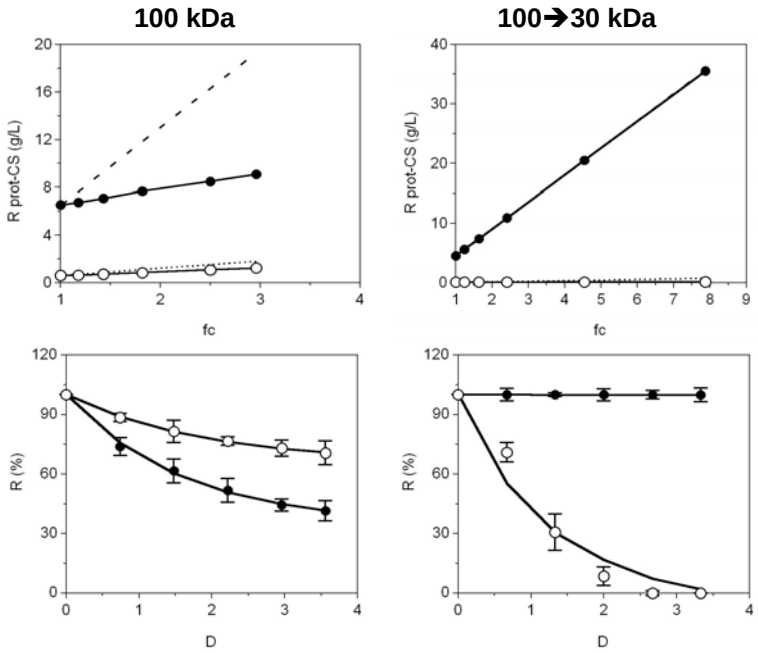

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TABLES

Table 1. Experimental domains and codification of independent variables in the factorial rotatable designs executed to study the cartilage hydrolysis by alcalase and the subsequent chemical treatment of the hydrolyzates in alkaline hydroalcoholic solutions.

Coded values	Natural values			
	Alcalase hydrolysis		Chemical treatment	
	T (ºC) : *T*	pH	NaOH (M): *N*	EtOH (v): *E*
-1.41	30.0	6.0	0.10	0.30
-1	37.3	6.9	0.20	0.46
0	55.5	9.0	0.45	0.85
+1	72.7	11.1	0.70	1.24
+1.41	80.0	12.0	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 2. Second order equations describing the effect of *T* and *pH* on alcalase cartilage hydrolysis, CS production and I_p-index (coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R^2_{adj}) and F-values (F_1, F_2, F_3 and F_4) is also shown. S: significant; NS: non-significant.

Parameters	H_m	v_m	CS	I_p
b_0 (intercept)	13.00	0.264	7.34	86.87
b_1 (T)	-	-	-1.07	-
b_2 (pH)	-	-	-2.21	-17.38
b_{12} (TxpH)	-3.72	-	-2.04	-
b_{11} (T^2)	-3.01	-0.095	-1.11	-30.35
b_{22} (pH^2)	-6.28	-0.140	-1.72	-11.84
R^2_{adj}	0.720	0.776	0.812	0.939
F1	7.72 $[F_9^3 = 3.86] \Rightarrow S$	21.80 $[F_{10}^2 = 4.10] \Rightarrow S$	11.33 $[F_7^5 = 3.97] \Rightarrow S$	62.20 $[F_9^3 = 3.86] \Rightarrow S$
F2	0.50 $[F_3^8 = 8.85] \Rightarrow S$	0.26 $[F_2^8 = 19.37] \Rightarrow S$	0.68 $[F_4^8 = 6.04] \Rightarrow S$	0.39 $[F_3^8 = 8.85] \Rightarrow S$
F3	2.91 $[F_4^9 = 6.00] \Rightarrow S$	0.51 $[F_4^{10} = 5.96] \Rightarrow S$	2.09 $[F_4^7 = 6.09] \Rightarrow S$	2.08 $[F_4^9 = 6.00] \Rightarrow S$
F4	4.44 $[F_4^5 = 6.26] \Rightarrow S$	0.18 $[F_4^6 = 6.16] \Rightarrow S$	3.54 $[F_4^3 = 6.59] \Rightarrow S$	2.94 $[F_4^5 = 6.26] \Rightarrow S$

Table 3. Second order equations describing the effect of *N* and *E* on selective precipitation of CS (coded values according to criteria defined in Table 1). The coefficient of adjusted determination (R^2_{adj}) and F-values (F_1, F_2, F_3 and F_4) is also shown. S: significant.

Parameters	CS	I_p
b_0 (intercept)	9.14	89.24
b_1 (*N*)	1.56	16.09
b_2 (*E*)	3.85	38.50
b_{12} (*N*x*E*)	-	-
b_{11} (N^2)	-2.36	-22.18
b_{22} (E^2)	-2.31	-22.16
R^2_{adj}	0.843	0.842
F1	17.11 $[F_8^4 = 3.84] \Rightarrow S$	17.03 $[F_8^4 = 3.84] \Rightarrow S$
F2	0.56 $[F_4^8 = 6.04] \Rightarrow S$	0.56 $[F_4^8 = 6.04] \Rightarrow S$

SUPPLEMENTARY MATERIAL

Table A. Parametric estimations corresponding to the Weibull equation [4] applied to the enzymatic hydrolysis kinetics at 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 summarized.

Experimental conditions	H_m (%)	v_m (% min^{-1})	τ (min)	β	R^2	p-value
T:-1 (37.3ºC) / pH:-1 (6.9)	-	-	-	-	-	-
T:1 (72.7ºC) / pH:-1 (6.9)	-	-	-	-	-	-
T:-1 (37.3ºC) / pH:1 (11.1)	14.86 ± 0.88	0.045 ± 0.006	71.9 ± 10.3	0.63 ± 0.03	0.996	<0.001
T:1 (72.7ºC) / pH:1 (11.1)	-	-	-	-	-	-
T:-1.41 (30.0ºC) / pH:0 (9.0)	4.99 ± 0.11	0.049 ± 0.004	51.8 ± 2.5	1.46 ± 0.13	0.967	<0.001
T:1.41 (80.0ºC) / pH:0 (9.0)	9.04 ± 1.10	0.140 ± 0.086	6.0 ± 3.4	0.27 ± 0.05	0.951	<0.001
T:0 (55.0ºC) / pH:-1.41 (6.0)	-	-	-	-	-	-
T:0 (55.0ºC) / pH:1.41 (12.0)	1.00 ± 0.12	0.010 ± 0.006	12.2 ± 1.8	0.54 ± 0.21	0.932	<0.001
T:0 (55.0ºC) / pH:0 (9.0)	13.39 ± 0.08	0.231 ± 0.006	13.4 ± 0.4	0.67 ± 0.02	0.995	<0.001
T:0 (55.0ºC) / pH:0 (9.0)	13.40 ± 0.11	0.209 ± 0.007	14.1 ± 0.5	0.63 ± 0.02	0.994	<0.001
T:0 (55.0ºC) / pH:0 (9.0)	9.14 ± 0.28	0.420 ± 0.071	2.9 ± 0.7	0.38 ± 0.06	0.919	<0.001
T:0 (55.0ºC) / pH:0 (9.0)	14.45 ± 0.11	0.207 ± 0.006	14.8 ± 0.4	0.61 ± 0.02	0.996	<0.001
T:0 (55.0ºC) / pH:0 (9.0)	14.75 ± 0.13	0.252 ± 0.010	12.3 ± 0.5	0.61 ± 0.02	0.992	<0.001

Table B. Optima values of the two independent variables (T_{opt} and pH_{opt}) to obtain the maximum responses from the equations defined in Table 1 and for the different dependent variables evaluated.

	H_m	v_m	CS	I_p
T_{opt} (ºC)	55.0	55.0	59.2	53.6
pH_{opt}	9.0	9.0	7.34	7.43
Y_{max}	13.0	0.264	8.08	93.1

Table C. Summary of the independent variables (*NaOH: N*, *EtOH: E*) in the response surface design with the corresponding experimental (Y_e) and predicted (Y_p) results of selective precipitation of CS from *P. glauca* wastes. Natural values of experimental conditions are in brackets.

Independent variables		CS (g/L)		I_p (%)	
X_1: *N*	X_2: *E*	Y_e	Y_p	Y_e	Y_p
-1 (0.20)	-1 (0.46)	0.00	-0.94	0.00	-9.7
1 (0.70)	-1 (0.46)	0.00	2.18	0.00	22.5
-1 (0.20)	1 (1.24)	8.97	6.76	89.3	67.3
1 (0.70)	1 (1.24)	8.84	9.88	90.5	99.5
-1.41 (0.10)	0 (0.85)	0.00	2.24	0.00	22.5
1.41 (0.80)	0 (0.85)	8.92	6.64	90.2	67.8
0 (0.45)	-1.41 (0.30)	0.00	-0.88	0.00	-9.1
0 (0.45)	1.41 (1.40)	9.13	9.98	90.3	99.5
0 (0.45)	0 (0.85)	9.75	9.14	89.6	89.2
0 (0.45)	0 (0.85)	9.20	9.14	89.3	89.2
0 (0.45)	0 (0.85)	8.89	9.14	89.0	89.2
0 (0.45)	0 (0.85)	8.75	9.14	89.0	89.2
0 (0.45)	0 (0.85)	9.13	9.14	89.3	89.2

Table D. Parametric estimates from UF-DF purification data of CS and proteins fitted to the equation [6]. Determination coefficients (R^2) are also shown. NS: non-significant.

UF-DF sequence		CS	Proteins
100 kDa	R_0	64.84 ± 8.30	34.34 ± 2.75
	R_f	34.65 ± 8.52	65.66 ± 2.97
	s	0.378 ± 0.203	0.472 ± 0.087
	R^2	0.998	0.999
	R_{3D}	54.81	27.30
100→30 kDa	R_0	0.00 ± 0.00	100.0 ± 31.20
	R_f	100.0 ± 1.80	0.00 ± 0.00
	s	1.00 ± 0.00	0.107 (NS)
	R^2	1.000	0.962
	R_{3D}	0.0	93.14