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Optimization of extraction and purification process of hyaluronic acid from fish eyeball.

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Headline: Purification of hyaluronic acid from fish eyeball.

21 **ABSTRACT**

22 The goal of the present work is to optimize the different steps for obtaining highly purified
23 hyaluronic acid (HA) from fish eyeball. The extraction and purification process of HA from
24 vitreous humour of fish, among other biological materials, is based on the succession of: 1) a
25 step of protein electrodeposition, previous or simultaneous with a diafiltration process in total
26 recirculation, 2) a selective recovery in hydroalcoholic solution of impure sediments obtained by
27 alcoholic exhaustive precipitation, 3) an alkaline treatment under hydroalcoholic solution and
28 controlled conditions of alkalinity, temperature, proportion of ethanol and time that it
29 precipitates HA and solubilizes proteins, and 4) HA recovery by alkaline suspension of the
30 precipitate in hydroalcoholic phosphate monosodium that it dissolves HA, neutralizes the extract
31 and leaves insoluble proteins in the sediment. Thus, HA with high purity (more than 99.5%),
32 useful for clinical and cosmetic applications, are obtained by means of low-cost process using a
33 waste material.

34

35 **Keywords:** Hyaluronic acid; fish by-products; bioprocessing; environment; food processing;
36 downstream processing.

37

38 **INTRODUCTION**

39 HA is a polymer formed by repeating disaccharide units of N-acetyl-D-glucosamine and
40 glucuronic acid. This glycosaminoglycan is present in tissues as cartilage, sinovial fluid, skin,
41 rooster combs, umbilical cord and vitreous humour and sinovial fluid, besides in the cell wall of
42 bacteria such a *Streptococcus zooepidemicus* (Shiedlin et al., 2004; Yamada and Kawasaki,
43 2005; Vázquez et al., 2009). In recent years, an increasing interest has been reported due to its
44 numerous applications as cosmetic and pharmaceutical compound (Nerem, 2006; Kim et al.,
45 2008; DeAngelis, 2008; Zhang et al., 2008). The habitual sources for its industrial production are
46 rooster crest, bovine synovial liquid, bovine vitreous humour and, with rising offer, bacterial

47 cultivations (Shiedlin et al., 2004; Huang et al., 2008; Vázquez et al., 2010). However, vitreous
48 humour from eyeball of certain fishes also contains appreciable concentrations of HA that it
49 could establish rational uses of these waste materials by upgrading. It would help to reduce
50 environmental pollution on coastal areas. This substrate would also avoid the risk of bovine
51 spongiform encephalopathy (BSE) that bovine origin generates. Furthermore, commercial prices
52 of HA obtained from animal sources like vitreous humour is much higher than that obtained by
53 fermentation. In Table 1, HA concentrations from various sources are summarized.
54
55 The main problem of HA purification is the elimination of the proteins that are being part of
56 proteoglycan matrix and they are potentially allergenic in many applications of the product. The
57 final concentration of proteins in the preparations should be around of ~5-10 μg of protein per
58 mg of HA for clinical uses that imply injection, descending the demands of purity in non
59 injectable performances (perfusion, topical application, oral administration). With a source as
60 vitreous humour of swordfish, it means to take the relationship protein/HA from an initial value
61 of ~45 until a final value of ~0.01.
62
63 The fundamentals of many of the purification procedures reported in recent literature are already
64 described in Rodén et al. (1972). These stages can be summarized in the following terms:
65
66 a) In aqueous solution, proteins and HA precipitate together when ethanol is added in the
67 appropriate proportion. A part of the proteins, variable according to the source, are not
68 solubilised to the ethanol concentrations to those HA still remains in solution. It makes that
69 fractional precipitation can be employed as a potential resource of partial purification.
70
71 b) A part of the proteins, variable according to the source, can be also hydrolyzed by means of
72 protease action (papain, alkalase, trypsin, pronase). Thus, the subsequent treatment of the

hydrolysates with ethanol leaves on hydroalcoholic solution a part of the hydrolysis products, whereas the remains of the proteases are still distributed between the soluble fraction and the sediment.

An example of the application of these properties, economically viable with rich sources, it is in the patent of Cullis-Hill (1989) that improves other previous works and that it is based on the recurrent execution of alcoholic precipitations and enzymatic hydrolysis, repeated as many times as necessary to achieve the suitable purity. On the other hand, in this work ethanol is used with 2% (w/v) of acetic acid what develops the irreversible denaturalization of the proteins in the precipitation steps.

Other interesting works that use technical of chromatographic separation (Kitagawa et al., 1990) or reactions with salts of quaternary ammonium (Hildesheim, 1987) do not constitute, however, precedent of the process that we are proposing. In same circumstances they are the procedures that, still using ultrafiltration techniques (Yoshizawa, 1990), are applied in terms and with concrete objectives different from those are presented in this article. According to our knowledge, no works of extraction and purification of HA from vitreous humour of fishes have been reported until now.

In the present study, a process for HA recovery and purification from vitreous humour of selected species of fishes is described. Thus, a combination of steps of ultrafiltration-diafiltration system, protein electrodeposition, selective resolubilization in hydroalcoholic medium and selective precipitation in alkaline hydroalcoholic solution is studied and optimized.

MATERIALS AND METHODS

Vitreous humour preparation

Eyeballs from swordfish (*Xiphias gladius*) and shark (*Prionace* sp.) were obtained from the fish port of Vigo and frozen at –20ºC. Subsequently, these frozen eyeballs were undergone to two or three serial cuts. The fragments were defrosted on a warp of parallel threads of nylon (meshes with knots elevate the losses and time of process besides generating a bigger proportion of impurities) that allows the leak of vitreous humour and it retains optic capsule including lens. Subsequently, this material was homogenized, in order to complete the deconstruction of the humour, and clarified by centrifugation at 6,000 × *g* for 15 min. Three clear phases were obtained: sediment of impurities, a little fraction of lipid supernatant (easily removed by aspiration) and a majority interface of viscous vitreous humour.

Ultrafiltration-diafiltration system

Ultrafiltration-diafiltration was performed by means of plate polysulfone membranes (*Millipore Minitan System*) of 60 cm^2 with cut-off at 100, 300 and 675 kD, using an assembly with total recirculation at 35ºC. A pack of 4 plate membranes (surface total area= 240 cm^2) were employed.

Electrodeposition system

The electrodeposition device was performed by means of two platinum electrodes of 50 cm-length and prepared in spiral/cylindric format. The electric current established between both electrodes was variable in the range of 10-40 mA.

Alcoholic precipitation and selective recovery of the precipitate

121 The retentates obtained from electrodeposition and diafiltration steps were salted with NaCl
122 0.5M and slowly precipitated with ethanol 99-100% under intensive agitation to avoid the
123 formation of floccules.
124
125 This hydroalcoholic solution is incubated to ~5ºC and sediment is spontaneously precipitated in
126 3-5 hours of incubation. The corresponding clear supernatant is drained by means of a peristaltic
127 pump and it is rejected. The sediment, including HA and a protein fraction, is redissolved, by
128 intensive agitation, adding the volume of water that is necessary to obtain a appropriate
129 water:ethanol relationship to quantify HA and to maximize the unsolubilized protein fraction
130 (see section 3 of results and discussion). The suspension, in the appropriate relationship of
131 water:ethanol, is diluted with a hydroalcoholic solution (with the same relationship) until a
132 volume approximately equivalent to 1/5 of the initial retentate. Subsequently, it is clarified by
133 centrifugation (6,000 × *g* for 15 min) being now the sediment rejected (it only contains insoluble
134 protein) and the supernatant recovered.
135
136 ***Alkaline process on hydroalcoholic solution***
137 Experimental plan implied a rotatable design of two variables (see below): *S*, or NaOH
138 concentration in the reaction mixture, with domain [0.45; 0.85 M], and *E*, or volumes of ethanol
139 per volume of retentate, with domain [0.6; 0.9]. The corresponding tests were carried out adding
140 to the previous hydroalcoholic extract, slowly and with vigorous agitation at 5ºC for 1-5 h,
141 hydroalcoholic solutions of NaOH in the required proportions to obtain reaction mixtures with
142 the pre-established values of *S* and *E*.
143
144 When the agitation is interrupted, a mass of cottony aspect precipitates and it can be separated by
145 centrifugation at 6,000 × *g* for 15 min. The discarded supernatant contains the protein fraction
146 solubilised by the treatment. The alkaline sediment that contains HA together with an insoluble

147 protein fraction is firstly redissolved in a small volume of water:ethanol (1:0.75) solution, adding
148 as well an aqueous solution of NaH_2PO_4 0.5M:ethanol (1:0.75) in order to neutralize or to take
149 the pH in a established value. It should be pointed out that the use of acids as HCl or acetic for
150 this purpose presents the risk of reducing the average molecular mass of HA causing losses in
151 the retentate at 300 kD (Tømmeraas and Melander, 2008). The homogeneous resolution obtained
152 is centrifuged (6,000 × *g* for 15 min) and the corresponding supernatant with HA is collected.
153 The protein sediment is washed with water:ethanol (1:0.75) and the supernatant is joined with
154 the previous one.
155
156 ***Analytical methods***
157 HA assay was a slight modification of the method of Van Den Hoogen et al. (1998) following
158 the proposal and mathematical corrections defined by Murado et al. (2005). Proteins were
159 determined by the method of Lowry et al. (1951). HA molecular weight was determined by size-
160 exclusion chromatography on HPLC by means of an Ultrahydrogel Linear column (Waters,
161 USA) with 0.1M $NaNO_3$ as mobile phase (flow= 0.6 mL/min) and a refractive-index detector.
162 The column was calibrated with polystyrene standards (Sigma) of varying molecular weights
163 (32, 77, 150, 330, 990 and 2600 kD).
164
165 ***Experimental design and statistical methods***
166 In all cases that the joint effect of two variables was studied, an approach using rotatable designs,
167 with central quintuple replication, was carried out (Akhnazarova and Kafarov, 1982; Box et al.,
168 2005). Experimental domain and coding criteria are given in Table 2. The results of the factorial
169 designs were fitted to equations of the type:
170
171 $$Z = b_0 + b_1 X + b_2 Y + b_{12} XY + b_{11} X^2 + b_{22} Y^2 \quad (1)$$
172

Statistical significance of the coefficients was verified by means of Student´s *t*-test ($\alpha<0.05$), and model consistency by means Fisher´s *F*-test ($\alpha<0.05$) applied to following mean squares ratios:

	the model is acceptable if
F_1 = Model / Total error	$F_1 \geq F_{den}^{num}$
F_2 = (Model + Lack of fitting) / Model	$F_2 \leq F_{den}^{num}$
F_3 = Total error / Experimental error	$F_3 \leq F_{den}^{num}$
F_4 = Lack of fitting / Experimental error	$F_4 \leq F_{den}^{num}$

Although it is a common practice to limit this test to the F_1 or F_1 and F_2 quotients, it should be pointed out that F_3 and F_4 are essential to avoid the introduction of irrelevant variables or variable combinations in this type of empiric models.

RESULTS AND DISCUSSION

1: Initial diafiltration-concentration process

Initially, clarified vitreous humour is diafiltrated using a system with total recirculation and a value of dilution flow between the half and the third part of permeation flow. When two membranes of cut-off at 675 and 100 kD are sequentially applied to a volume V_0, we can obtain:

a) A retentate from 675 kD with a lower volume than $V_0/10$, that it contains, at least, 75% of HA total and approximately 16% of the initial protein.

b) A permeate with an approximate volume of $2V_0$ that is diafiltrated at 100 kD until a retentate volume between $V_0/10$ and $V_0/15$. This retentate contains the 25% remaining and a 29% of initial protein. The corresponding permeate, with the remaining protein fraction and without HA, is rejected. Although the ratio protein/HA increases in these second retentates with regard to the corresponding value in the raw material, the elimination of this protein in the subsequent steps is slightly more efficient with retentates than using raw materials.

When a cut-off at 300 kD is only used, it is possible to achieve a retentate with an approximate volume of $V_0/12$ that it contains 96% of HA and 46% of the initial protein.

2: Protein electrodeposition

It was carried out inserting electrodeposition device into the clarified vitreous humour and establishing between both electrodes an initial electric current of 10 mA. This value was gradually increased until 40 mA for 1 min and was maintained in this level for 30 min. As consequence of this current step, in few seconds a deposit not very soluble in water and soluble in NaOH 0.5M was formed in the anode. This precipitate produces a strongly positive reaction of Lowry-proteins. The fact that the electrode washing with distilled water generates a suspension with an approximate pH 4.2 suggests that the process implies the interchange of electrons towards anode from carboxyl groups of the proteins with net negative charge (cathode reaction: $H^+ + e^- = H$). These proteins precipitate when they approach to the isoelectric point. On the other hand, the deposit detached from the electrode remains unsolubilized for at least one hour and it can be removed by centrifugation. Though the effect of ferric metals in the breakdown of HA has been repeatedly reported (Wong et al., 1981; Hawkins and Davies, 1996; Balogh et al., 2003), the use of platinum electrodes did not affect to the proportions of HA retained at 300 and 675 kD.

The interest of the protein fraction so removed (approximately 0.3 g of protein per liter of vitreous humour, with a current of 40 mA for 15 min) comes from its effects on the diafiltration efficiency. Deposits obtained with same electric current values in raw vitreous humour and with retentates from diafiltration at 675 kD reduced to a volume of $V_0/5$, indicating that these are non filterable materials to this cut-off and contribute to increase transmembrane pressure with the progress of the process. In fact, when electrodeposition begins after a time period of enough

diafiltration so that the permeate flow falls to 50% of the initial value, recoveries of this flow until 90% of initial value are observed.

Therefore, electrodeposition can be carried out as a previous or simultaneous operation to the diafiltration. Although in Faraday's laws the solute concentration implied in the electrode reactions are not present in the mathematical equations, the protein amount deposited by unit of time increases with the retentate concentration (data not shown). This effect is easily understandable since the progress of the diafiltration eliminates chemical species of low molecular mass, able to compete with non filterable proteins in the anode reaction. A previous deposition process, followed or not by a centrifugation step, can be combined with diafiltration, case in which a prefilter (*e.g.*, nylon mesh of 40-100 μm) should be used. In all cases, deposition efficiency increases with a brief wash the anode in NaOH solution when accumulated protein layer reduces the electric current to inadequate values.

3: Alcoholic precipitation and selective recovery of the precipitate

In Figure 1 the joint effect of ethanol and NaCl on retentates precipitation are depicted. This response was evaluated by means of HA concentration and recovery proteins in extracts obtained by redissolution of the corresponding sediments in water:etanol (1:0.75). The recovery of HA is little affected by salt concentration and increases asymptotically with the proportion of ethanol. However, high concentrations of both variables produces drops, slight but consistent, from a maximum value of HA recovery. On the other hand, recovery of proteins, much more affected by salt concentration, falls when salt concentration increasing at any considered level of ethanol. Meanwhile, the response to the ethanol loses the asymptotic nature, falling from a maximum when salt concentration decreases.

247 Figure 1 shows the appropriate range for the precipitation process. These experimental profiles
248 revealed that proportions of ethanol no lower than 1.5 volumes per volume of retentate and salt
249 concentrations higher than 1.5 M in the retentate should be used for optimal HA recovery. Lower
250 values of ethanol can lead to losses of HA and lower values of salt do not affect to HA recovery
251 but they led to extracts with higher protein concentrations.
252
253 Regarding the sediment redissolution, a convenient water:ethanol relationship is 1:0.75 in an
254 approximately equivalent volume to 1/5 of retentate. Higher proportions of ethanol present the
255 risk of HA losses, mainly in retentates with high concentration ratios. However, lower
256 proportions do not affect to the HA recovery but they contribute unnecessarily to redissolve
257 proteins (data not shown).
258
259 Finally, another possible repetition of this step (Figure 2) implies the addition of NaCl to the
260 retentate until a concentration 0.5M and 0.5 volumes of ethanol. In this alternative, scarcer
261 protein sediment to the previous proposed procedure is obtained, whereas the whole of HA
262 remains in solution in the supernatant. Though the consumption of ethanol can decrease with
263 regard to the precedent procedure without losses in the recovery of HA, the supernatant that
264 continues to the subsequent stage is more diluted and it contains higher proportions of proteins.
265
266 ***4: Alkaline process***
267 The joint effect of NaOH and ethanol concentrations on HA recovery after a treatment of 10 h at
268 5ºC was evaluated by means of a second order experimental design following the approach of
269 Akhnazarova and Kafarov (1982). When independent variables are coded in such way that both
270 natural domains become the codified domain [–1;1]:
271
272 S (molar concentration of NaOH): [0.450 ; 0.850] $\rightarrow$ [–1;1]

273 E (ethanol volumes per water volume): [0.600 ; 0.900] → [–1;1]

274

275 HA recovery (as %) is satisfactorily fitted to the following empirical equation:

276

277 $$HA = 93.93 - 4.46\ S - 35.25\ SE - 39.12\ S^2 - 5.25\ E^2 \quad (2)$$

278

279 whose coefficients were statistically significant (t-Student test, $\alpha = 0.05$), and its consistency was
280 proven by means of *F*-Fisher test applied to the relationships F_1, F_2, F_3 and F_4 ($\alpha = 0.05$). Inside
281 the studied interval, the maximum of this equation (2), whose response surface is showed in
282 Figure 3, is placed in the maximum value of ethanol proportion (0.9), with 0.558 M of NaOH.

283

284 On the other hand, in Figure 4 (left) the percentage of HA recovery is shown at different times,
285 operating in the maximum of the equation (2). The values that decay until 97% in 10 hours are
286 satisfactorily fitted to a first order kinetics equation (with t in hours):

287

288 $$HA = 100 \cdot \exp\left(-0.00317 \cdot t\right) \quad (3)$$

289

290 Figure 4 (right) reveals, moreover, that the effect of the treatment on the proteins distribution
291 between supernatant and sediment hardly varies after first hour, being able to be considered
292 practically immediate.

293

294 Thus, equations (2) and (3) are able to use for determining the most appropriate conditions in the
295 alkaline treatment, that can be established, at 5ºC, in 0.9 volumes of ethanol, NaOH 0.56 M for
296 1-5 hours.

297

Finally, the extracts from alkaline process can be treated by diafiltration at convenient cut-off membrane to achieve simultaneously the HA concentration and the phosphate dilution required. Furthermore, the soluble proteins remainders, to concentrations in the range of 0.02-0.04 mg/mL, as well as the salts are efficiently removed in this step. If an ulterior purification is still required, it can return to the selective redissolution of the alcoholic precipitate (Figure 3) in similar terms to those described previously.

5: Testing the proposal methodology

Example 1

A volume of 2.5 L of swordfish humour vitreous (*Xiphias gladius*) clarified by centrifugation was undergone, under soft shaking, to electric current of 40 mA with platinum electrodes. Anode, a mesh of 3 × 0.5 cm, was washed after 10 min by immersion in NaOH 0.5N and this operation was repeated twice before diafiltration beginning. Table 2 shows the main parameters of the process step that are described next.

Diafiltration was carried out using membrane of cut-off at 300 kD with total recirculation assembly and nylon mesh of 60 μm using as prefilter. Pressure and dilution flow with distilled water were maintained constant at 40-50 psi and with a 50% of permeation flow, respectively. Electric current of 40 mA was also applied for 8 periods of 15 min. The operation was interrupted one time for washing the membrane (15 min with NaOH 0.1M at 45ºC without pressure) and prefilter and it was maintained until to reach a retentate volume of 260 mL.

In 250 mL of retentate, 21.9 g of NaCl (1.5 M) were dissolved. Subsequently, 375 mL of 99% ethanol were slowly added under magnetic and intense shaking at 5ºC. This agitation system was maintained for 30 min and it was afterwards left in rest to the same temperature overnight. In these conditions, compact sediment and a clarified and rejected supernatant of 425 mL were

obtained. The sediment was mixed with 60 mL of water:ethanol (1:0.75) under vigorous agitation, until to get a fine and homogeneous suspension that it was centrifuged (6,000 × *g* for 15 min), recovering now the supernatant. This last sediment was washed with 20 mL of water:ethanol (1:0.75) joining the corresponding supernatant with the previous one.

75 mL from the whole of the supernatants were mixed with NaOH 0.56M in water:ethanol (1:0.9) solution. After 2 h of intense agitation at 5ºC, the mixture was centrifuged (6,000 × *g* for 15 min) at the same temperature being discarded the supernatant. The sediment was redissolved in a total volume of 40 mL with water:ethanol (1:0.75) and the aqueous solution of NaH_2PO_4 0.5M:ethanol (1:0.75) necessary for obtaining a pH-value of 7.25. The redissolution was centrifuged (6,000 × *g* for 15 min), the supernatant was recovered and the sediment was washed with early solutions until similar pH-value, gathering both supernatants.

Finally, an aliquot of 57 mL from the supernatants were diafiltrated at 100 kD until to obtain 30 mL of retentate with the characteristics specified in Table 3. In this retentate, the molecular weight of HA was 1600 kD.

Example 2

A volume of 2.5 L of shark vitreous humour (*Prionace sp.*) was perfomed in similar way to example 1 with the differences in the work volumes and HA and proteins concentrations that are specified in Table 3. It should be pointed out that the most advantageous ratio protein:AH is not only translated in a final extract of more volume, concentration and purity, but also in a faster and more efficient process. In this case, the molecular weight of HA in the final solution was 2000 kD.

Example 3

A volume of 0.3 L of veal vitreous humour with an initial concentration of 0.258 mg/mL of HA was also handled in a similar way to example 1. In the different fractions, same volumetric relationships with initials were maintained. Thus, a sample I of 12 mL with 6.35 mg/mL of HA and 99.4% of purity was obtained.

CONCLUSIONS

A set of different physical and chemical processes, including protein electrodeposition, separation by membrane (ultrafiltration and diafiltration), as well as selective precipitation and redissolution performance have been optimized in order to extract and to purify HA from humour vitreous of eyeball from fish processing wastes. Solutions of HA with more than 99% of purify were obtained in the optimal conditions proposed.

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FIGURE CAPTIONS

Figure 1: HA recovery (up) and protein presented (down) in redissolutions of precipitates obtained with different proportions of NaCl (molarity in the retentate) and ethanol (volumes per volume of retentate).

Figure 2: Flow diagram of the different operations developed in the preparation of highly purify HA from vitreous humour of fish eyeball. Discontinuous lines (B) show possible repetitions in the stages in order to increase purify of the final samples.

Figure 3: Joint effect of NaOH concentration and ethanol proportion on HA recovery (%) in alkaline treatments at 5ºC. Independent variables are codified according the criteria specified in the text. Response surface corresponding to the equation (2).

Figure 4: HA recovery (left) and remain and removed protein (right) by alkaline treatment in the maximum of the equation (3), with different times of incubation. Keys, ○ (left): HA; ○ (right): remain protein in the extract; ▽: removed protein in the sediment; △: removed protein in the supernatant. Dotted line to the left shows the fits of the HA data to the equation (2).

TABLE CAPTIONS

Table 1: Concentrations of HA in various sources.

Table 2: Experimental domain and codification of two independent variables in the factorial rotatable design.

Table 3: Main parameters of the process steps, called according to the Figure 2, that are described in examples 1 (*X. gladius*) and 2 (*Prionace sp.*). In volume column, the values into open brackets are the aliquots used in the subsequent step.

TABLES

Table 1

SOURCE	HA (g per liter or Kg)	REFERENCE
Rooster combs	8-45	Nakano et al., 1994
Bovine synovial liquid	15-40	Cullis-Hill, 1989
Pig synovial liquid *	0.5-6	PRESENT WORK
Bovine vitreous humour	0.3	Gherezghiher, 1987
Pig vitreous humour	0.04	PRESENT WORK
Bacterial cultures	2-6	Johns et al., 1994 ; Cooney et al., 1999
Vitreous humour of swordfish(a)	0.055	PRESENT WORK
Vitreous humour of shark(b)	0.3	PRESENT WORK

* The farm animals systematically provided concentrations closed to the specified minimum, and very often they practically lacked synovial liquid in their articulations.
(a) *Xiphias gladius*
(b) *Prionace sp.*

Table 2

Variables		**Experimental matrix in coded values**								
X		-1	1	-1	1	$-2^{1/2}$	$2^{1/2}$	0	0	0
Y		-1	-1	1	1	0	0	$-2^{1/2}$	$2^{1/2}$	0

If we define Vn: natural value, with domain [m;M]
Vc: coded value, with domain $[-2^{1/2}; 2^{1/2}]$
We can write Vo: natural value at the center of the domain = (m+M)/2
ΔVn: Increment of natural value corresponding to an unitary increment of coded value = $(M-m)/(2\times2^{1/2})$

Codification: $Vc = (Vn-Vo)/\Delta Vn$ Decodification: $Vn = Vo + (\Delta Vn \times Vc)$

Table 3

Steps	Volume mL		HA mg / mL		Protein-Lowry mg / mL		HA purity* %	
	Ex. 1	Ex. 2	Ex. 1	Ex. 2	Ex. 1	Ex. 2	Ex. 1	Ex. 2
Raw material	2500	2500	0.055	0.283	2.1	2.78	2.55	9.24
Retentate 300 kDa	260 (250)	416 (400)	0.508	1.617	9.6	7.68	5.03	17.39
Supernatant 2	80 (75)	200 (180)	1.540	3.072	0.52	0.42	74.76	87.97
Supernatant 4	60 (57)	125 (122)	1.848	4.202	0.038	0.022	97.99	99.48
Simple I	30	100	3.370	4.818	0.009	0.007	99.73	99.85

*Purity (%) was calculated as: $Purity\,(\%) = \frac{HA\ concentration}{HA\ concentration + Protein\ concentration} \times 100$

FIGURE 1

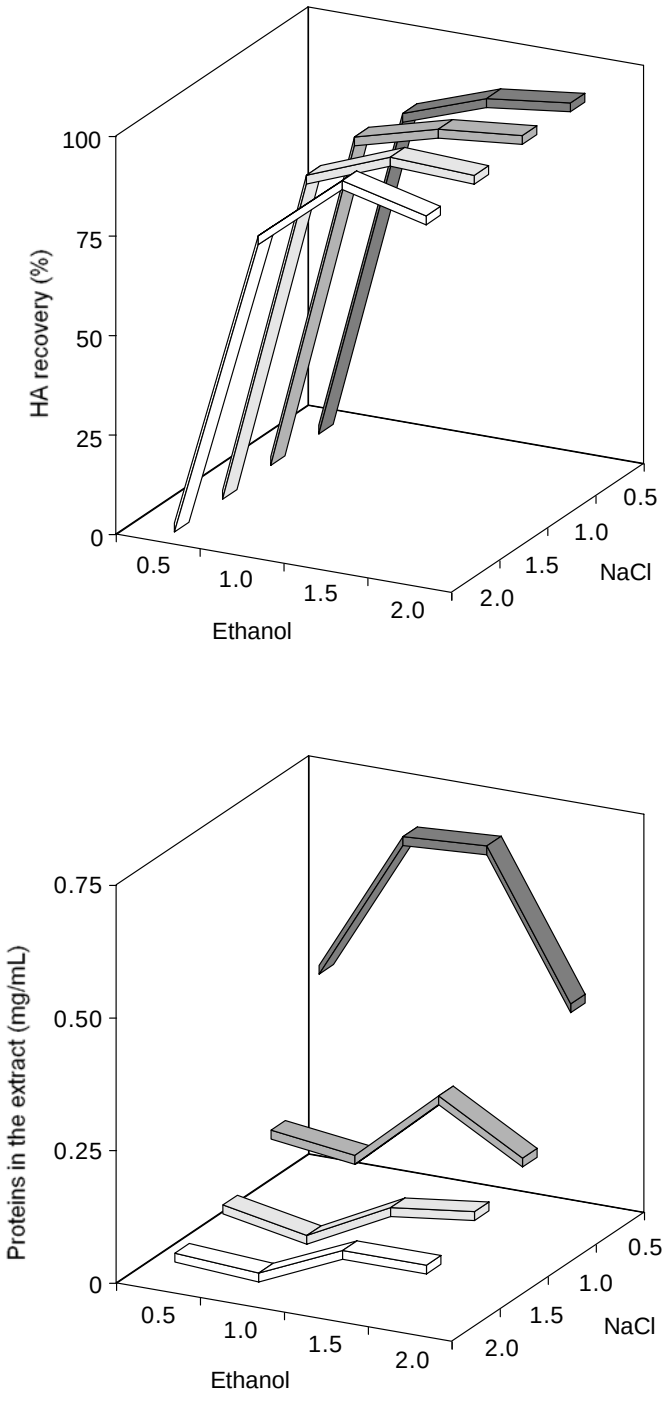

HA recovery (%)
100
75
50
25
0
0.5
1.0
1.5
2.0
Ethanol
0.5
1.0
1.5
2.0
NaCl
Proteins in the extract (mg/mL)
0.75
0.50
0.25
0
0.5
1.0
1.5
2.0
Ethanol
0.5
1.0
1.5
2.0
NaCl

FIGURE 2

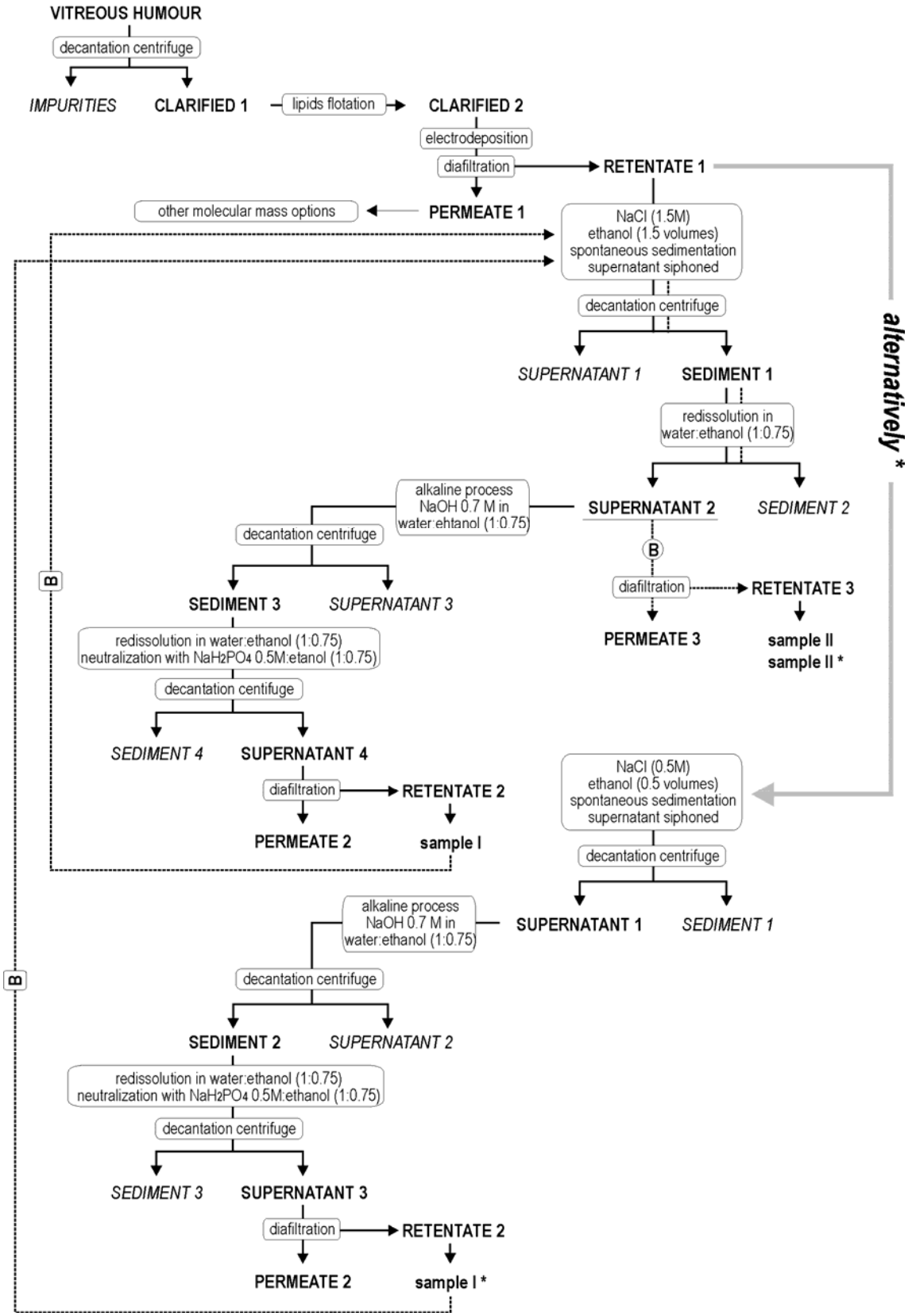

VITREOUS HUMOUR
decantation centrifuge
IMPURITIES
CLARIFIED 1
lipids flotation
CLARIFIED 2
electrodeposition
diafiltration
RETENTATE 1
other molecular mass options
PERMEATE 1
NaCl (1.5M)
ethanol (1.5 volumes)
spontaneous sedimentation
supernatant siphoned
decantation centrifuge
SUPERNATANT 1
SEDIMENT 1
redissolution in water:ethanol (1:0.75)
alkaline process
NaOH 0.7 M in water:ehtanol (1:0.75)
SUPERNATANT 2
SEDIMENT 2
decantation centrifuge
B
diafiltration
RETENTATE 3
SEDIMENT 3
SUPERNATANT 3
PERMEATE 3
sample II
sample II *
redissolution in water:ethanol (1:0.75)
neutralization with NaH2PO4 0.5M:etanol (1:0.75)
decantation centifuge
SEDIMENT 4
SUPERNATANT 4
diafiltration
RETENTATE 2
PERMEATE 2
sample I
alternatively *
NaCl (0.5M)
ethanol (0.5 volumes)
spontaneous sedimentation
supernatant siphoned
decantation centrifuge
alkaline process
NaOH 0.7 M in water:ethanol (1:0.75)
SUPERNATANT 1
SEDIMENT 1
decantation centrifuge
SEDIMENT 2
SUPERNATANT 2
redissolution in water:ethanol (1:0.75)
neutralization with NaH2PO4 0.5M:ethanol (1:0.75)
decantation centrifuge
SEDIMENT 3
SUPERNATANT 3
diafiltration
RETENTATE 2
PERMEATE 2
sample I *

FIGURE 3

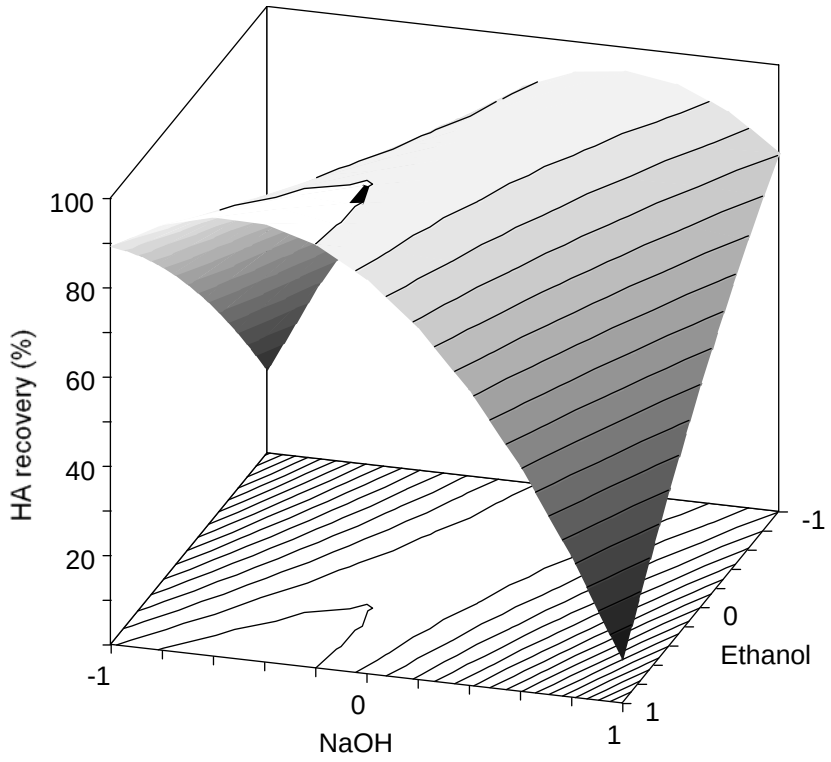
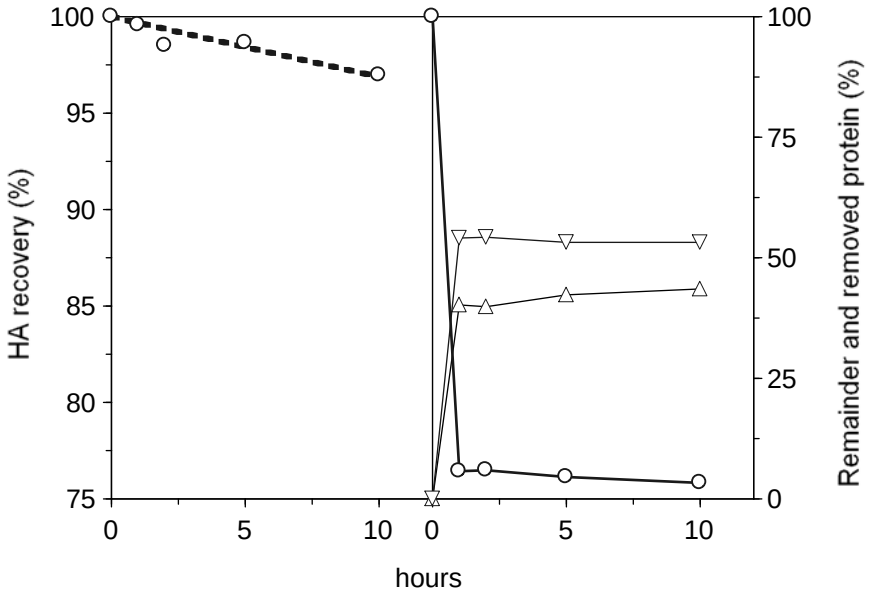


FIGURE 4


100
95
90
85
80
75
HA recovery (%)
0
5
10
0
5
10
hours
100
75
50
25
0
Remainder and removed protein (%)