1 **Optimization of extraction and purification process of hyaluronic acid from**

2 **fish eyeball.**

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18 **Headline:** Purification of hyaluronic acid from fish eyeball.

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ABSTRACT

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

INTRODUCTION

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

73 hydrolysates with ethanol leaves on hydroalcoholic solution a part of the hydrolysis products,

74 whereas the remains of the proteases are still distributed between the soluble fraction and the

75 sediment.

76

77 An example of the application of these properties, economically viable with rich sources, it is in

78 the patent of Cullis-Hill (1989) that improves other previous works and that it is based on the

79 recurrent execution of alcoholic precipitations and enzymatic hydrolysis, repeated as many times

80 as necessary to achieve the suitable purity. On the other hand, in this work ethanol is used with

81 2% (w/v) of acetic acid what develops the irreversible denaturalization of the proteins in the

82 precipitation steps.

83

84 Other interesting works that use technical of chromatographic separation (Kitagawa et al., 1990)

85 or reactions with salts of quaternary ammonium (Hildesheim, 1987) do not constitute, however,

86 precedent of the process that we are proposing. In same circumstances they are the procedures

87 that, still using ultrafiltration techniques (Yoshizawa, 1990), are applied in terms and with

88 concrete objectives different from those are presented in this article. According to our

89 knowledge, no works of extraction and purification of HA from vitreous humour of fishes have

90 been reported until now.

91

92 In the present study, a process for HA recovery and purification from vitreous humour of

93 selected species of fishes is described. Thus, a combination of steps of ultrafiltration-diafiltration

94 system, protein electrodeposition, selective resolubilization in hydroalcoholic medium and

95 selective precipitation in alkaline hydroalcoholic solution is studied and optimized.

96

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 $6,000 \times g$ 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 ($6,000 \times g$ 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 $6,000 \times g$ 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   $Z = b_0 + b_1 X + b_2 Y + b_{12} XY + b_{11} X^2 + b_{22} Y^2$ (1)

172

173 Statistical significance of the coefficients was verified by means of Student´s t-test ($\alpha<0.05$), and

174 model consistency by means Fisher´s F-test ($\alpha<0.05$) applied to following mean squares ratios:

175

	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	$F_4 \leq F_{den}^{num}$

176

177 Although it is a common practice to limit this test to the F_1 or F_1 and F_2 quotients, it should be

178 pointed out that F_3 and F_4 are essential to avoid the introduction of irrelevant variables or

179 variable combinations in this type of empiric models.

180

181 **RESULTS AND DISCUSSION**

182 *1: Initial diafiltration-concentration process*

183 Initially, clarified vitreous humour is diafiltrated using a system with total recirculation and a

184 value of dilution flow between the half and the third part of permeation flow. When two

185 membranes of cut-off at 675 and 100 kD are sequentially applied to a volume V_0, we can obtain:

186

187 a) A retentate from 675 kD with a lower volume than $V_0/10$, that it contains, at least, 75% of HA

188 total and approximately 16% of the initial protein.

189

190 b) A permeate with an approximate volume of $2V_0$ that is diafiltrated at 100 kD until a retentate

191 volume between $V_0/10$ and $V_0/15$. This retentate contains the 25% remaining and a 29% of

192 initial protein. The corresponding permeate, with the remaining protein fraction and without HA,

193 is rejected. Although the ratio protein/HA increases in these second retentates with regard to the

194 corresponding value in the raw material, the elimination of this protein in the subsequent steps is

195 slightly more efficient with retentates than using raw materials.

196

197   When a cut-off at 300 kD is only used, it is possible to achieve a retentate with an approximate

198   volume of $V_0/12$ that it contains 96% of HA and 46% of the initial protein.

199

200   *2: Protein electrodeposition*

201   It was carried out inserting electrodeposition device into the clarified vitreous humour and

202   establishing between both electrodes an initial electric current of 10 mA. This value was

203   gradually increased until 40 mA for 1 min and was maintained in this level for 30 min. As

204   consequence of this current step, in few seconds a deposit not very soluble in water and soluble

205   in NaOH 0.5M was formed in the anode. This precipitate produces a strongly positive reaction of

206   Lowry-proteins. The fact that the electrode washing with distilled water generates a suspension

207   with an approximate pH 4.2 suggests that the process implies the interchange of electrons

208   towards anode from carboxyl groups of the proteins with net negative charge (cathode reaction:

209    $H^+ + e^- = H$). These proteins precipitate when they approach to the isoelectric point. On the other

210   hand, the deposit detached from the electrode remains unsolubilized for at least one hour and it

211   can be removed by centrifugation. Though the effect of ferric metals in the breakdown of HA has

212   been repeatedly reported (Wong et al., 1981; Hawkins and Davies, 1996; Balogh et al., 2003),

213   the use of platinum electrodes did not affect to the proportions of HA retained at 300 and 675

214   kD.

215

216   The interest of the protein fraction so removed (approximately 0.3 g of protein per liter of

217   vitreous humour, with a current of 40 mA for 15 min) comes from its effects on the diafiltration

218   efficiency. Deposits obtained with same electric current values in raw vitreous humour and with

219   retentates from diafiltration at 675 kD reduced to a volume of $V_0/5$, indicating that these are non

220   filterable materials to this cut-off and contribute to increase transmembrane pressure with the

221   progress of the process. In fact, when electrodeposition begins after a time period of enough

222   diafiltration so that the permeate flow falls to 50% of the initial value, recoveries of this flow

223   until 90% of initial value are observed.

224

225   Therefore, electrodeposition can be carried out as a previous or simultaneous operation to the

226   diafiltration. Although in Faraday's laws the solute concentration implied in the electrode

227   reactions are not present in the mathematical equations, the protein amount deposited by unit of

228   time increases with the retentate concentration (data not shown). This effect is easily

229   understandable since the progress of the diafiltration eliminates chemical species of low

230   molecular mass, able to compete with non filterable proteins in the anode reaction. A previous

231   deposition process, followed or not by a centrifugation step, can be combined with diafiltration,

232   case in which a prefilter (*e.g.*, nylon mesh of 40-100 μm) should be used. In all cases, deposition

233   efficiency increases with a brief wash the anode in NaOH solution when accumulated protein

234   layer reduces the electric current to inadequate values.

235

236   *3: Alcoholic precipitation and selective recovery of the precipitate*

237   In Figure 1 the joint effect of ethanol and NaCl on retentates precipitation are depicted. This

238   response was evaluated by means of HA concentration and recovery proteins in extracts obtained

239   by redissolution of the corresponding sediments in water:etanol (1:0.75). The recovery of HA is

240   little affected by salt concentration and increases asymptotically with the proportion of ethanol.

241   However, high concentrations of both variables produces drops, slight but consistent, from a

242   maximum value of HA recovery. On the other hand, recovery of proteins, much more affected

243   by salt concentration, falls when salt concentration increasing at any considered level of ethanol.

244   Meanwhile, the response to the ethanol loses the asymptotic nature, falling from a maximum

245   when salt concentration decreases.

246

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): $[0.600 ; 0.900] \rightarrow [-1;1]$

274

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

276

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

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      $$HA = 100 \cdot \exp(-0.00317 \cdot t) \qquad (3)$$

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

298   Finally, the extracts from alkaline process can be treated by diafiltration at convenient cut-off

299   membrane to achieve simultaneously the HA concentration and the phosphate dilution required.

300   Furthermore, the soluble proteins remainders, to concentrations in the range of 0.02-0.04 mg/mL,

301   as well as the salts are efficiently removed in this step. If an ulterior purification is still required,

302   it can return to the selective redissolution of the alcoholic precipitate (Figure 3) in similar terms

303   to those described previously.

304

305   ***5: Testing the proposal methodology***

306   *Example 1*

307   A volume of 2.5 L of swordfish humour vitreous (*Xiphias gladius*) clarified by centrifugation

308   was undergone, under soft shaking, to electric current of 40 mA with platinum electrodes.

309   Anode, a mesh of 3×0.5 cm, was washed after 10 min by immersion in NaOH 0.5N and this

310   operation was repeated twice before diafiltration beginning. Table 2 shows the main parameters

311   of the process step that are described next.

312

313   Diafiltration was carried out using membrane of cut-off at 300 kD with total recirculation

314   assembly and nylon mesh of 60 μm using as prefilter. Pressure and dilution flow with distilled

315   water were maintained constant at 40-50 psi and with a 50% of permeation flow, respectively.

316   Electric current of 40 mA was also applied for 8 periods of 15 min. The operation was

317   interrupted one time for washing the membrane (15 min with NaOH 0.1M at 45ºC without

318   pressure) and prefilter and it was maintained until to reach a retentate volume of 260 mL.

319

320   In 250 mL of retentate, 21.9 g of NaCl (1.5 M) were dissolved. Subsequently, 375 mL of 99%

321   ethanol were slowly added under magnetic and intense shaking at 5ºC. This agitation system was

322   maintained for 30 min and it was afterwards left in rest to the same temperature overnight. In

323   these conditions, compact sediment and a clarified and rejected supernatant of 425 mL were

324   obtained. The sediment was mixed with 60 mL of water:ethanol (1:0.75) under vigorous

325   agitation, until to get a fine and homogeneous suspension that it was centrifuged (6,000 × g for

326   15 min), recovering now the supernatant. This last sediment was washed with 20 mL of

327   water:ethanol (1:0.75) joining the corresponding supernatant with the previous one.

328

329   75 mL from the whole of the supernatants were mixed with NaOH 0.56M in water:ethanol

330   (1:0.9) solution. After 2 h of intense agitation at 5ºC, the mixture was centrifuged (6,000 × g for

331   15 min) at the same temperature being discarded the supernatant. The sediment was redissolved

332   in a total volume of 40 mL with water:ethanol (1:0.75) and the aqueous solution of NaH_2PO_4

333   0.5M:ethanol (1:0.75) necessary for obtaining a pH-value of 7.25. The redissolution was

334   centrifuged (6,000 × g for 15 min), the supernatant was recovered and the sediment was washed

335   with early solutions until similar pH-value, gathering both supernatants.

336

337   Finally, an aliquot of 57 mL from the supernatants were diafiltrated at 100 kD until to obtain 30

338   mL of retentate with the characteristics specified in Table 3. In this retentate, the molecular

339   weight of HA was 1600 kD.

340

341   *Example 2*

342   A volume of 2.5 L of shark vitreous humour (*Prionace sp.*) was perfomed in similar way to

343   example 1 with the differences in the work volumes and HA and proteins concentrations that are

344   specified in Table 3. It should be pointed out that the most advantageous ratio protein:AH is not

345   only translated in a final extract of more volume, concentration and purity, but also in a faster

346   and more efficient process. In this case, the molecular weight of HA in the final solution was

347   2000 kD.

348

349   *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)/ΔVn   Decodification: Vn = Vo + (ΔVn$\times$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: $Purity\,(\%) = \dfrac{HA\ concentration}{HA\ concentration + Pr\,otein\ concentration} \times 100$
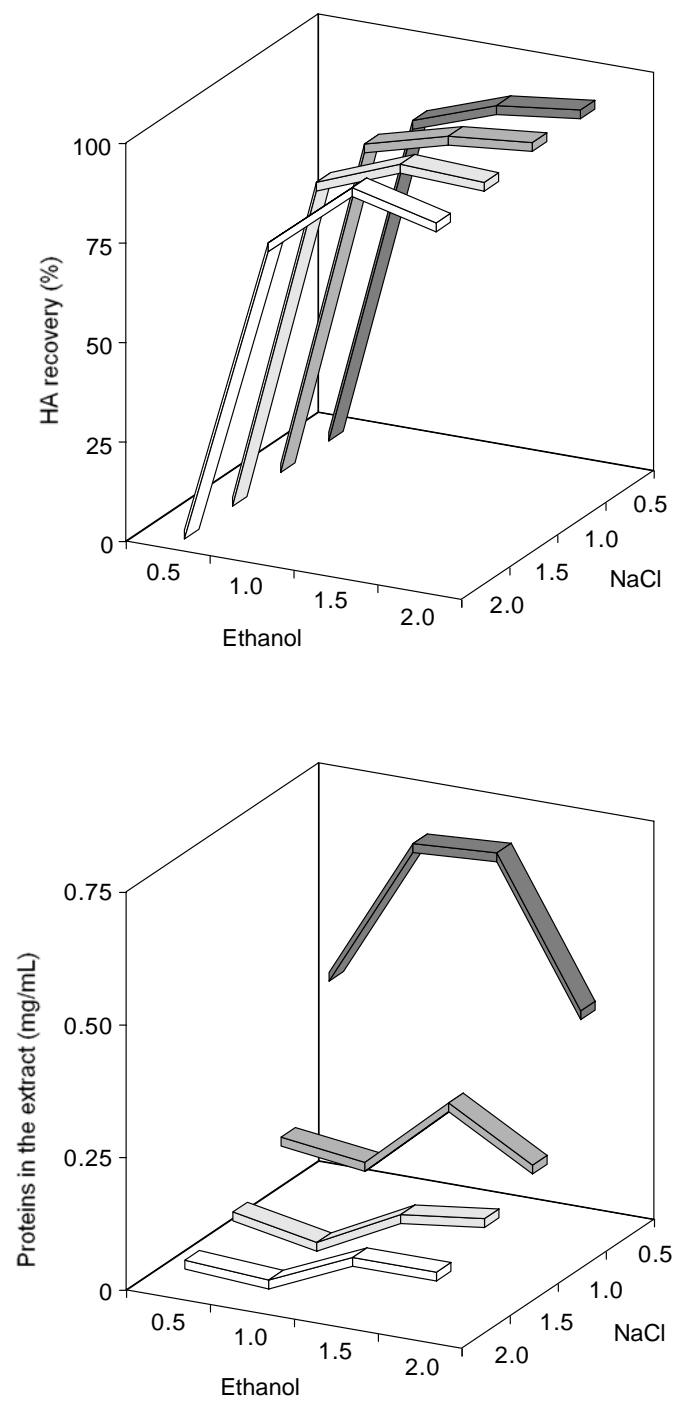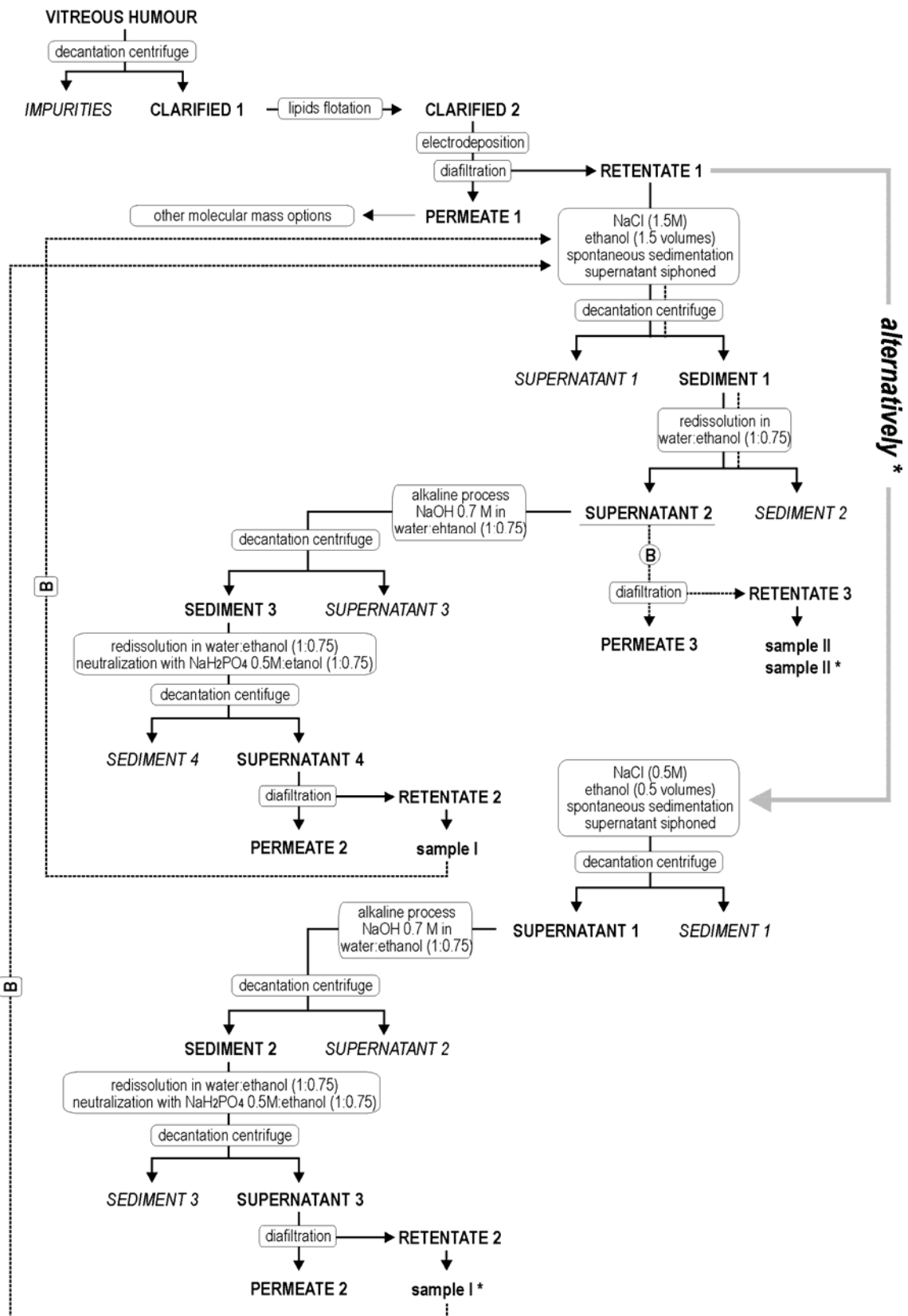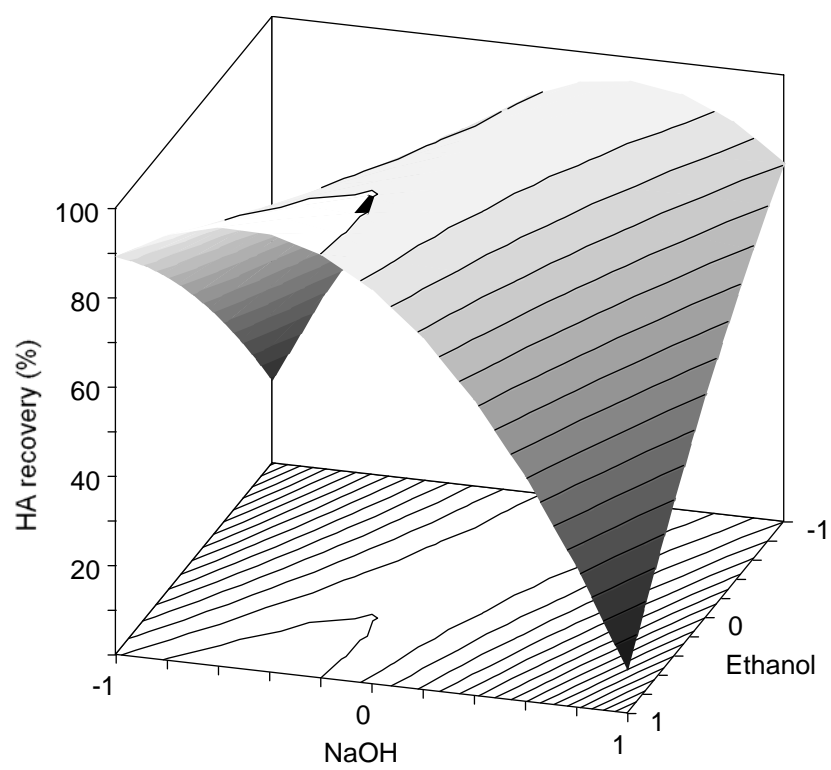
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4