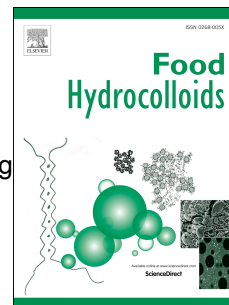# Accepted Manuscript

Insoluble soybean polysaccharides: Obtaining and evaluation of their O/W emulsifying properties

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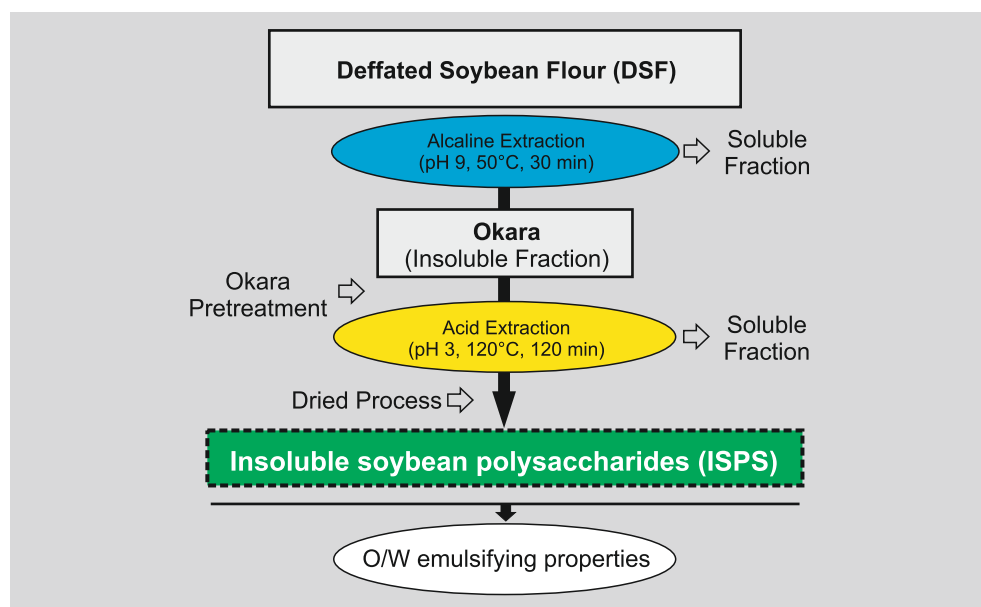
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Insoluble soybean polysaccharides: Obtaining and evaluation of their O/W

emulsifying properties

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22 **Abstract**

23 The aims of this work were to obtain different samples of insoluble soybean polysaccharides (ISPS)

24 from defatted soy flour and to study their potential application as O/W emulsifier. In this regard, the

25 insoluble residue (okara) resulting from an aqueous extraction (60ºC, pH 9.0), was submitted to an

26 acidic extraction (pH 3.5, 120℃) without or with a pretreatment (high pressure homogenization or

27 sonication treatment). The insoluble residues of these extractions were dried (oven, 70℃ or

28 vacuum post-treatment with 2-propanol, 40℃) yielding different ISPS samples. Aqueous

29 dispersions of ISPS samples (1-2% w/w, pH 3 and 7), were used to prepare coarse and fine O/W

30 emulsions. Emulsion stability against creaming and coalescence processes, and the rheological

31 behavior were analyzed. ISPS samples obtained by okara pretreatment and vacuum dried post-

32 treatment with 2-propanol allow to produces emulsions with high values of flocculation degree,

33 increasing the stability of the particle size, and allowing the formation of stronger gel-like emulsions.

34 These pretreatments expose internal sites of the polysaccharide and protein structures, increasing

35 their superficial hydrophobicity and, therefore, allow a strong absorption of the macromolecules at

36 the oil-water interface and/or the formation of external layers, increasing the rigidity of the interfacial

37 film and contributing to the formation of hydrated flocs, Also, these treatments could solubilize

38 certain compounds in okara that would interfere negatively in the formation of the interfacial film.

39 Particularly, sample obtained by high pressures homogenization of the okara presented the best

40 emulsifying properties and it was not significantly affected by variations in the pH of the emulsion.

41 The results of this research work demonstrate a high potential of application of the ISPS samples as

42 O/W emulsifier, under acid and neutral conditions, increasing the added value of an important by-

43 product of the soybean industry.

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47   1. Introduction

48   Proteins and polysaccharides play a key role in the structure formation and stabilization of oil-in

49   water (O/W) emulsions. Proteins are commonly used as emulsifiers due to its ability to prevent

50   droplet aggregation and coalescence (Dickinson, 2009; Phillips & Williams, 2001). On the other

51   hand, polysaccharides are often incorporated to stabilize the emulsion droplets against gravitational

52   separation by increasing the viscosity of the aqueous phase or by the formation of a gel (Chuah,

53   Kuroiwa, Kobayash, & Nakajima, 2009; Liu, Verespej, Alexander, & Corredig, 2007). Various

54   studies report that polysaccharides may also interact with protein adsorbed at the interface

55   enhancing the emulsion stability. In this way, only polysaccharides with sufficient hydrophobic

56   character to adsorb strongly at the oil-water interface or those directly complexed with proteins are

57   capable of making and stabilizing O/W emulsions (Akhtar, Dickinson, Mazoyer, & Langendorff,

58   2002; Evans, Ratcliffe, & Williams, 2013; Neirynck et al., 2007).

59   Soybean proteins are widely recognized for their low price, high nutritional quality and versatile

60   functional properties (Wang, Johnson, & Wang, 2004). The first step in the production of protein

61   isolate is an extraction under alkaline conditions (pH 7.5–9, up to 80°C) (Pearson, 1983; Berk,

62   1992). The insoluble residue left after this alkaline extraction is called 'okara'. Most okara is treated

63   as an industrial waste, because it contains about 80% moisture and spoils quickly (Maeda, 2000).

64   However, this by-product has various components that can be isolated in fractionating steps to

65   produce high-value products such as proteins and carbohydrates that can be used as food

66   additives. In fact, the soluble fraction obtained by heating okara in weak acidic conditions (pH 3-3.5,

67   120°C) allows the extraction of the soluble soybean polysaccharides (SSPS) (Nakamura, Fujii,

68   Tobe, Adachi, & Hirotsuka, 2012). SSPS has various functions, such as dispersion, stabilization,

69   emulsification, and adhesion (Maeda, 2000; Porfiri, Cabezas, & Wagner, 2016). Furthermore, this

70   acid extraction generates an insoluble fraction containing a complex polysaccharide with high

71   protein content. There is no specific literature about this fraction, named insoluble soybean

72   polysaccharides (ISPS) in the present research.

73 In recent years, the concepts of applying modification by mechanical treatments in carbohydrates

74 polymers research have gained much attention. For example, high hydrostatic pressure treatments

75 are used to break binding forces (hydrogen bridges and hydrophobic forces) of the highly branched

76 regions in pectin materials without the degradation of the main chain (covalent bonds) (Michel, &

77 Autio, 2001; Chen et al., 2012). Moreover, the exerted shear stresses of a sonication process are

78 large enough to alter the molecular conformation of the polysaccharide and/or to disrupt entangled

79 structures (Ogutu, Mu, Elahi, Zhang, & Sun, 2015). These mechanical treatments can not only alter

80 the particle size and surface area of these polymers but also improve their functional properties

81 (Chen, Gao, Yang, & Gao, 2013).

82 The aims of this research were to evaluate and compare the chemical composition and the O/W

83 emulsifying properties of ISPS samples obtained under different extraction conditions.

84 2. Materials and methods

85 2.1 Material

86 Defatted solvent-free soy flour (DSF), prepared under controlled conditions (not thermally inactived

87 to avoid protein denaturation), was provided by Terminal 6 S.A. (Santa Fe, Argentina). Refined

88 sunflower oil (Molinos Cañuelas, Argentina) was purchased in a local supermarket. The chemical

89 reagents used in this work were of analytical grade.

90 2.2 Obtaining samples of insoluble soybean polysaccharides

91 2.2.1 Preparation and treatment of the okara

92 Defatted soybean flour was ground to a particle size lesser than 150 μm. ISPS was prepared by

93 adapting the extraction method of Porfiri et al. (2016). The defatted soy flour was extracted by

94 adding 11 times the weight of distilled water, adjusting the dispersion to pH 9.0 with 5M NaOH, and

95 gently stirring during 30 min at 60ºC. The residue obtained after centrifugation (7000 ×g, 4℃, 15

96 min), called *okara* (Furuta, Takahashi, Tobe, Kiwata, & Maeda, 1998), was suspended in distilled

97 water at a concentration of 25.0 % w/w and adjusted at pH 3.5 with 38% HCl. As shown in Figure 1,

98 portions of okara dispersion were subjected separately to the following homogenization processes:

99 I) High pressure valve homogenization: dispersion was subjected to a treatment in a two-valve high

100 pressure homogenizer in 3 cycles, at 1000 bar and 100 bar in the first and second valve,

101 respectively (GEA Niro Soavi).

102 II) High intensity ultrasonic homogenization: dispersion was subjected to a treatment in a probe-

103 type ultrasonic homogenizer (Sonics Vibra Cells- 7070 J, 75% power, 3 minutes -30'' on, 15'' off-).

104 2.2.2.1 Particle size determination of okara dispersions

105 De Brouckere (D[4,3]) mean diameters of particles of the okara dispersions, without and with

106 previous homogenization treatment, were determined by laser diffraction with a particle size

107 analyzer (Malvern Mastersizer 2000E, Malvern Instruments Ltd., Worcestershire, U.K.). Refractive

108 indexes of 1.33 and 1.45 were used for water (dispersant) and okara particles, respectively (Preece

109 et al., 2015). Samples were diluted in the water bath of the dispersion system (Hydro 2000MU).

110 2.2.2 Obtaining and drying procedure of acid insoluble fractions

111 Okara dispersions, without and with previous homogenization treatment, were heated in autoclave

112 at 120°C for 2 h. After that, they were centrifuged at 7000×g at 4°C for 15 min and wet insoluble

113 fractions were obtained. These fractions were dried by the following procedures:

114 a) Forced-air drying oven at 70° ± 2°C to a constan t weight, obtaining the samples: ISPS-U-O

115 (untreated okara, oven dried), ISPS-V-O (valve homogenized okara, oven dried) and ISPS-S-O

116 (sonicated okara, oven dried) (Figure 1).

117 b) Vacuum drying post-treatment with 2-propanol (Kalapathy & Proctor, 2001). The high moisture

118 content (> 87%, w/w) of the wet insoluble fractions did not allow the use of a vacuum drying oven

119 because of the high processing times required without a previous moisture reduction. Therefore, in

120 order to reduce these moisture content, the insoluble fractions were dispersed in an equal volume

121 of 2-propanol, the pH was adjusted to 3.5 and allowed to settle for 4 hs. The pellet was collected,

122 centrifuged, dispersed in 70% 2-propanol, stirred for 30 min and centrifuged. The washing was

123 repeated with 100 % 2-propanol. The precipitate was dried in a vacuum drying oven at 40°C

124 (pressure less than 50 mm Hg). This process allows obtaining the samples: ISPS-U-P (untreated

125 okara, treated with propanol), ISPS-V-P (valve homogenized okara, treated with propanol) and

126 ISPS-S-P (sonicated okara, treated with propanol) (Figure 1).

127 2.2.3 Okara and ISPS samples yields

128 Yields (w/w %) were calculated as the weight of dried okara or ISPS sample obtained with 100

129 grams of DSF (Equation 1). The okara dispersion without previous homogenization was oven-dried

130 at 70°C to obtain the dried okara sample.

131 Yield (%)= (Okara or ISPS sample produced (g) / Initial weight of DSF (g)) x 100   (1)

132 2.3 Chemical composition

133 The proportions of neutral monosaccharides were determined after hydrolysis with pure

134 trifluoroacetic acid (90 min, 120°C), in order to d etect cellulosic and fiber materials (Morrison, 1988).

135 Hydrolyzates were derivatized to the alditol acetates (Albersheim, Nevins, English, & Karr, 1967)

136 and analyzed by gas-liquid chromatography (GLC) using a capillary column (30 m x 0.25 mm)

137 coated with SP-2330 (0.20 mm) on a HP-5890 Gas Chromatograph equipped with a flame

138 ionization detector (FID). Nitrogen was used as the carrier gas, with a flow rate of 1 ml/min and a

139 split ratio of 100:1. Chromatography runs were programmed starting at 200°C, 2°C/min to 230°C

140 (hold for 20 min), while the injector and detector were set at 240°C. The percentage of the different

141 monosaccharides was calculated by considering that the FID responses are proportional to the

142 molecular weight of the alditol acetates. Every hydrolysis step was carried out in duplicate.

143 Uronic acid contents of the sample were determined by a colorimetric method using m-

144 hydroxydiphenyl, and expressed as galacturonic acid (GalA) (Blumenkrantz, & Asboe-Hansen,

145 1973). Degree of esterification was expressed as percent methoxy groups as determined by a

146 titration method (Lira-Ortiz et al., 2014).

147 The protein content of samples was determined by Micro-Kjeldahl (N$\times$ 6.25) using a colorimetric

148 procedure for determining the nitrogen content calibrated with ammonium sulphate (Nkonge, &

149 Balance, 1982). Total carbohydrates were determined by difference. The lipid amount was

150 negligible given the process for obtaining the soybean flour used as starting material.

151 2.4 FTIR analysis

152 Infrared spectra of the ISPS samples were registered in the range of 850 to 2150 cm^{-1} on a Fourier-

153 Transform Infrared Analyzer (FTIR) Shimadzu IR-Affinity (Shimadzu Co., Japan) equipped with an

154 attenuated total reflectance diamond module (GladiATR, Pike Technologies, USA). IR spectra were

155 measured as an average of 45 scans, a resolution of 4.0 cm^{-1}.

156 2.5 Preparation of aqueous dispersions of ISPS samples

157 Aqueous dispersions were prepared by dissolving the ISPS samples (1.0 and 2.0 w/w) in 8 mM

158 sodium citrate buffer (pH 3.0 and 7.0), with the addition of 0.02% w/v sodium azide to retard the

159 microbial growth. To promote dispersion, samples were allowed to stand overnight and

160 subsequently were stirred (25℃, 50 rpm, 30 min) us ing a magnetic stirrer.

161 2.6 Surface and interfacial tension

162 ISPS samples (0,1 g protein/ml) were dispersed in 8 mM sodium citrate buffer (pure water, pH 3.0

163 and 7.0). These dispersions were left overnight at room temperature to fully hydrate, and then

164 mixed by using an Ultraturrax T-25 homogenizer using a 25 N-10 G dispersing tool (10,000 rpm, 2

165 min, IKA Labortechnik GmbH & Co, Germany). The du Noüy ring (platinum ring) method was used

166 to determine the surface (air – aqueous phase) and interfacial (sunflower oil air - aqueous phase)

167 tensions of each dispersion at 25 ℃ using a LAUDA Ring/Plate Tensiometer model TD (Lauda Dr.

168 R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany). As a result of adsorption of the

169 surface active compounds, the surface (or interfacial) tension decreased from the value for the

170 clean interface $\gamma 0$ to a value γ. Therefore, the interfacial and surface pressure at equilibrium (π^i_e and

171 π^s_e, respectively, where i stands for interfacial and s represents surface) were calculated as:

172 $\pi^i_e = \gamma^i 0 - \gamma^i$ (mN/m)   (2)

173 $\pi^s_e = \gamma^s 0 - \gamma^s$ (mN/m)   (3)

174 where the symbols 0 and e represent the initial and equilibrium stages, respectively.

175 2.7 Interfacial rheology

176 Rheological properties of the oil-water interface generated by the ISPS samples were studied by

177 using an AR-G2 rheometer (TA Instruments, New Castle, DE) equipped with a du Noüy ring

178 (platinum ring) geometry. For this, 30 ml of aqueous dispersion of 0.5% of ISPS (8 mM sodium

179 citrate buffer, pH 3.0 and 7.0) were placed in a beaker (6.7 cm in diameter) and the ring was

180 lowered to make contact with the surface. In order to increase the repeatability the gap was zeroed

181 and kept constant at the position of 10900 μm. The same volume of sunflower oil was carefully

182 placed above the aqueous phase, generating an oil-water interface. Oscillatory shear

183 measurements were conducted at constant frequency of 0.1 Hz and strain of 5%, which was

184 measured to be within the linear viscoelastic regime. The temperature was set to 25 ºC and the

185 experimental data were obtained by recording G´ and G´´ as a function of time immediately after

186 interface generation (Cabezas, Pereira Ortiz, Wagner & Porfiri, 2017).

187 2.8 Preparation of the O/W emulsions

188 2.8.1 Coarse O/W emulsions

189 Refined sunflower oil (oil mass fraction, $\Phi_m = 0.3$) and the different ISPS aqueous dispersions in a

190 range of 1.0–2.0 % w/w were used to prepare emulsions. Coarse emulsions were prepared at room

191 temperature in an Ultraturrax T-25 homogenizer using a 25 N-10 G dispersing tool (25,000 rpm, 2

192 min, IKA Labortechnik GmbH & Co, Germany) (Cabezas, Madoery, Diehl, & Tomas, 2012). The

193 behavior of these emulsions as a function of the storage time was analyzed for 90 min.

194 2.8.2 Fine O/W emulsions

195 Coarse emulsions previously obtained with 2% of different ISPS samples (ISPS-U-P, ISPS-V-P and

196 ISPS-S-P) were homogenized in a two-valve high pressure homogenizer (400 bar, 1 cycle, GEA

197 Niro Soavi). The behavior of these fine emulsions as a function of the storage time was analyzed for

198 28 days.

199 2.9 Evaluation of the emulsifying properties of the ISPS samples

200 2.9.1 Particle size determination of emulsions

201 De Brouckere (D[4,3]) mean diameters of particles were determined by laser diffraction with a

202 particle size analyzer (Malvern Mastersizer 2000E, Malvern Instruments Ltd., Worcestershire,

203 U.K.). D[4,3] are more sensitive to an increment of droplet size than Sauter mean diameters (D[3,2])

204 (Cortés-Muñoz, Chevalier-Lucia, & Dumay, 2009). The refractive indexes for water and sunflower

205 oil were 1.33 and 1.47, respectively. Samples were diluted in the water bath of the sample

206 dispersion unit (Hydro 2000MU).

207 Furthermore, D[4,3] were measured after 2 min of sonication at 40 W with the ultrasonic probe of

208 the dispersion system. The purpose of this treatment was break down the flocs that would be

209 present in the emulsions (Porfiri et al., 2016). Subsequently, flocculation degree (FD) and

210 coalescence index (CI) were analyzed. The FD was calculated by Equation (2):

211 $$FD = \frac{D[4,3] - D[4,3]_{sonic}}{D[4,3]_{sonic}} \qquad (4)$$

212 Where D[4,3] and D[4,3]$_{sonic}$ are the volume-weighted diameters, measured before and after the

213 sonication treatment, respectively.

214 The CI of coarse and fine emulsions were calculated by Equation (3) and (4), respectively:

215 $$CI = \frac{D[4,3] - D[4,3]_{initial}}{D[4,3]_{initial}} \times 100 \qquad (5)$$

216 Where D[4,3]$_{initial}$ and D[4,3] are the volume-weighted diameters measured in initial and stored (90

217 minutes) emulsions, respectively.

218 $$CI = \frac{D[4,3]_{sonic} - D[4,3]_{sonic,initial}}{D[4,3]_{sonic,initial}} \times 100 \qquad (6)$$

219 Where D[4,3]$_{sonic,initial}$ and D[4,3]$_{sonic}$ are the volume-weighted diameters measured in initial and

220 stored (28 days) emulsions after the sonication treatment, respectively.

221 2.9.2 Creaming kinetics of the coarse O/W emulsions

222 Coarse O/W emulsions tend to separate a "serum" lower layer and a "creamed" upper layer. The

223 kinetics of creaming of these emulsions was determined by manually measuring the change in the

224 height of the serum layer over time using 10 ml graduated and transparent cylinder (Mc Clements,

225 1999). The percentage of serum layer is then given by the Equation (5):

226 $\% \ Serum \quad layer = \dfrac{V_{Sl} - V_E}{V_E} \times 100$ (7)

227 Where V_E is the volume of the emulsion in the graduated cylinder and V_{Sl} is the volume of the

228 serum layer

229 2.9.3 Optical characterization of fine O/W emulsions

230 The destabilization process of the fine emulsions was analyzed from the backscattering (%BS)

231 profiles of each emulsion obtained during 28 days at 24°C (Cabezas et al., 2012). The %BS was

232 measured using a vertical scan analyzer (QuickScan, Beckman-Coulter, USA).

233 2.9.4 Light Microscopy

234 Micrographs of the emulsions were obtained after being diluted 10 times with an optical microscope

235 operating at 400 × magnification and fitted with a digital camera (Canon A570 IS; Malaysia) at 4×

236 optical zoom.

237 2.9.5 Rheology

238 Oscillatory rheology of the fine emulsions was studied using an AR-G2 rheometer (TA Instruments;

239 New Castle, DE, USA) with parallel-plate geometry (gap 1000 μm, diameter 40 mm). Temperature

240 (24°C) was controlled with a water bath (Julabo ACW 100, Julabo Labortechnik, Germany)

241 associated with the rheometer. Experimental data were obtained by recording the storage or elastic

242 modulus (G') and the loss or viscous modulus (G'') as a function of oscillation frequency (0.1-100

243 Hz range) within the linear viscoelasticity range previously determined by stress-sweeps (strain 1

244 %).

245 2.9.6 Statistical analysis

246 Data were evaluated using analysis of variance (ANOVA) with the Statgraphics Centurion XV

247 software (StatPoint Inc. 2005, USA). Assays were conducted at least in triplicate. Least significance

248 difference (LSD) values were used to differentiate mean values, and significance as defined at

249 $P < 0.05$.

250 3. Results and Discussion

251 3.1 Yields and chemical composition of okara and ISPS samples

252 The extraction yields and the chemical analysis of the okara and the six ISPS samples were

253 determined (Table 1). Okara has a high content of carbohydrates and proteins, which are insoluble

254 in alkaline conditions. Subsequently, this product was subjected to an acid extraction (2 hours, pH

255 3, 120℃) allowing obtaining six samples of insoluble soybean polysaccharides (ISPS) under

256 different conditions (Figure 1). According to previous works on soybean meal, this intense heating

257 process (120℃, 2h) would dissociate the quaternary structures of the protein and denatures their

258 subunits (Zhang et al., 2013). ISPS samples have higher protein content than okara due to the

259 solubilization of the soybean soluble polysaccharides under acid condition at high temperature and

260 pressure (Furuta et al., 1998). ISPS yield was lower in samples drying post-treatment with 2-

261 propanol with respect to the oven dried ones. This may be due to the differential solubility of

262 different components in the alcoholic and aqueous media, allowing to eliminate low molecular

263 weight polysaccharide or peptides fragments. Furthermore, it was noted that fractions without a

264 pretreatment of okara (ISPS-U) had a higher extraction yield, mainly those that were dried in an

265 oven (Table 1). The lower yield of the ISPS-V and ISPS-S samples indicates that both

266 pretreatments (valve homogenization and sonication) increased the percentage of soluble

267 compounds in acid media during the autoclaving treatment, mainly proteinaceous material. This

268 observation would be related to the weakening or rupture of non-covalent bonds (Van der Waals

269 forces, hydrogen bridges and/or hydrophobic forces) of polysaccharide/protein aggregates by the

270 high pressure (Mozhaev, Heremans, Frank, Masson, & Balny, 1996) or ultrasound waves

271 treatments to the okara (Wang, Cheung, Leung, & Wu, 2010). The downsizing of the structures was

272 evident in the smaller De Brouckere (D[4,3]) mean diameters of the valve homogenized (29.1 ±

273 0.3 μm) or sonicated (34.4 ± 1.1 μm) okara-dispersions in relation to the untreated okara (64.8 ± 0.8

274 μm).

275 FTIR spectra presented the characteristic bands of the protein region, particularly amide I (AI, C-O

276 stretching, ~1636 cm^{-1}) and amide II (AII, N-H deformation, ~1537 cm^{-1}) and the bands

277 corresponding to carbohydrate region (900-1200 cm^{-1}). FTIR spectra show that the intensity of

278 these spectral bands was substantially modified as a consequence of the homogenization

279 processes (Figure 2). In this sense, it was observed an increase of the band intensities of the ISPS

280 samples obtained with a previous homogenization treatment (ISPS-V and ISPS-S), respect to that

281 obtained without pretreatment (ISPS-U). In contrast to the previously mentioned, these untreated

282 samples presented higher protein content than the homogenized samples under both drying

283 methods. This behavior would confirm the rupture of non-covalent bonds of the

284 polysaccharide/protein aggregates by the high pressure and ultrasound waves. These processes

285 allow to expose internal sites of the polysaccharide and protein structures, modifying characteristics

286 of the ISPS samples as their superficial hydrophobicity and, therefore, their functional

287 characteristics. On the other hand, the intensity of the different bands of the oven-dried samples

288 (ISPS-O) were significantly lower than those vacuum-dried samples post-treatment with 2-propanol

289 (ISPS-P). The oven-dried treatment considerably reduces the unfolding effect on the molecular

290 structures of the different homogenization processes. According to previous works, a decrease of

291 the absorbance over the entire spectrum would be associated with an aggregation process

292 (Ingrassia, Palazolo, Risso, & Wagner, 2016). The sugar compositions of the okara and the six

293 ISPS samples are detailed in Table 1. A lower content of galactose and arabinose, and a higher

294 content of mannose and glucose are observed in the ISPS samples than those observed in okara.

295 This observation is due to the acid extraction of the soluble soybean polysaccharide (SSPS) which

296 mainly consists of galactose and arabinose (Nakamura, Furuta, Kato, Maeda, & Nagamatsu, 2003).

297 Furthermore, although significant differences were observed to the sugar composition a common

298 polysaccharide matrix in the different ISPS samples would be identified. These fractions were

299 composed mainly of glucose (> 42%), galacturonic acid (> 20%) and galactose (> 13%). The high

300 glucose content can be related to residues of cellulose present in the starting material, and different

301 hemicelluloses described in the literature as part of the cotyledon (arabinogalactan, glucomannan

302 and xyloglucan) (O'Toole, 2004; Redondo-Cuenca, Villanueva-Suárez, & Mateos-Aparicio, 2008).

303 The insolubility of the samples in alkaline and acid conditions could infer the presence of

304 carbohydrates covalently attached to proteins in a glycoprotein matrix, for example forming

305 arabinogalactan-proteins and glucomannan-proteins (Aspinall, & Whyte, 1964; Fincher, Stone, &

306 Clarke, 1983). This matrix, as observed in different gums and pectins, can lead surface-active

307 compound potentially applicable in the formulation of emulsions (Dickinson, 2009).

308 3.2 Evaluation of the emulsifying properties of the ISPS samples

309 3.2.1 Particle size determination of O/W coarse emulsions

310 The particle size analysis (D[4,3]) allowed to evaluate the destabilization by coalescence of the

311 different emulsions (Figure 3). Oven-dried samples (ISPS-O, Figure 3.a, b, c) generally presented

312 smaller particle sizes than those dried post-treatment with 2-propanol (ISPS-P, Figure 3.d, e, f),

313 however, this difference was considerably reduced by increasing the concentration of the different

314 emulsifiers to 2%. It should be noted that at this concentration ISPS-P samples were in all cases

315 more stable against coalescence than ISPS-O samples with values of CI<3, and a similar behavior

316 at the different pH values.

317 Particles higher than 80 μm in emulsions with 1% of fractions dried post-treatment with 2-propanol

318 could be generated by the bridging flocculation mechanism. This mechanism loses effect by

319 increase the fraction concentration, however, it may be causing the difference in particle size

320 between the ISPS-O and ISPS-P emulsions. The size of individual droplets and the flocculation

321 degree could not be determined applying low energy sonication with the ultrasonic probe of the

322 dispersion system. This methodology was not possible to use in coarse emulsions given that the

323 energy applied caused emulsion destabilization, mainly due to the tendency to coalesce of the

324 higher droplets (Porfiri et al., 2016). However, the presence of flocs was probed through the

325 microscope (data not shown).

326 3.2.2 Creaming stability of O/W coarse emulsions

327 Stability against this process increased for ISPS samples obtained from pretreated okara (ISPS-V,

328 ISPS-S), particularly those dried post alcohol precipitation. In this sense, the oven dried samples

329 produces emulsions with a final serum layer higher than 32% and 17% at concentrations of 1% and

330 2%, respectively (Figure 4.a, b, c). In contrast, samples dried post-treatment with 2-propanol (ISPS-

331 P) showed less than 15% of final serum layer at addition of 2% (Figure 4.d, e, f). Particularly,

332 emulsions prepared with ISPS-V-P provided a serum layer of 4.2% at pH 3 and was not detectable

333 at pH 7 (Figure 4.e). As it was previously suggested according the compositional characterization of

334 the ISPS, both homogenization processes and the alcoholic treatment expose internal sites of the

335 polysaccharide and protein structures, increasing their superficial hydrophobicity and, therefore,

336 allow a strong absorption of the macromolecules at the oil-water interface. In addition, the

337 homogenization treatments could solubilize certain compounds in okara as low molecular weight

338 polysaccharide or peptides fragments. These compounds would generate a competitive adsorption

339 with ISPS in the interface during the emulsification process.

340 On the other hand, emulsions formulated at acidic and neutral conditions showed no significant

341 differences using the ISPS-P samples at 2% as emulsifying agents, demonstrating versatility at

342 different pH environments.

343 3.2.3 Selection of dispersions

344 The analysis of the coarse emulsions allowed selecting the ISPS dispersions with potential

345 application as emulsifying agents. Conditions that generated coarse emulsions with greater stability

346 against the coalescence and creaming processes were selected. In this regard, fine emulsions were

347 prepared with the ISPS-P samples in a concentration of 2%.

348 3.2.4 Functional characterization of the selected ISPS dispersions

349 The effects of the ISPS samples on surface (π^s_e) and interfacial (π^i_e) pressure at equilibrium are

350 shown in Table 2. All of the dispersions examined showed an increase in these values. In these

351 sense, although significant differences were observed in the measurements, the nominal values of

352 these parameters demonstrate a similar activity of the three ISPS samples in the analyzed

353 conditions. This behavior shows that ISPS has emulsification potential and should be able to form a

354 protective film around the oil droplets during the homogenization process and to stabilize an oil-in-

355 water emulsion.

356   Interfacial G´ and G´´ versus time are shown in Figure 5. In all cases, the adsorption of the sample

357   to the interface started immediately after the interface generation and G´ values were significantly

358   higher than G´´. This indicates the formation of gel-like interfacial films. At the different pH, ISPS-V-

359   P and ISPS-S-P presented a faster arrange of macromolecules at the water/oil interface, promoting

360   a stronger gel-like structure of the interfacial film (higher G') in comparison with the ISPS-U-P

361   sample.

362   The similar π^s_e and π^i_e values could be related to the formation of a predominantly proteic interfacial

363   film. On the other hand, the increased exposure of active sites showed by the FTIR analysis (Figure

364   2), particularly in ISPS-V and ISPS-S samples, would also allow the contribution of the

365   polysaccharides either at the interface or in the formation of external layers, significantly modifying

366   the rigidity of the interfacial film (for example, by increasing the values of G'). This better activity of

367   the polysaccharides would be in direct relation with the increase of hydrophobic zones generated

368   during the homogenization treatments (Ngouémazong, Christiaens, Shpigelman, VanLoey, &

369   Hendrickx, 2015).

370   3.2.4 Optical characterization of fine O/W emulsions

371   The variation over time of the backscattering (%BS) values enable to discriminate between the

372   particle migration (sedimentation, creaming) and particle size variation (flocculation, coalescence)

373   processes (Cabezas et al., 2012). The %BS profiles were obtained for the fine O/W emulsions

374   during 28 days at 24°C (Figure 6). These emulsions showed constant values of %BS along the

375   length of the tube during 28 days of storage, indicating a high stability against the destabilization

376   processes of creaming and/or coalescence.

377   3.2.5 Particle size determination of O/W fine emulsions

378   D[4,3] values before (Figura 5.a) and after (Figura 5.b) deflocculation by low energy sonication,

379   allowed to analyze the effect of the destabilization process by coalescence and/or flocculation.

380   ISPS samples produce emulsions with initial D[4,3] values in a range between 8.0 and 10.1 μm.

381   High FD values (>5.7) were obtained immediately after preparing these six emulsions (Figure 7.c),

382   and the presence of flocs was confirmed by optical microscopy (Figure 8). Particularly, emulsions

383   with pretreated okara samples (ISPS-V-P, ISPS-S-P) generated the largest flocs (FD>8.1).

384   Most emulsions showed D[4,3] values almost constant along time, which indicates that the initially

385   flocs do not disintegrate during storage. In contrast, the microstructure of such flocs was modified

386   during storage depending on the type of emulsifier. This can be seen by analyzing the

387   corresponding coalescence index (Figure 7.d): emulsions with pretreated okara (ISPS-V-P and

388   ISPS-S-P, CI<1.3%) showed an increased resistance to coalescence in relation to those with not-

389   pretreated okara (ISPS-U-P, CI>7.0%). The presence of hydrated flocs doing a gel-like structure,

390   especially at higher FD values, would avoid the coalescence of the droplets due to the lower

391   mobility of the system. In different protein samples, a positive relationship between the flocculation-

392   creaming rates with surface hydrophobicity was detected (Wang, Li, Jiang, Qi, & Zhou, 2014). The

393   unfolding structures of the ISPS-V-P and ISPS-S-P samples would increase the interfacial activity of

394   these protein/polysaccharide systems (possibly forming a glycoprotein matrix) at the interfacial level

395   and/or by formation of external layers, increasing the rigidity of the interfacial film (Figure 5) and

396   contributing to the formation of hydrated flocs (Figure 7.c and 8),

397   3.2.6 Rheological properties of O/W fine emulsions

398   Storage (G') and viscous modulus (G'') were analyzed as a function of oscillation frequency (0.1-

399   100 Hz, strain 1%). The evolution of these rheological parameters during storage (28 days),

400   particularly at 1 Hz, was used to compare the fine emulsions (Figure 9). Storage modulus were

401   higher than loss modulus (G'>>G") maintaining this behavior in a wide range of frequencies. This

402   observation indicates the formation of a strong gel-like material, which is directly related to the

403   presence of the mentioned hydrated flocs (Mc Clements, 1999). Samples with pretreated okara

404   (Figure 9.c-f), showed higher G' and G'' values and a better rheological stability during the storage

405   time than the sample without pretreatment (Figure 9.a, b). Both homogenization processes and the

406   alcoholic treatment generates emulsions with high values of flocculation degree (FD $_{ISPS-V \text{ or } ISPS-S}$ >

407   FD $_{ISPS-U}$, Figure 7.c), increasing the stability of the particle size (CI $_{ISPS-V \text{ or } ISPS-S}$ < CI $_{ISPS-U}$, Figure

408   7.d), and allowing the formation of stronger gel-like emulsions (Figure 9). Particularly, ISPS-V-P

409 gives emulsions with greater physical (Figure 6 and 7) and rheology (Figure 9) stability over storage

410 time and it was not significantly affected by variations in the pH of the emulsion.

411 4. Conclusion

412 The present research analyzed different insoluble fractions in both alkaline (pH 9, 60℃, 30 min)

413 and acidic conditions (pH 3, 120℃, 90 min) of defa tted solvent-free soy flour. These fractions,

414 called insoluble soybean polysaccharides (ISPS), have a high content of polysaccharides and

415 protein. The compositional analyses of these samples allow inferring the presence of carbohydrates

416 covalently attached to proteins in a glycoprotein matrix (arabinogalactan-proteins and

417 glucomannan-proteins). High pressure homogenization and ultrasonic sonication pretreatments on

418 the alkaline-insoluble residue (okara) and an alcoholic treatment expose internal sites of the

419 polysaccharide and protein structures, increasing their superficial hydrophobicity and, therefore,

420 allow a strong absorption of the macromolecules at the oil-water interface and/or the formation of

421 external layers, increasing the rigidity of the interfacial film and contributing to the formation of

422 hydrated flocs, Also, these treatments could solubilize certain compounds in okara (low molecular

423 weight polysaccharide or peptides fragments) that would interfere negatively in the formation of the

424 interface during the emulsification process. These behaviors were evidenced by the formation of

425 stronger gel-like emulsions with higher stability of the particle size,   which is associated directly with

426 the formation of hydrated flocs. It should be noted that variations in pH (acid and neutral conditions)

427 did not cause significant differences in the activity of the samples with an okara pretreatment (ISPS-

428 V or ISPS-S). Particularly, the sample obtained by high pressures homogenization of the okara

429 (ISPS-V-P) presented the best emulsifying properties. The results of this research work

430 demonstrate a high potential of application of the ISPS samples in the production of O/W

431 emulsions, under acid and neutral conditions, increasing the added value of an important by-

432 product of the soybean protein isolates or soluble soybean polysaccharides (SSPS) industries.

433 Acknowledgements

438

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541   explosion with dilute acid soaking. *Journal of Food Engineering*, 119, 56–64

542

543 **Tables:**

544 **Table 1:** Extraction yield and chemical analysis of the insoluble soybean polysaccharide samples

Sample	Sample/DSF Yield (W/W %)	Total Carbohydrates (DWB%)	Sugar Composition (Mol %)								Protein (DWB%)	Ash (DWB%)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Gal A		
Okara	21.88[d]	62.85[e]	3.27[d]	2.02[b]	16.25[d]	4.53[a]	1.63[a]	29.06[d]	22.93[a]	20.30[ab]	33.57[a]	3.58[b]
ISPS-U-O	13.32[c]	54.11[c]	3.12[d]	2.14[c]	6.44[c]	6.70[bc]	3.67[c]	16.73[c]	40.11[b]	21.10[b]	42.77[d]	3.12[a]
ISPS-V-O	12.56[b]	59.17[d]	2.71[bc]	1.94[b]	5.46[b]	7.51[d]	3.82[c]	13.89[a]	44.49[de]	20.17[a]	37.16[b]	3.67[b]
ISPS-S-O	12.52[b]	58.13[d]	2.80[bc]	2.02[b]	6.57[c]	7.17[cd]	3.66[c]	14.69[b]	42.01[c]	21.08[b]	38.10[b]	3.77[b]
ISPS-U-P	11.53[a]	50.83[a]	2.98[cd]	2.19[c]	5.01[a]	7.43[d]	3.60[c]	14.98[b]	43.66[cd]	20.15[a]	46.08[f]	3.09[a]
ISPS-V-P	11.45[a]	55.93[c]	2.07[a]	1.68[a]	5.08[a]	6.72[bc]	3.85[c]	14.44[b]	46.01[e]	20.15[a]	40.86[c]	3.21[a]
ISPS-S-P	11.21[a]	52.20[b]	2.52[b]	2.00[b]	5.17[ab]	6.27[b]	3.16[b]	15.65[bc]	45.01[de]	20.23[ab]	44.24[e]	3.56[b]

545

546 Mean values of three determinations; ISPS: insoluble soybean polysaccharide; DSF: Defatted solvent-free soy flour; Rha: rhamnose; Fuc:

547 fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; Gal A: galacturonic acid. Values with different letters in each

548 column are significantly different (P<0.05).

549

550 **Table 2:** Surface (π^s_e) and interfacial (π^i_e) pressure at equilibrium of 8 mM sodium citrate buffer
551 dispersion with 0.1% of different ISPS-P samples at pH 3.0 and 7.0. Mean values (n=4).
552

pH	π^s_e (mN/m)			π^i_e (mN/m)		
	ISPS-U-P	ISPS-V-P	ISPS-S-P	ISPS-U-P	ISPS-V-P	ISPS-S-P
3.0	19.07[a]	19.61[ab]	20.17[bc]	7.56[a]	8.64[c]	7.82[ab]
7.0	20.57[c]	20.32[c]	20.69[c]	8.04[b]	9.04[d]	8.58[c]

553
554
555 Values with different letters in each parameter are significantly different (P<0.05)

556 **Figures:**

557 **Figure 1:** Flow diagram of the process used for producing different insoluble soybean

558 polysaccharide fractions.

559

560 **Figure 2:** Fourier transform infrared spectra of the 850-2150 cm^{-1} region of a) ISPS-U, b) ISPS-V

561 and c) ISPS-S samples. Abbreviations are described in Figure 1. Mean values (n=4).**Figure 3:** De

562 Brouckere mean diameters (D[4,3]) of O/W emulsions (Φ_m = 0.3) obtained in Ultra-Turrax

563 homogenizer with the addition of different concentrations (1 – 2% w/w) of insoluble soybean

564 polysaccharide samples at pH 3 and 7. Abbreviations are described in Figure 1. Mean values (n=3).

565 Values with different letters are significantly different (P<0.05).

566

567 **Figure 4:** Serum layer (%) of O/W emulsions (Φ_m = 0.3) obtained in Ultra-Turrax homogenizer with

568 the addition of different concentrations (1 – 2% w/w) of insoluble soybean polysaccharide samples

569 at pH 3 and 7. Abbreviations are described in Figure 1. ND: not detectable. Mean values (n=3).

570 Values with different letters at each concentration (1%: underlined letters and 2%: not underlined

571 letters) are significantly different (P<0.05).

572

573 **Figure 5:** Storage (G') and viscous modulus (G'') at the oil-water interface as a function of time (0 –

574 60 minutes, 0.1 Hz, strain 5%) using 8 mM sodium citrate buffer with 0.5% of different ISPS-P

575 samples at a) pH 3.0 and b) 7.0. Abbreviations are described in Figure 1. Mean values (n=3).

576 Values with different letters in each viscoelastic moduli (G': not underlined letters and G'':

577 underlined letters) are significantly different (P<0.05).

578 **Figure 6:** Back scattering (%BS) profiles as a function of the tube length with storage time (28 day),

579 for O/W emulsions (Φ_m = 0.3) obtained in ultrasound homogenizer with the addition of 2% of

580 different insoluble soybean polysaccharide samples at pH 3 and 7. Abbreviations are described in

581 Figure 1. Mean values (n=3).

582

583 **Figure 7:** De Brouker mean diameters without (D[4,3]) and with a subsequent deflocculating

584 process by low energy sonication (D[4,3] defloculated), Flocculation degree (FD) and coalescence

585 index (CI) for O/W emulsions (Φ_m = 0.3) obtained in ultrasound homogenizer with the addition of 2%

586 of different insoluble soybean polysaccharide samples at pH 3 and 7. Abbreviations are described

587 in Figure 1. Mean values (n=3). Values with different letters in each parameter are significantly

588 different (P<0.05).

589

590 **Figure 8:** Optical microscopy images of fine emulsions stabilized with 2 % of different insoluble

591 soybean polysaccharide samples after 1 day of storage at pH 3 and 7. Abbreviations are described

592 in Figure 1. Bar = 20 μm.

593

594 **Figure 9:** Storage (G') and viscous modulus (G'') as a function of oscillation frequency (0.1-100 Hz,

595 strain 1%) for O/W emulsions (Φ_m = 0.3) obtained in ultrasound homogenizer with the addition of

596 2% of different insoluble soybean polysaccharide samples after 1 and 28 days of storage at pH 3

597 and 7. Abbreviations are described in Figure 1. Mean values (n=3). Values with different letters in

598 each viscoelastic moduli (G': underlined letters and G'': not underlined letters) are significantly

599 different (P<0.05).
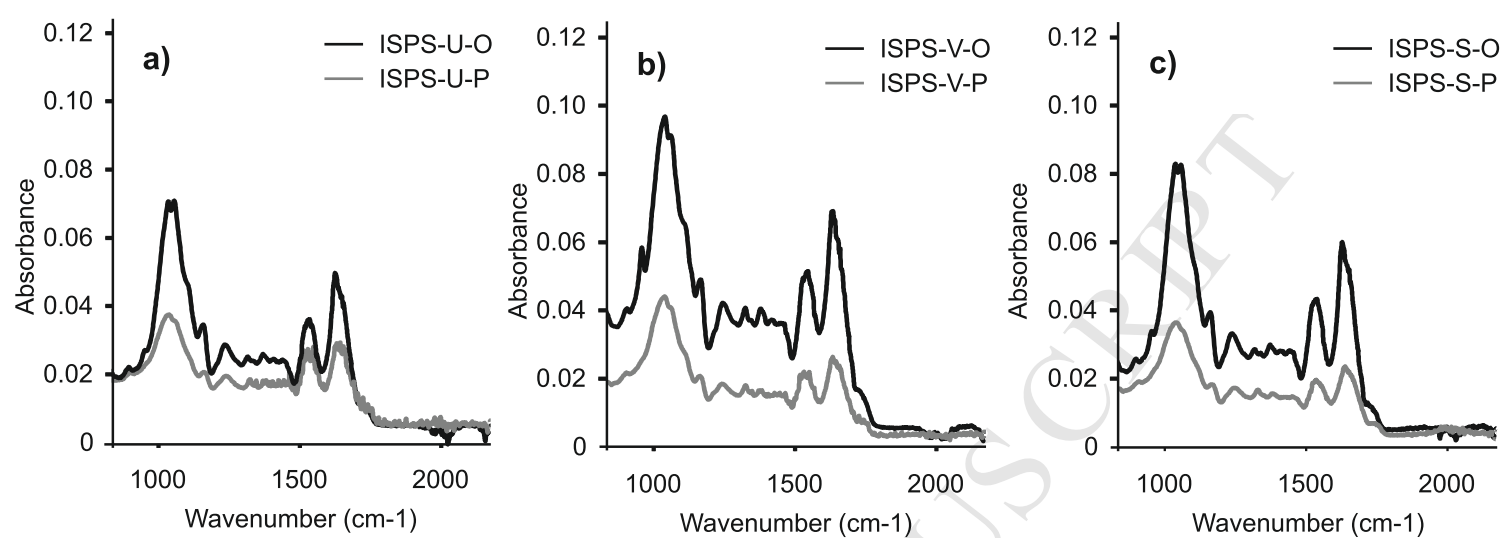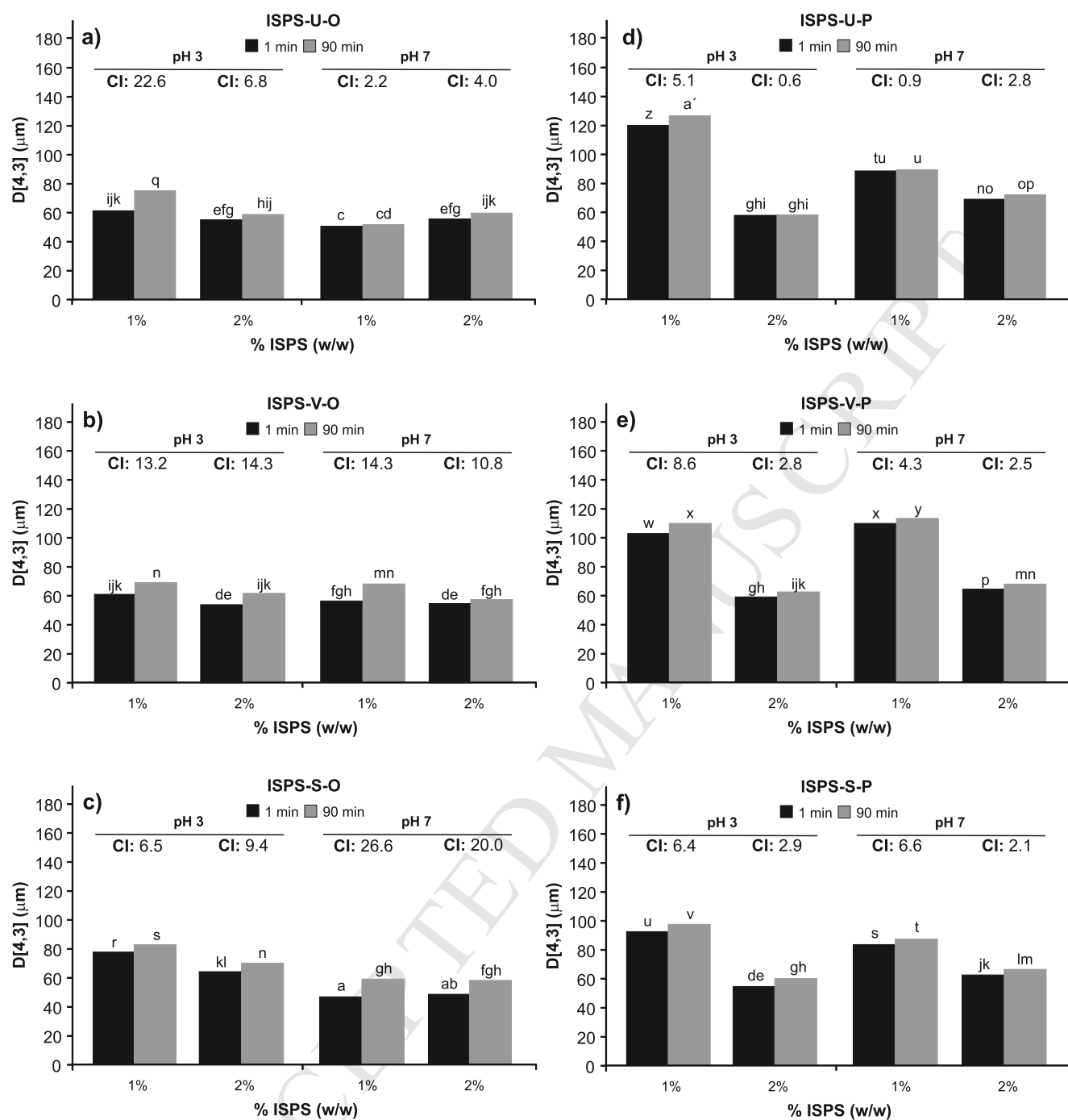
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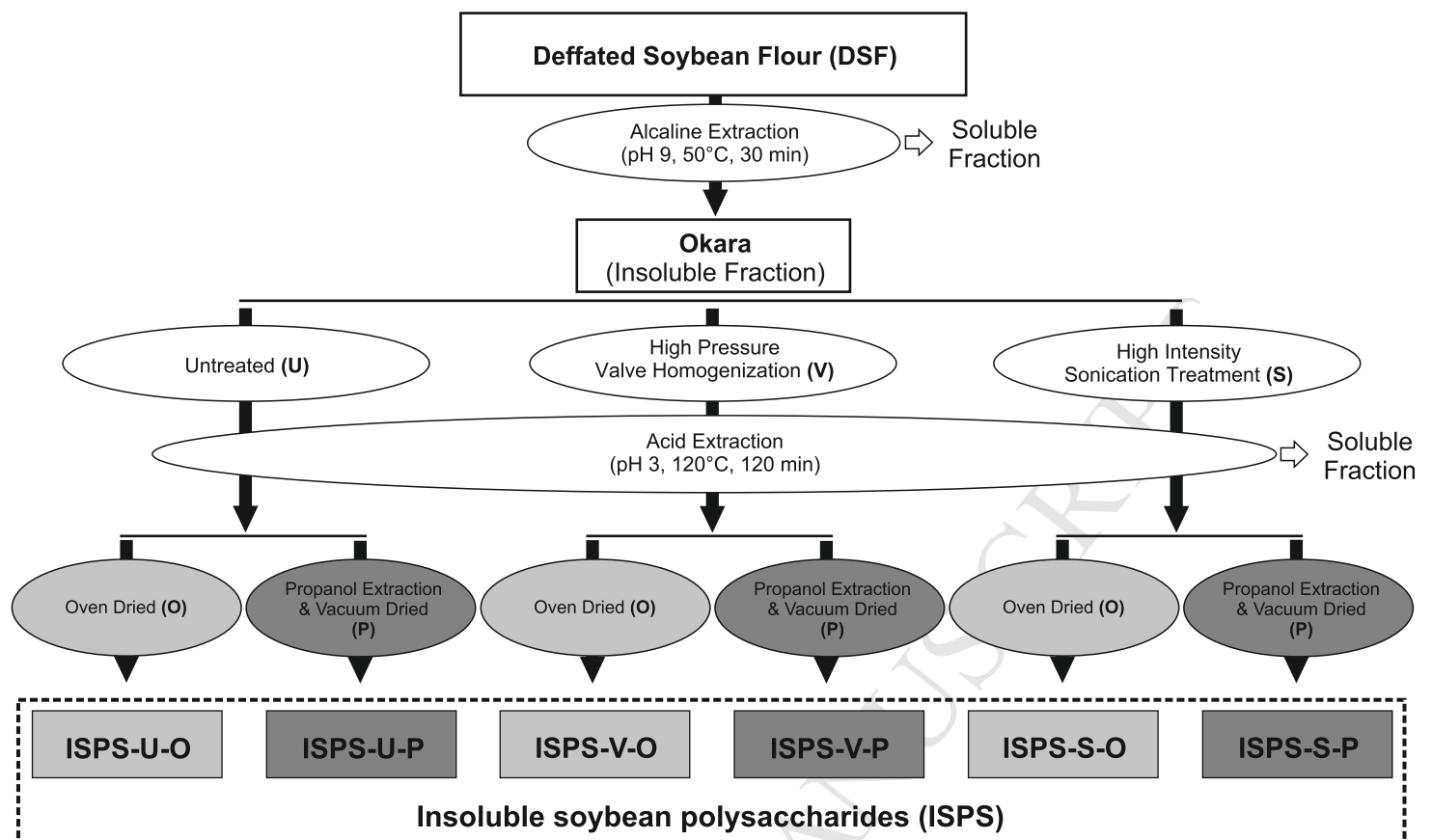
Figure 1

Figure 2
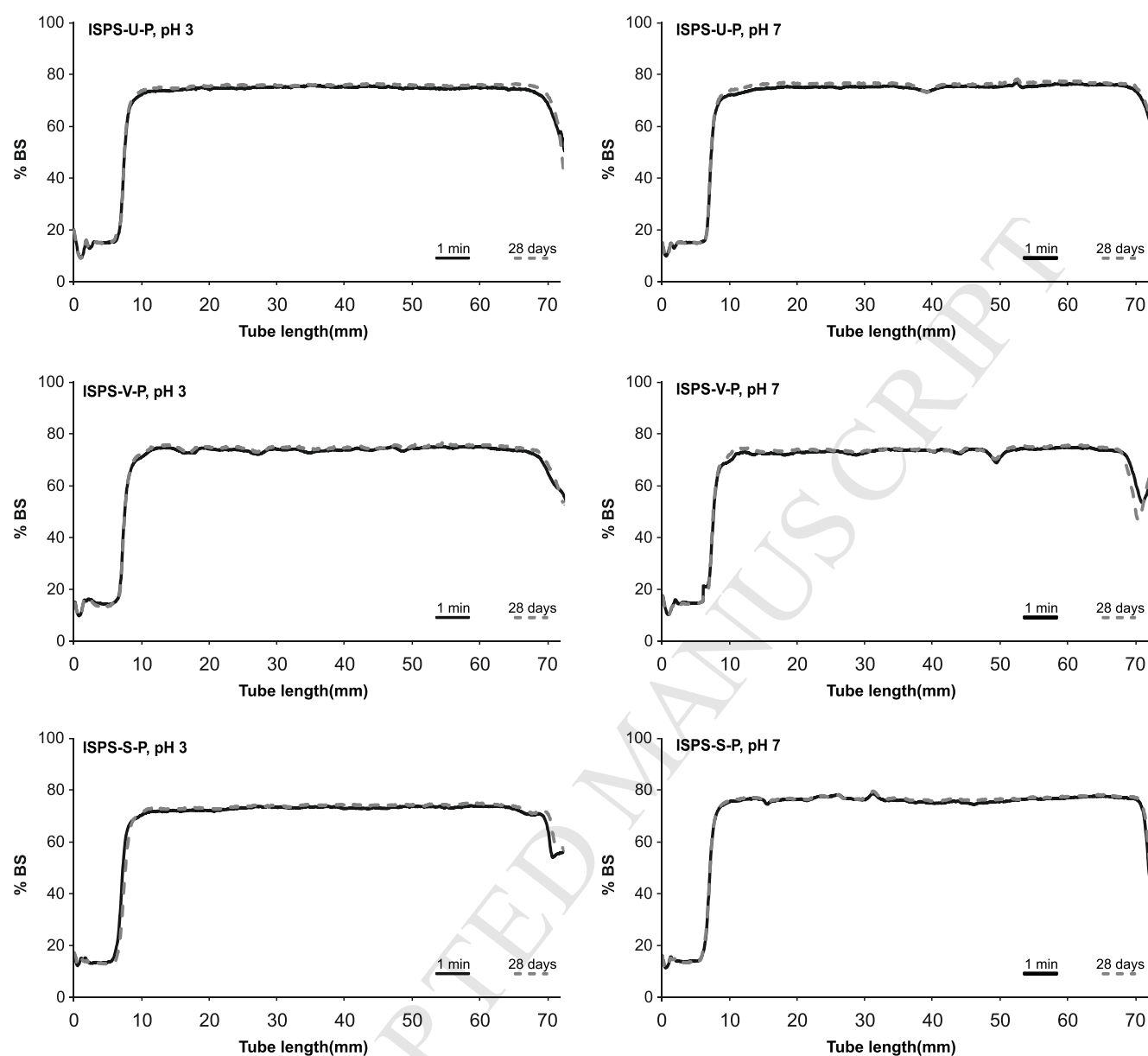
Figure 3
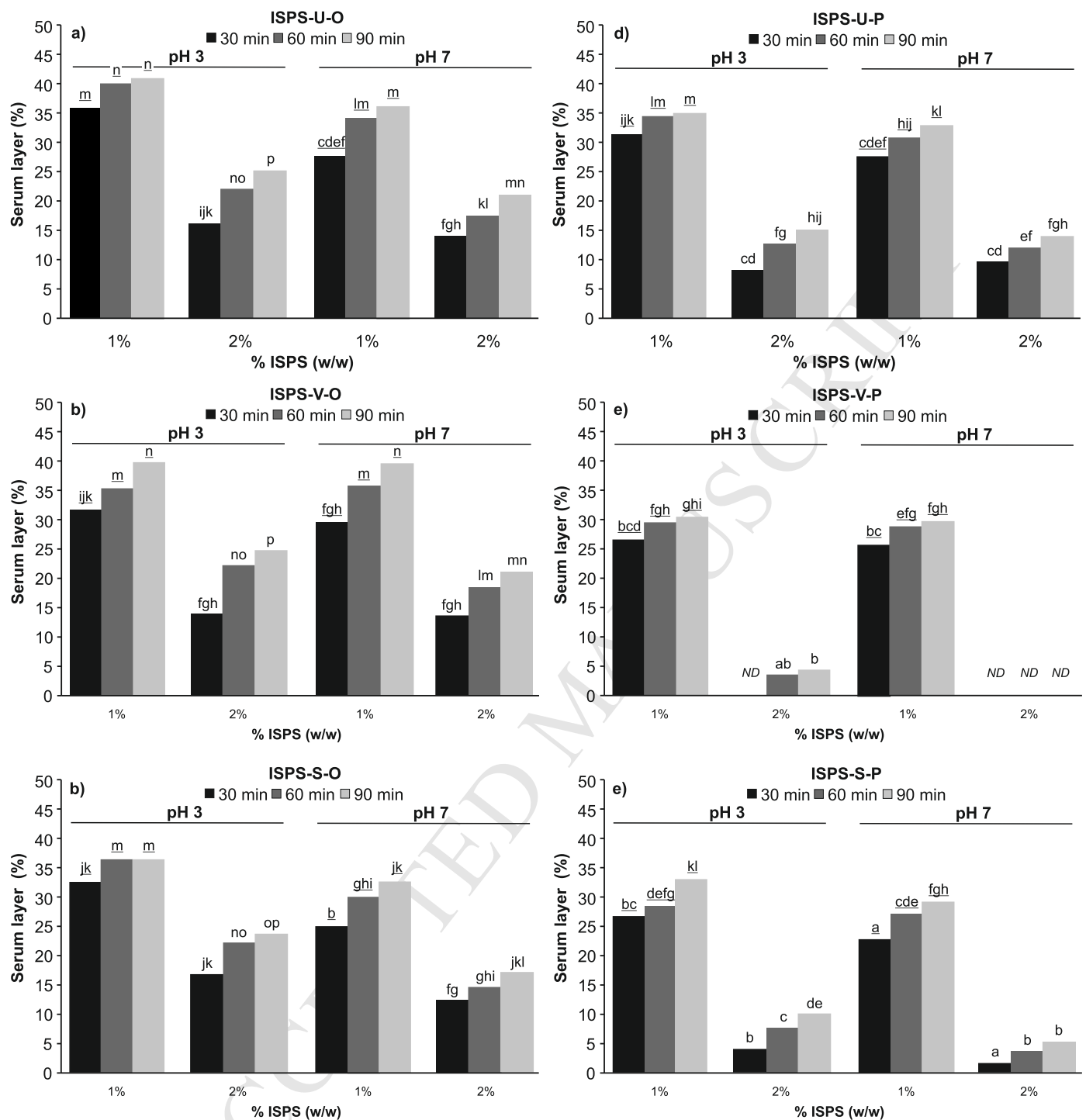
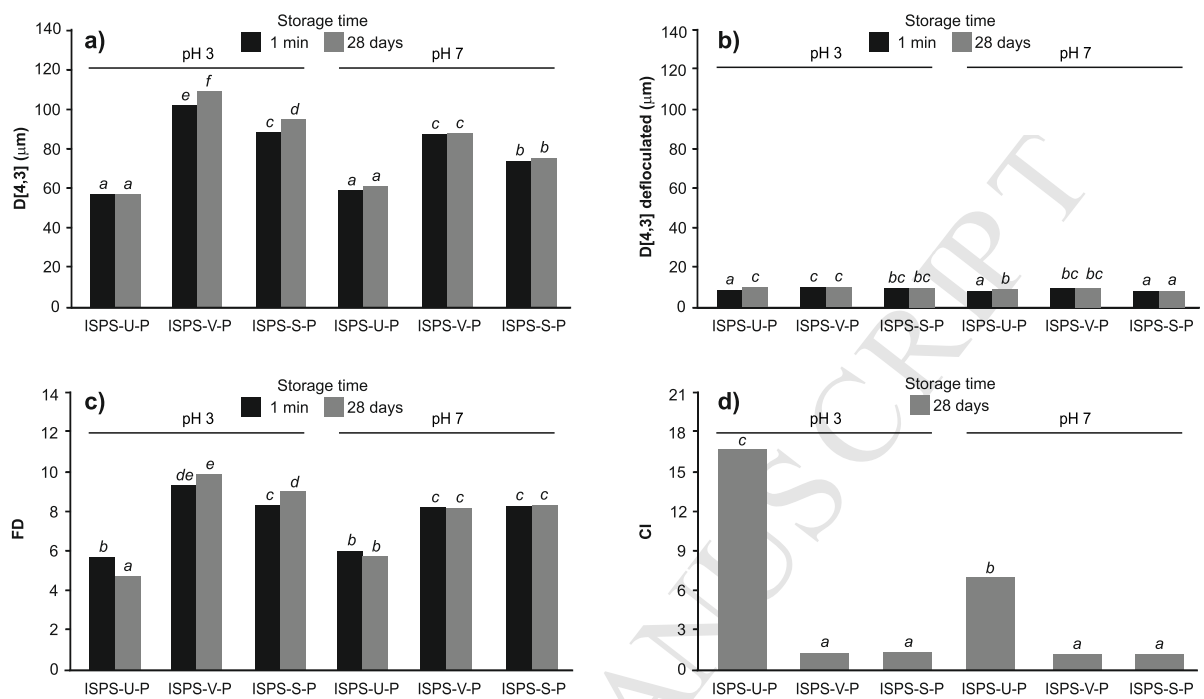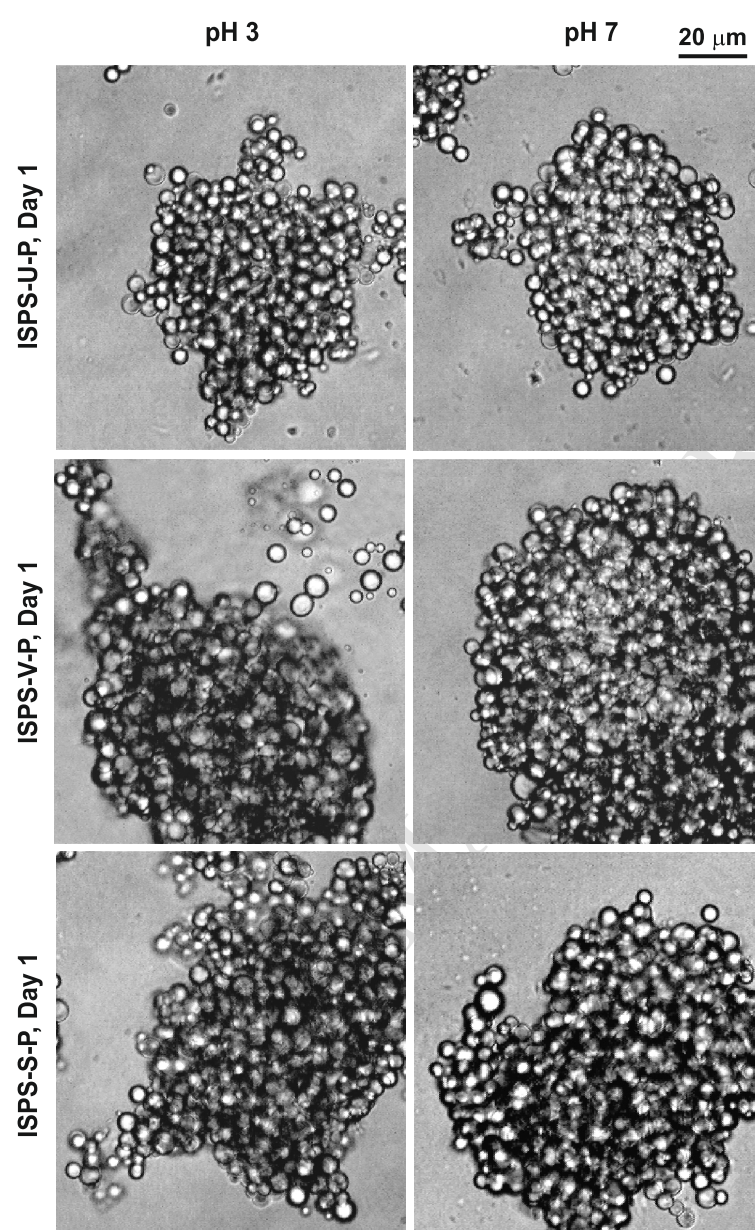**Figure 4**
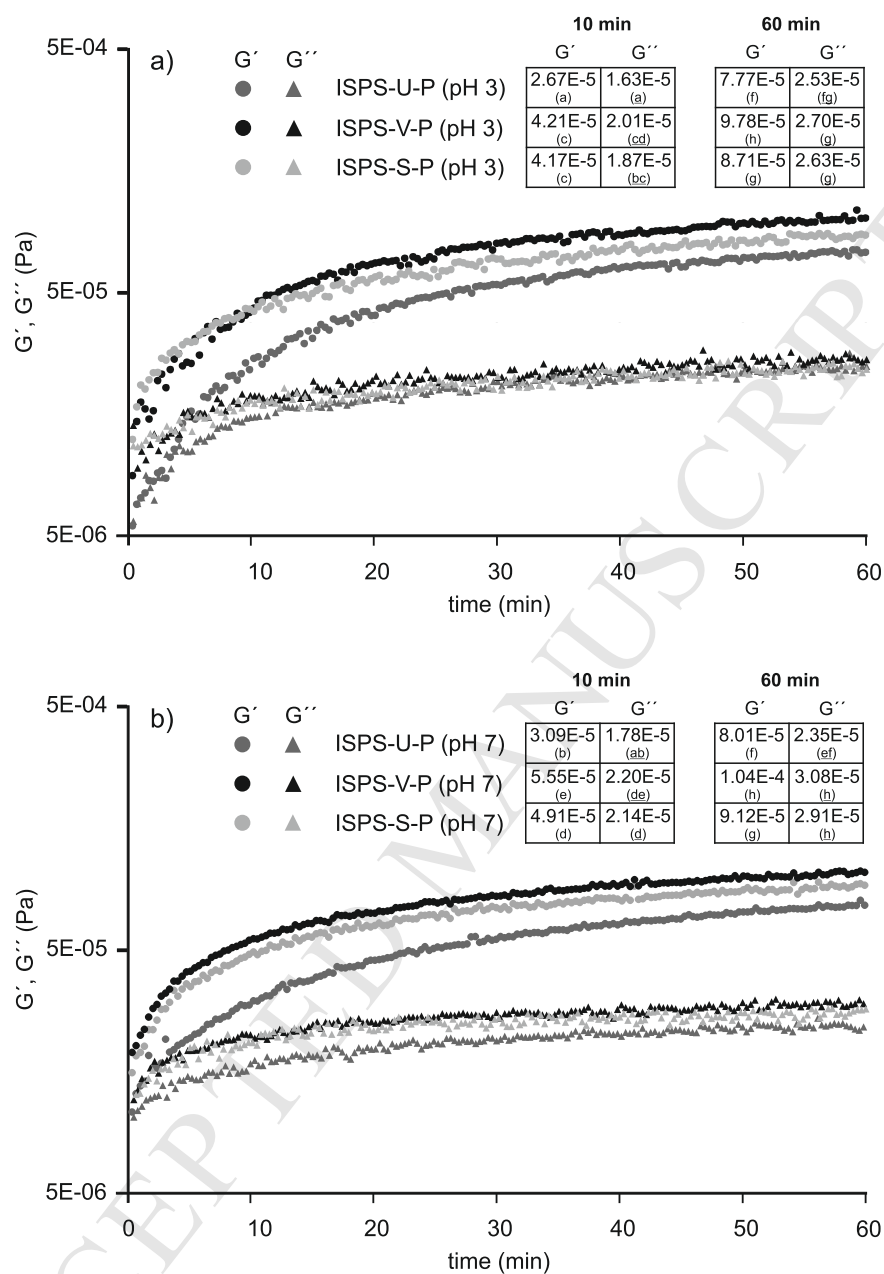
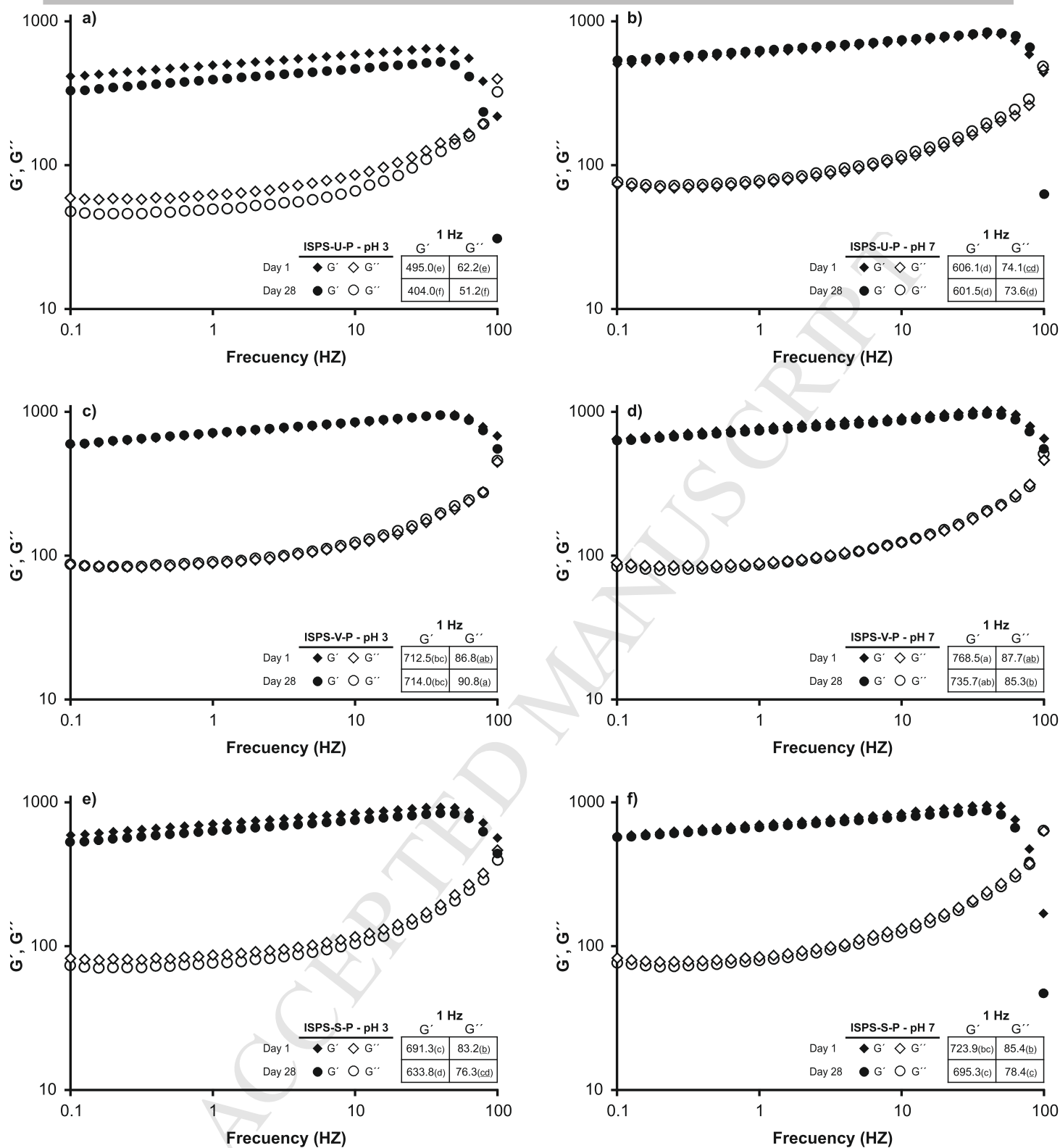**Figure 5**

Figure 6

Figure 7

Figure 8

Figure 9

Insoluble soybean polysaccharides: Obtaining and evaluation of their O/W emulsifying properties

1. Insoluble soybean polysaccharides samples (ISPS) were extracted from defatted soybean flour.
2. ISPS samples are composed mainly of acid and alkaline-insoluble polysaccharides and proteins.
3. ISPS samples were effective in the formulation and stabilization of acidic and neutral O/W emulsions.
4. The results are of interest to the manufacturing of foods by using residual materials of the food industry.