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# SUSTAINABLE EXTRACTION OF PROTEINS AND BIOACTIVE SUBSTANCES FROM POMEGRANATE PEEL (*PUNICA GRANATUM* L.) USING PRESSURIZED LIQUIDS AND DEEP EUTECTIC SOLVENTS

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19 ABSTRACT

20 Pomegranate peel is a source of proteins, bioactive peptides, and phenolic
21 compounds. The simultaneous extraction of these compounds required the use of
22 polluting solvents and reagents that are non-suitable. This work targets the development
23 of green methodologies based on pressurized liquids (PLE) or deep eutectic solvents
24 (DES) for the extraction of these compounds. Extracts were digested with different
25 proteolytic enzymes and different functionalities (antioxidant, hypocholesterolemia, and
26 antihypertensive capacities) were evaluated. Highly antioxidant and
27 hypocholesterolemic extracts and hydrolysates were obtained using PLE while high
28 antihypertensive capacity was observed in the hydrolysates from proteins extracted
29 using DES. Peptides and polyphenols were identified by HPLC-ESI-Q-TOF/MS.
30 Higher amounts of peptides were shown in hydrolysates from DES extracts while
31 hydrolysates from PLE extracts presented higher amounts of phenolic compounds.
32 Some peptides were assigned to proteins from *Punica granatum*. Both green methods
33 improved the extraction of bioactive compounds from pomegranate peel compared to
34 the non-sustainable method.

35

36 **Keywords:** pomegranate peel; pressurized liquid extraction (PLE); deep eutectic
37 solvents (DES); peptide; polyphenol; mass spectrometry.

38

39

40 ## 1. Introduction

41 Wastes and coproducts are generated within different phases of the food cycle
42 (Kumar et al., 2017). They are currently discarded in landfills or incinerated. Alternative
43 destinations are their processing into biogas, their composting into biofertilizers
44 (Banerjee et al., 2017) or their use in animal feeding (Kumar et al., 2017). Nevertheless,
45 these food wastes can contain valued substances such as proteins and bioactive
46 compounds. The growing consumption of proteins urge for new proteins sources and
47 food coproducts could help to support this demand (Aiking, 2011). Proteins can also be
48 sources of bioactive peptides. Bioactive peptides and, in general, bioactive compounds
49 can provide beneficial effects in terms of health promotion and reduction of the
50 incidence of disorders (Nazir et al., 2019; Santos et al., 2019). Bioactive compounds can
51 be employed for the development of nutraceuticals and functional foods or in medicinal
52 and pharmaceutical preparations (Kumar et al., 2017; Ran et al., 2019).

53 Methods currently employed to extract proteins and bioactive compounds are
54 associated to a high solvent and energy consumption, risk of thermal degradation of
55 heat-labile components, and long extraction times (Kumar et al., 2017; Banerjee et al.,
56 2017). Thus, there is a great interest in the development of efficient extraction methods
57 that are more environmentally friendly and that can minimize the degradation of target
58 compounds (Duarte et al., 2014; Sumere et al., 2018). No much progress has taken place
59 in relation with the extraction of proteins that require volatile organic solvents and
60 polluting reagents.

61 Pressurized Liquid Extraction (PLE) uses high pressures and temperatures
62 enabling a reduced extraction time, less solvent consumption, high extraction yields,
63 and use of completely safe solvents (Žlabur et al., 2018). PLE has been used to extract

64 phenolic compounds but it has been scarcely employed for the extraction of proteins
65 (Herrero et al., 2015; Ameer et al., 2017).

66 Deep eutectic solvents (DES) are environmentally friendly, easily synthesized,
67 biodegradable, non-volatile, non-toxic, highly stable, and have low cost (Benvenutti et
68 al., 2019). They are constituted by two or more compounds acting as either hydrogen
69 bond donors (HBD) or hydrogen bond acceptors (HBA) (Bai et al., 2017; Jiang et al.,
70 2018). The HBA is often a quaternary ammonium salts like choline chloride, whereas
71 the HBD comprises amines, carboxylic acids, alcohols, polyols, acid amides or
72 carbohydrates (Benvenutti et al., 2019). The mixture of these compounds, at a suitable
73 ratio, results in hydrogen bond interactions and the formation of a solvent with lower
74 melting point than those corresponding to its individual components (Ozturk et al.,
75 2018; Rajha et al., 2019a). Different DES have been previously employed in the
76 extraction of proteins (Bai et al., 2017; Grudniewska et al., 2018; Wahlström et al.,
77 2017) or phenolic compounds (Rajha et al., 2019a; Rajha et al., 2019b; Gullón et al.,
78 2019; Pal & Jadeja, 2019; Ozturk et al., 2018; Djaoudene & Louaileche, 2018).

79 Pomegranate peel represents about 40-50% of the whole fruit weight
80 (Kharchoufi et al., 2018; Kaderides et al., 2019; Rajha et al., 2019a). Phenolic fraction
81 has been the most studied within the pomegranate peel (Sumere et al., 2018; Kaderides
82 et al., 2019; Smaoui et al., 2019) while the protein fraction has been hardly considered.
83 Pomegranate peel proteins were extracted by our research group using conventional
84 solvents and high intensity focused ultrasounds (HIFU) (Hernández-Corroto et al.,
85 2019). Results demonstrated that both proteins and phenolic compounds were
86 responsible for bioactive properties observing potential synergic effects among them.

87 This work proposes the development of green analytical methods for the
88 extraction of high-added-value compounds from pomegranate peel using sustainable

89 techniques based on pressurized liquids and deep eutectic solvents. Extracted
90 compounds were identified using HPLC-ESI-Q-TOF/MS and their functionality and
91 potential synergies were also investigated.

92

93

94 **2. Materials and methods**

95 *2.1 Chemicals and samples*

96 All reagents were of analytical grade and water was purified in a Milli-Q system
97 from Millipore (Bedford, MA, USA). Hydrochloric acid (HCl), acetonitrile (ACN),
98 acetic acid (AA), and urea were from Scharlau (Barcelona, Spain). Sodium chloride
99 (NaCl) and phosphate buffer (PB) were from Merck (Darmstadt, Germany). DL-
100 dithiothreitol (DTT), albumin from bovine serum (BSA), thermolysin, sodium
101 tetraborate, β-mercaptoethanol, o-phthaldialdehyde (OPA), L-glutathione (GSH), 2,2'-
102 azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS), potassium
103 persulphate, 1,10-phenantroline, ferrous sulphate, hydrogen peroxide ($H_2O_2$), bovine
104 pancreatic cholesterol esterase (CEase), p-nitrophenylbutylrate (p-NPB), taurocholic
105 acid, oleic acid, phosphatidylcholine, sodium taurocholate hydrate, angiotensin
106 converting enzyme (ACE**),** N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic) acid
107 (HEPES), and tripeptide hippuryl-histidyl-leucine (HHL) were obtained from Sigma-
108 Aldrich (Saint Louis, MO, USA). Bradford reagent (Coomassie Blue G-250), Laemmli
109 buffer, Tris/glycine/SDS running buffer, Mini-Protean precast gels, Bio-Safe Coomassie
110 G-250 stain, and Precision Plus Protein All Blue standards were acquired at Bio-Rad
111 (Hercules, CA, USA). Ethanol (EtOH) was from Thermo Fisher Scientific (Waltham,
112 MA, USA). Cholesterol assay kit, which contained the assay buffer, the cholesterol
113 reagent, the enzyme mix, and the dye reagent, was obtained from BioAssay Systems
114 (Hayward, CA, USA). Alcalase 2.4 L FG was donated by Novozymes Spain S.A.
115 (Madrid, Spain). Pomegranates were purchased in a local market.

116 Polyphenols standards (punicalagin, gallic acid, ellagic acid, and punicalin) were
117 acquired in Sigma-Aldrich.

118

119 *2.2 Preparation of deep eutectic solvents (DES)*

120 Different DES were prepared according to Rajha et al. (2019a). HBA and HBD
121 components of DES are described in Table 1. All DES contained water ($3^{th}$ component)
122 to control solvent viscosity. The three components were mixed at a 1:1:3 molar ratio.
123 Mixtures were heated in a water bath at 80 ºC with agitation until a clear liquid was
124 obtained.

125

126 *2.3 Extraction of proteins and bioactive compounds from pomegranate peels*

127 Pomegranates peels were dried to 50 ºC for 48 h. Dried peels were ground in a
128 mortar and, next, in a domestic mill. Finally, they were stored at -20 ºC until use. After
129 extraction, proteins in extracts were evaluated by Bradford assay and separated by SDS-
130 PAGE (polyacrylamide gel electrophoresis) following a procedure previously described
131 (Hernández-Corroto et al., 2019).

132

133 *2.3.1 Pressurized Liquid Extraction (PLE)*

134 Extraction of proteins and bioactive substances was performed using an
135 accelerated solvent extractor system (ASE 150, Dionex, Sunnyvale, CA, USA).
136 Solvents were degassed in an ultrasound bath for 30 min. In every extraction, 2 g of
137 dried pomegranate peels were mixed with 8 g sand and put into a 10 mL stainless steel
138 extraction cell. A circular cellulose filter (2.5 cm, Whatman) was placed at the bottom
139 of the extraction cell to prevent suspended particles from entering the collection bottles.
140 Before extraction, the oven was preheated for 6 min. Optimal extraction conditions
141 were: extraction pressure, 1500 psi; extraction solvent, 70% (v/v) EtOH; extraction
142 temperature, 120 ºC; static extraction time, 3 min; extraction time, 12 min; and one

143 static cycle. The extracts were evaporated in a centrifugal concentrator (Eppendorf AG,
144 Hamburg, Germany) and pellets were stored at -20ºC.
145

146 *2.3.2 Extraction with DES*

147 Extractions were firstly carried out by mixing 150 mg of dried pomegranate
148 peels with 5 mL of the DES grouped in Table 1 using a High Intensity Focused
149 Ultrasounds (HIFU) probe (model VCX130, Sonics Vibra-Cell, Hartford, CT, USA) for
150 1 min at 30% amplitude (Hernández-Corroto et al., 2019). After DES selection, an
151 incomplete factorial experimental design of second order, based on three levels, was
152 employed to optimize HIFU and other extraction conditions. Four different factors such
153 as molar ratio between DES components (1:1, 1:2, and 1:3), molar ratio of water (10,
154 15, and 20), HIFU amplitude (30%, 45%, and 60%), and extraction time (1, 8, and 15
155 min) were used. The response was the protein content (mg protein/g peel), which was
156 determined by the Bradford method. Twenty nine experiments were conducted in a
157 randomized order, corresponding to twenty four points of the factorial design and five
158 additional center points to consider the experimental errors. The experimental design
159 and data analysis were carried out by Box-Behnken design with Statgraphics Centurion
160 XVII software (Statpoint Technologies, Inc., Warranton, VA, USA). Experimental data
161 were fitted to a quadratic model using a second-order polynomial model equation:

162 Protein content (mg protein/g peel) = $\beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$

163 where $\beta_0$ is the constant, $\beta_i$ is the linear regression coefficient, $\beta_{ii}$ is the quadratic
164 regression coefficient, and $\beta_{ij}$ is the interaction regression coefficient, while $X_i$ and $X_j$
165 are the independent variables. The determination coefficient ($R^2$) and the analysis of

166 variance (ANOVA) at a confidence level of 95% were employed to evaluate the fitting
167 of data to the polynomial model equation.

168 Extracts were next centrifuged (10 min at 4000 xg) and supernatants were
169 collected. Proteins in supernatants were precipitated with cold EtOH (15 mL, 4 ºC, 24
170 h) and centrifuged (10 min, 4000 xg). The pellet was purified again using the same
171 procedure. The resulting pellet was dried at room temperature and stored at -20 ºC.

172

173 *2.4 Enzymatic digestion of proteins*

174 Protein hydrolysis was carried out using two different enzymes (alcalase and
175 thermolysin) under optimal conditions (Hernández-Corroto et al., 2019).

176 Evaporated extracts obtained by PLE were dissolved in a 5 mM borate buffer
177 (pH 9.0) at a concentration of 5 mg/mL, when digesting with the alcalase enzyme, and
178 in a 5 mM phosphate buffer (pH 7.5) at the same concentration, when digesting with the
179 thermolysin enzyme. For these purposes, the HIFU probe was employed for 5 min at
180 30% amplitude. The alcalase/substrate ratio was 0.3 AU/g protein while the
181 thermolysin/substrate ratio was 0.1 g enzyme/g substrate. Solutions were incubated in a
182 Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 50 ºC for 2 h, for the
183 digestion with alcalase, and at 70 ºC for 1 h, for the digestion with thermolysin. After
184 hydrolysis, the temperature of both solutions was raised to 100 ºC and kept at this
185 temperature for 10 min to stop the reaction. Resulting solutions were centrifuged (10
186 min, 6000 rpm) and supernatants, containing peptides, were stored at -20 ºC.

187 Proteins extracted using DES were dissolved in a 100 mM borate buffer (pH
188 9.0), for the digestion with both enzymes, at a concentration of 5 mg/mL. For this

189 purpose, the HIFU probe was employed for 15 min at 30% amplitude. Digestions were
190 next carried at the conditions described before.

191 Peptide content in all hydrolysates was determined following the OPA method
192 described in Hernández-Corroto et al. (2018).

193

194 *2.5 Evaluation of the functionality of extracts and hydrolysates*

195 Antioxidant capacity was determined by the evaluation of the capacity of
196 samples to inhibit the formation of hydroxyl radicals and to scavenge free radicals
197 (Hernández-Corroto et al., 2018). Extracts and hydrolysates of two independent
198 experiments were analyzed by triplicate.

199 Hypocholesterolemic capacity was determined by the evaluation of the capacity
200 of samples to inhibit the cholesterol esterase (CEase) enzyme and the cholesterol
201 micellar solubility. Both procedures were previously described in Hernández-Corroto et
202 al. (2019). Antihypertensive capacity was evaluated using a methodology previously
203 described in the same work. Extracts and hydrolysates of two independent experiments
204 were analyzed by triplicate.

205

206 *2.6 Identification of peptides and phenolic compounds by RP- and HILIC-HPLC-*
207 *ESI-Q-TOF*

208 Peptides and phenol compounds were identified using a 6530 series high
209 sensitivity mass spectrometry Quadrupole-Time-of-Flight (Q-TOF) coupled to a High-
210 Performance Liquid Chromatograph (HPLC), model 1100, both from Agilent
211 Technologies. Extracts obtained by PLE were filtered using regenerated cellulose
212 syringe filters from Sartorious (Barcelona, Spain) (for the analysis of peptides) or

213 nonsterile hydrophobic PTFE syringe filters from Labbox (Barcelona, Spain) (for the
214 analysis of polyphenols). Both filters had a pore size of 0.45 µm. Extracts obtained
215 using DES passed through a solid phase extraction C18 columns from Isolute (Uppsala,
216 Sweden) to remove salts. RP-HPLC separation was carried out in an Ascentis Express
217 Peptide ES-C18 column (100 mm x 2.1 mm, 2.7 µm particle size) with a guard column
218 (5 mm x 2.1 mm, 2.7 µm particle size), both from Supelco (Bellefonte, PA, USA).
219 HILIC separation was carried out in an Ascentis Express column (100 mm x 2.1 mm,
220 2.7 µm particle size) with a guard column (5 mm x 2.1 mm, 2.7 µm particle size), also
221 from Supelco. Chromatographic conditions for the separation of peptides by RP-HPLC
222 and their detection by MS were the described in Hernández-Corroto et al. (2019).
223 Chromatographic conditions for the separation of peptides by HILIC were: mobile
224 phase A, 65 mM ammonium acetate in water; mobile phase B, ACN; injection volume,
225 15 µL; flow rate, 0.3 mL/min; column temperature, 25 ºC. The optimized elution
226 gradient was: 95–78% B in 25 min, 78–60% B in 5 min, 60% B for 5 min, and a
227 reversed gradient from 60 to 95% B in 5 min to recover initial eluting conditions.

228 MS/MS spectra of peptides were analyzed using the *de novo* tool of PEAKS
229 Studio Version 7 software from Bioinformatics Solutions Inc. (Waterloo, Canada).
230 Peptide sequences were accepted if the average local confidence (ALC, expected
231 percentage of correct amino acids in the peptide sequence) was equal or higher to 90%.
232 Since the *de novo* tool cannot differentiate between I and L amino acids, only isoforms
233 with L are shown although both isoforms are equally possible. Peptide sequences were
234 also analyzed by PEAKS DB (database search tool) using FASTA database that
235 included protein sequences from *Punica granatum* organism extracted from UNIPROT
236 database. Peptides sequences were associated to a protein if the error tolerance was less
237 than 10 ppm and the mass tolerance was 0.5 Da for the fragments. Peptides and proteins

238 with a -10lgP equal or higher to 15 and peptides with ALC equal or higher to 90%
239 confirmed the confidence between them.

240 Phenolic compounds were identified by comparison of their retention times and
241 MS spectra with those corresponding to standards. Polyphenol standards were dissolved
242 in water or MeOH at a concentration of 100 µM. Rest of polyphenols were assigned
243 according to their fragmentation pattern. All samples were injected, at least, by
244 triplicate.

245

246 *2.7 Statistical analysis*

247 Statistical analysis was performed using Statgraphics Centurion XVII software
248 (Statpoint Technologies, Inc., Warranton, VA, USA). Values were expressed as mean ±
249 standard deviation. The analysis of variance (ANOVA) was performed using a
250 significant level of 0.05.

251

252 **3. Results and discussion**

253 *3.1. Optimization of the extraction of proteins from pomegranate peels using green*
254 *methods*

255 Two green methodologies were developed to extract proteins and bioactive
256 compounds from pomegranate peel. First methodology employed PLE and second
257 methodology used DES.

258

259 *3.1.1. Pressurized Liquid Extraction (PLE)*

260 Different parameters were optimized for the extraction of proteins from
261 pomegranate peel: concentration of extracting solvent, temperature, static cycles, static
262 time, and presence of additives in the solvent.

263 EtOH was employed as extracting solvent. Protein content in extracts is
264 displayed in Table 2. Extraction yield increased at higher percentages of EtOH up to
265 70% (v/v), which was selected for further optimizations. Different temperatures were
266 next tried observing that the protein extraction yield improved at higher temperatures. A
267 temperature of 120 ºC was selected as optimum. Afterwards, different static cycles and
268 times were employed, although no significant differences were observed in the
269 extraction yield. Moreover, two additives (DTT and urea) were added to the optimum
270 extracting solvent to denature proteins and promote their extractability although no
271 significant affect was observed.

272 The extract obtained under optimal conditions was analyzed by SDS-PAGE (see
273 Fig.S1A). Different bands corresponding to proteins from 25 and 150 kDa were
274 observed. Under these conditions, it was possible the extraction of 9 ± 1 mg proteins/g
275 pomegranate peel which is lower than the amount of proteins extracted by the non-
276 sustainable methodology (15 ± 2 mg/g) (Hernández-Corroto et al., 2019).

277

278 *3.1.2. Extraction using DES*

279 Eight different DES were firstly employed (Table 1). The protein content in
280 extracts is shown in Table 3. The highest protein extraction yield was obtained with
281 ChCl:urea, ChCl:EG, ChCl:AA, and NaOAc:urea DES. Since urea is a reagent that
282 usually interferes in the assay employed for the estimation of proteins (Bradford assay),
283 the protein content determined in extracts obtained with ChCl:urea and NaOAc:urea

284 DES are likely overestimated. Extracts were analyzed by SDS-PAGE (Fig.S1B)
285 observing main bands from 100 to 250 kDa and, in some cases, additional bands at
286 lower molecular weights. From these results, the ChCl:AA DES was selected.

287 A Box-Behnken experimental design was employed to optimize HIFU and other
288 extraction conditions using the protein extraction yield as response variable. Factors
289 employed in the 29 experiments and protein content in extracts are grouped in Table 4.
290 The second-order polynomial model best fitting collected variables to predict the
291 protein content is,

292 $$\text{Protein content (mg protein/g peel)} = 25.4 - 1.57 X_1 - 0.157 X_2 - 0.225 X_3 - 0.278 X_4 +$$
293 $$0.221 X_1^2 + 0.0410 X_1 X_2 - 0.0123 X_1 X_3 + 0.0425 X_1 X_4 + 0.00248 X_2^2 - 0.00443 X_2 X_3$$
294 $$+ 0.0198 X_2 X_4 + 0.00332 X_3^2 + 0.00583 X_3 X_4 - 0.0149 X_4^2$$

295 where $X_1$ is the molar ratio of acetic acid, $X_2$ is the molar ratio of water, $X_3$ is the
296 amplitude of the HIFU probe, and $X_4$ is the extraction time. The mathematical model
297 enabled to predict the 92% of the response variability. An ANOVA determined the
298 suitable fitting of data to the model (p-value of the lack-of-fit > 0.05). The effect of
299 explanatory variables on the protein extraction yield is displayed in a response surface
300 3-D contour plot at different acetic acid concentrations (Fig.1). The higher was the ratio
301 of water and acetic acid in the solvent, the lower was the protein extraction yield while
302 the probe amplitude and the extraction time were positively correlated with the
303 extraction yield. Optimal conditions for the extraction of proteins were: a
304 ChCl:AA:$H_2O$ DES at 1:1:10 molar ratio using an HIFU amplitude of 60% for 11 min.
305 Under these conditions, it was possible the extraction of 20 ± 1 mg protein/g peel. This
306 is more than twice the amount of proteins extracted by PLE (9 ± 1 mg/g) and higher

307 than those extracted by the non-sustainable methodology (15 ± 2 mg/g) (Hernández-
308 Corroto et al., 2019).

309

310 *3.2. Fitting of protein extracts to release peptides by enzymatic hydrolysis*

311 Extracted proteins were next hydrolyzed with alcalase and thermolysin, under
312 previously optimized conditions, in order to obtain peptides (Hernández-Corroto et al.,
313 2019). For that purpose, the extract obtained by PLE was evaporated and next dissolved
314 in a suitable digestion buffer.

315 Since the extract obtained using ChCl:AA DES showed a pH < 3 and alcalase
316 and thermolysin enzymes activity at this pH was very low, it was necessary the
317 precipitation of proteins and their solubilization in a more suitable buffer. Proteins were
318 precipitated with EtOH (Bai et al., 2017) and the resulting pellet was dissolved in 100
319 mM borate buffer (pH 9), since the pellet could not be dissolved in a buffer with a lower
320 concentration.

321 Peptide content in the hydrolysates of the extract obtained by PLE was 0.3 ± 0.1
322 mg/mL, when using alcalase enzyme, and 0.58 ± 0.02 mg/mL, when using thermolysin,
323 while the peptide content in the hydrolysates obtained from proteins extracted using the
324 DES was 0.9 ± 0.1 mg/mL, in the case of alcalase enzyme, and 0.8 ± 0.1 mg/mL, in the
325 case of thermolysin. These peptide concentrations are lower than the observed in the
326 non-sustainable method (2.9 ± 0.1 mg/mL and 2.2 ± 0.1 mg/mL in the hydrolysates
327 obtained with alcalase and thermolysin, respectively). In the case of the extract obtained
328 by PLE, the lower peptide concentration can be attributed to the lower protein
329 concentration of the PLE extract, while in DES hydrolysates, the lower peptide
330 concentration can be because only part of the extracted proteins precipitated with EtOH.

331 Indeed, extracted proteins decreased from 20 to 5.2 mg protein/g peel after precipitation
332 of proteins with EtOH.

333

334 *3.3. Identification of peptides in the hydrolysates by HPLC-ESI-Q-TOF*

335 Peptides present in hydrolysates were analyzed by RP-HPLC-ESI-Q-TOF.
336 Fig.2A shows the total ion chromatogram (TIC) of the hydrolysates obtained by PLE (a)
337 or with the DES (b) using alcalase enzyme and the mass spectra corresponding to three
338 peptides. Higher amounts of peptides and intensity were observed in the hydrolysates
339 from proteins extracted using the DES which can be explained taking into account the
340 higher peptide content in hydrolysate obtained from the DES extract. Moreover, 23
341 different peptides were identified in this extract, while only 14 peptides were found in
342 the hydrolysate from proteins obtained by PLE (see Table 5). Fig.2B compares the TICs
343 of hydrolysates obtained using thermolysin. Again, more intense signals were observed
344 for the hydrolysate obtained from the DES extract. In this case, the number of identified
345 peptides was 4, in the hydrolysate obtained from proteins extracted by PLE, and 20, in
346 the hydrolysate obtained from proteins extracted with the DES. A similar situation was
347 observed when the protein isolate obtained using the non-sustainable method was
348 analyzed. In this case, 26 peptides were identified in the hydrolysates obtained with
349 alcalase and 16 peptides in the case of the hydrolysate obtained with thermolysin.

350 Peptides contained between 4 and 9 amino acids, in hydrolysates from proteins
351 obtained by PLE, and between 4 and 13 amino acids, in hydrolysates obtained using
352 DES (using alcalase enzyme in both cases). Some peptides in the hydrolysate obtained
353 from the DES extract were found in BIOPEP database (Minkiewicz et al., 2008) such as
354 peptides KVLL, responsible for antioxidant activity, KVLI and KVIL, with ACE and
355 dipeptidyl peptidase III (DPP-III) inhibitor activities, respectively, and FEEI, with

356 antithrombotic activity. Peptides in the hydrolysate obtained from the PLE extract using
357 thermolysin contained only 4 amino acids while peptides in hydrolysates obtained from
358 the DES extract, using the same enzyme, presented between 4 and 11 amino acids.
359 Some of these peptides were described in BIOPEP database like ILSS and IISS, with
360 antioxidant and antibacterial activities, respectively, LLEK, with calpain inhibitor and
361 antioxidant activities, and ILEK, with antibacterial activities. Most peptides observed in
362 hydrolysates had a molecular weight below 1 kDa.

363 Peptides in hydrolysates obtained with alcalase presented a higher amount of
364 hydrophobic amino acids than peptides in hydrolysates obtained with thermolysin.
365 Amino acids leucine/isoleucine (L/I) and valine (V) highlighted within peptides
366 released from proteins obtained by PLE and DES. The presence of aromatic amino acids
367 was higher in hydrolysates obtained with alcalase than in hydrolysates obtained with
368 thermolysin. Hydrophobic and aromatic amino acids could contribute to antioxidant
369 capacity (Erdmann et al., 2008; Hernández-Corroto et al., 2018). Furthermore, acidic
370 amino acids were identified in hydrolysates from proteins obtained by PLE and using
371 the DES. The presence of these amino acids has been related to hypocholesterolemic
372 peptides (Hernández-Corroto et al., 2019; Prados et al., 2018). This fact could also
373 explain the acidic isoelectric points (pI) observed for identified peptides. Most
374 hydrolysates obtained with alcalase showed poor water solubility, which is related to
375 that fact that these peptides presented high content in hydrophobic and aromatic amino
376 acids. The presence of these amino acids have been linked to a high antioxidant
377 capacity. Unlike them, hydrolysates obtained with thermolysin showed a lower content
378 in hydrophobic amino acids and, thus, they presented a good water solubility (calculated
379 by Peptide2.0). This feature has been observed within hypocholesterolemic peptides
380 (Zanoni et al., 2017). Peptides in hydrolysates obtained with thermolysin also showed a

381 high amount in phenylalanine (F), tyrosine (Y), and lysine (K) as C-terminus amino
382 acids which seems to be a common characteristic in ACE inhibitory peptides (Erdmann
383 et al., 2008). These solubilities and isoelectric points were obtained using Innovagen's
384 peptide property calculator. Surprisingly, the highest percentage of peptides with F, Y,
385 or K as C-terminus amino acids was found in the hydrolysate obtained using
386 thermolysin from the DES extract.

387 Hydrolysates from proteins extracted using the DES, which contained higher
388 amounts of peptides, were also analyzed by HILIC. Only one peptide (HPVLV) was
389 identified in the hydrolysate obtained with alcalase while four additional peptides
390 (VTYDYYEL, LSGGPMVVAHE, MPVVAEH, and ARAR) were observed in the
391 hydrolysate obtained with thermolysin. Mass spectra are displayed in Fig.S2.

392 Some identified peptides in Table 5 were also found in hydrolysates obtained
393 from proteins extracted using the non-sustainable method (Hernández-Corroto et al.,
394 2019). Additional peptides, shown in Table S1, were also in common among peptides
395 when ALC was reduced at 80% or when these peptides appeared, at least, in one
396 replicate of the hydrolysates obtained by green methodologies. Higher amount of
397 common peptides was found in hydrolysates with alcalase.

398 Furthermore, some of these peptides could be assigned to proteins from *Punica*
399 *granatum*. Table 6 grouped the name of three proteins and the number of peptides from
400 Table 5 that were within their sequences. A more detailed description of these peptides
401 is in Table S2.

402

403 *3.4. Evaluation of the antioxidant capacity*

404 Protection capacity against oxidation damage of hydrolysates and extract was
405 evaluated. Fig.4 shows that all hydrolysates and extracts presented a high antioxidant
406 capacity. Extract and hydrolysates obtained by PLE presented higher antioxidant
407 capacity than the extract and hydrolysates obtained using the DES. Indeed, it was
408 necessary a 1:12 dilution in the extract obtained by PLE and in hydrolysates to avoid
409 signal saturation. No significant differences were observed between the antioxidant
410 capacity of hydrolysates obtained with alcalase and thermolysin enzymes and that of the
411 extract obtained by PLE. Probably, polyphenols were coextracted along with proteins
412 contributing to the antioxidant capacity while contribution of peptides released from
413 extracted proteins was minimal.

414 Hydrolysate obtained using alcalase from the DES extract showed a slightly
415 more antioxidant capacity than the hydrolysate obtained with thermolysin. This fact
416 could be justified taking into account the higher amount of hydrophobic amino acids in
417 peptides released with alcalasa. In addition, the assay evaluating the capacity to
418 scavenge ABTS radicals showed a higher antioxidant capacity for the hydrolysate
419 obtained with alcalase than for the non-hydrolyzed extract.

420 Antioxidant capacity for the protein isolate and its hydrolysates, obtained by the
421 non-sustainable method (77–83% inhibition of hydroxyl radical formation and 68–74%
422 scavenging of ABTS radicals, previous three times dilution of extracts) (Hernández-
423 Corroto et al., 2019), were similar to the observed when using the DES but much lower
424 than the observed when using PLE. The PLE method probably co-extracted phenolic
425 compounds that highly contribute to the observed antioxidant capacity while the extract
426 obtained using the DES and the extract from the non-sustainable method showed a
427 higher contribution of proteins to the antioxidant capacity.

428 *3.5. Evaluation of hypocholesterolemic capacity*

429 Hypocholesterolemic capacity of the extracts and hydrolysates were grouped in
430 the Fig.5. The hypocholesterolemic capacity of both extracts was always higher or
431 similar to that of hydrolysates. Hydrolysates obtained with thermolysin presented higher
432 capacity to inhibit cholesterol esterase enzyme and to reduce micellar cholesterol
433 solubility than the hydrolysates obtained with alcalase with the exception of the
434 hydrolysate obtained with thermolysin from the DES extract. This fact can be explained
435 taking into account the lower amount of hydrophobic amino acids observed in the
436 hydrolysates obtained with thermolysin. Furthermore, the extract obtained by PLE
437 showed the highest inhibition of cholesterol esterase. This fact could be attributed to the
438 intact proteins or to polyphenols co-extracted with proteins by PLE. The hydrolysis of
439 this extract resulted in a reduction of the capacity to inhibit cholesterol esterase that
440 could be due to two reasons. If capacity of the extract was due to the extracted proteins,
441 probably peptides released during the hydrolysis have no capacity to inhibit the
442 cholesterol esterase enzyme. If capacity of the extract was due to the presence of
443 phenolic compounds, probably they have been degraded under hydrolysis conditions.
444 Neither proteins nor phenolic compounds seem to contribute to the capacity to reduce
445 micellar cholesterol solubility that can be attributed just to the released peptides.

446 The capacity to reduce cholesterol esterase of the DES extract and its
447 hydrolysates was significantly lower than the observed for the PLE extract and
448 hydrolysates. This fact could be related to the lower amount of phenolic compounds in
449 the extract obtained by the DES. Different results were observed for the protein isolate
450 obtained by the non-sustainable method (Hernández-Corroto et al., 2019). Probably
451 proteins extracted by this method are different from those extracted using DES or PLE.

452

453 *3.6. Evaluation of antihypertensive capacity*

454 ACE inhibition capacity is shown in Fig.6. Hydrolysates obtained from proteins
455 extracted with the DES presented higher percentage of ACE inhibition than the
456 precursor extract. $IC_{50}$ values of these hydrolysates were 28 ± 1 μg/mL, when using
457 alcalase, and 23 ± 5 μg/mL, when using thermolysin. This fact demonstrated that main
458 contributors to antihypertensive capacity in hydrolysates were released peptides.
459 Peptides released from proteins extracted using DES showed a higher antihypertensive
460 capacity than those obtained from proteins extracted by the non-sustainable method (75
461 ± 8 μg/mL, when using alcalase, and 49 ± 3 μg/mL, when using thermolysin)
462 (Hernández-Corroto et al., 2019). Taking into account the higher peptide concentration
463 in hydrolysates from the non-sustainable method (2.9 ± 0.1 mg/mL and 2.2 ± 0.1
464 mg/mL in the hydrolysate obtained with alcalase and thermolysin, respectively), it is
465 possible to confirm that peptides released from the DES extract are much more
466 antihypertensive than those obtained by the non-sustainable method. This supports that
467 proteins extracted by each method were different.

468 On the other hand, hydrolysates from the extract obtained by PLE showed a
469 lower ACE inhibition percentage, which is likely related to the smaller peptide
470 concentration of hydrolysates. This inhibition percentage was even lower than the
471 observed for the extract obtained by the non-sustainable method.

472

473 *3.7. Identification of polyphenols in the hydrolysates by RP-HPLC-ESI-Q-TOF*

474 Since results have suggested that polyphenols could be coextracted in the
475 extraction of proteins using DES or PLE and contribute to the antioxidant and
476 hypocholesterolemic capacity, a study of its presence was next carried out. For that
477 purpose, hydrolysates were analyzed by RP-HPLC-ESI-Q-TOF. Fig.3 shows the

478 chromatogram obtained by RP-HPLC at 260 nm. Twenty different peaks were assigned
479 to polyphenols in hydrolysates from the extract obtained by PLE, confirming previous
480 suspicions. Furthermore, these polyphenols were previously identified and described in
481 the hydrolysate obtained by the non-sustainable method (Hernández-Corroto et al.,
482 2019). Mass spectra of identified polyphenols are in the Fig.S3. Especially interesting
483 was the presence of punicalin (peak 5) and punicalagin (peaks 11 and 13) due to its high
484 bioactivity (ref). Unlike these results, extraction with DES was more selective for
485 proteins and it hardly extracted polyphenols. Only three peaks, corresponding to
486 galloyl-HHDP-hexoside, ellagic acid-hexoside, and ellagic acid, with extremely reduced
487 intensities were observed in the Fig.3b. A similar situation was observed when
488 analyzing the hydrolysates obtained with thermolysin. These results demonstrated that
489 the PLE was less selective for pomegranate peel proteins and coextracted phenolic
490 compounds while the selected DES mainly extracted proteins.

491

492

493 **Conclusions**

494 Two different green methods, based on pressurized liquid extraction (PLE) and
495 on a deep eutectic solvent (DES), have been developed for the extraction of proteins and
496 bioactive peptides from pomegranate peel. Antioxidant and hypocholesterolemic
497 capacities observed in the PLE extract and its hydrolysates were attributed to the
498 coextraction of phenolic compounds and to the intact proteins. A high antihypertensive
499 capacity was observed in hydrolysates from proteins extracted by DES, which was
500 attributed to released peptides.

501 A higher number of peptides were identified in hydrolysates obtained from the
502 DES extract than in hydrolysates obtained from the PLE extract. Some of these peptides
503 were assigned to proteins from *Punica granatum*. Identified peptides showed common
504 features within antioxidant, hypocholesterolemic, and antihypertensive peptides. A
505 higher number and amount of phenolic compounds were observed in the hydrolysates
506 obtained from the PLE extract. Developed green methodologies enabled to obtain
507 extracts and hydrolysates from pomegranate peels with higher bioactivity than the
508 observed when using a previous non-sustainable method. Selection of the most suitable
509 extracting method will depend, in each case, on the desired bioactivity.

510

511

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517

518

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653

654 **Figure captions**

655 **Figure 1.** 3-D contour plot showing the effect of the extraction time (min), the HIFU
656 probe amplitude (%), and the molar ratio of water in the DES at different acetic acid
657 molar ratios (AA = 1, 2, and 3) on the protein content (mg protein/g peel).

658

659 **Figure 2.** TIC corresponding to the pomegranate peel hydrolysates obtained from the
660 PLE extract (a) and from the DES extract (b) using alcalase (A) and thermolysin (B)
661 and mass spectra of three common peptides.

662

663 **Figure 3**. Chromatograms monitorized at 260 nm corresponding to the hydrolysates
664 obtained with alcalase and thermolysin enzymes from PLE extract and from the DES
665 extract. Peaks identification: 1, HHDP-hexoside; 2, galloyl-hexoside; 3, gallloyl-
666 HHDP-gluconate; 4, gallic acid; 5, punicalin; 6, pedunculagin I; 7, pedunculagin III; 8,
667 digalloyl-hexoside; 9, gallocatechin; 10, valoneic acid dilactone; 11, punicalagin α; 12,
668 punicalagin isomer; 13, punicalagin β; 14, pedunculagin II; 15, galloyl-HHDP-
669 hexoside; 16, digalloyl-gallagyl-hexoside; 17, ellagic acid-hexoside; 18, ellagic acid-
670 pentoside; 19, ellagic acid; 20, ellagic acid-deoxyhexoside.

671

672 **Figure 4**. Capacity to inhibit the formation of hydroxyl radicals and to scavenge ABTS
673 free radicals of pomegranate peel extracts obtained by PLE or using a DES and their
674 hydrolysates obtained with alcalase or thermolysin enzymes. Significant differences are
675 indicated by a letter (a-c).

676

677 **Figure 5**. Capacity to inhibit cholesterol esterase enzyme and to decrease cholesterol
678 micellar solubility of pomegranate peel extracts obtained by PLE or using a DES and

679 their hydrolysates obtained with alcalase or thermolysin enzymes. Significant
680 differences are indicated by a letter (a-c).
681
682 **Figure 6**. Antihypertensive activity of pomegranate peel extracts obtained by PLE or
683 using the DES and their hydrolysates obtained with alcalase or thermolysin enzymes.
684 Significant differences are indicated by a letter (a-b).
685
686

687 Table 1. Hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) used in the
688 synthesis of deep eutectic solvent (DES).

| Component 1 (HBA) | Component 2 (HBD) | Abbreviation | Reference |
|---|---|---|---|
| Choline Chloride | Urea | ChCl:urea | Moore et al., 2016; Bai et al., 2017; Sanchez-Fernandez et al., 2017; Jiang et al., 2018; Pal & Jadeja, 2019 |
| Choline Chloride | Ethylene glycol | ChCl:EG | Xu et al., 2015; Bai, et al., 2017; Ozturk et al., 2018; Gullón et al., 2019 |
| Choline Chloride | Glycerol | ChCl:gly | Xu et al., 2015; Bai et al., 2017; Sanchez-Fernandez et al., 2017; Grudniewska et al., 2018; Ozturk et al., 2018 |
| Choline Chloride | Acetic acid | ChCl:AA | Bai et al., 2017 |
| Choline Chloride | Glucose | ChCl:gluc | Xu et al., 2015; Gullón et al., 2019 |
| Choline Chloride | Sorbitol | ChCl:sorb | Xu et al., 2015 |
| Choline Chloride | Citric acid | ChCl:CA | Lores et al., 2017 |
| Sodium acetate | Urea | NaOAc:urea | Wahlström et al., 2017 |

689
690

691 Table 2. Optimized parameters for the extraction of proteins from pomegranate peel by
692 PLE.

| Concentration of EtOH (%) | Temperature (ºC) | Cycles | Time (min) | Additives | Protein content (mg prot/g peel) |
|---|---|---|---|---|---|
| 100 | 120 | 1 | 3 | - | 4.5 ± 0.4 |
| 80 | 120 | 1 | 3 | - | 7.9 ± 0.8 |
| **70** | **120** | **1** | **3** | **-** | **9 ± 1** |
| 60 | 120 | 1 | 3 | - | 8 ± 1 |
| 50 | 120 | 1 | 3 | - | 5.4 ± 0.5 |
| 40 | 120 | 1 | 3 | - | 5.2 ± 0.4 |
| 30 | 120 | 1 | 3 | - | 5.3 ± 0.5 |
| 0 | 120 | 1 | 3 | - | 4.5 ± 0.6 |
| 70 | 21 | 1 | 3 | - | 6.2 ± 0.8 |
| 70 | 50 | 1 | 3 | - | 6.6 ± 0.6 |
| 70 | 100 | 1 | 3 | - | 8.3 ± 0.9 |
| **70** | **120** | **1** | **3** | **-** | **9 ± 1** |
| 70 | 150 | 1 | 3 | - | 8 ± 1 |
| **70** | **120** | **1** | **3** | **-** | **9 ± 1** |
| 70 | 120 | 3 | 3 | - | 9 ± 1 |
| 70 | 120 | 5 | 3 | - | 9 ± 1 |
| **70** | **120** | **1** | **3** | **-** | **9 ± 1** |
| 70 | 120 | 1 | 15 | - | 7.9 ± 0.9 |
| **70** | **120** | **1** | **3** | **-** | **9 ± 1** |
| 70 | 120 | 1 | 3 | 0.25% DTT | 8.7 ± 0.6 |
| 70 | 120 | 1 | 3 | 0.25% DTT + 3 M urea | 9 ± 1 |

693 Bold conditions were selected for next experiment
694

695 Table 3. Protein content of extracts obtained using different DES.

| DES | Protein content (mg prot/g peel) |
| --- | --- |
| ChCl:urea | 14 ± 1 |
| ChCl:EG | 13 ± 1 |
| ChCl:gly | 7.1 ± 0.2 |
| **ChCl:AA** | **15 ± 1** |
| ChCl:gluc | 5 ± 3 |
| ChCl:sorb | 4 ± 1 |
| ChCl:CA | 6 ± 2 |
| NaOAc:urea | 11 ± 5 |

696 Bold conditions were selected for next experiments.

697
698

699 Table 4. Optimization of different parameters for the extraction of proteins from
700 pomegranate peel using ChCl:AA DES.

| Experiment number | Variables | | | | | Response Variable |
|---|---|---|---|---|---|---|
| | ChCl (molar ratio) | AA (molar ratio) | $H_2O$ (molar ratio) | Amplitude (%) | Time (min) | Protein content (mg prot/g peel) |
| 1 | 1 | 3 | 20 | 45 | 8 | 16.6 ± 0.5 |
| 2 | 1 | 2 | 15 | 60 | 1 | 15 ± 1 |
| 3 | 1 | 2 | 10 | 45 | 15 | 16 ± 1 |
| 4 | 1 | 3 | 15 | 45 | 1 | 15 ± 2 |
| 5 | 1 | 2 | 15 | 45 | 8 | 17 ± 1 |
| 6 | 1 | 2 | 10 | 30 | 8 | 17.5 ± 0.4 |
| 7 | 1 | 2 | 15 | 45 | 8 | 17 ± 1 |
| 8 | 1 | 1 | 20 | 45 | 8 | 17.0 ± 0.9 |
| 9 | 1 | 2 | 15 | 45 | 8 | 17.0 ± 0.2 |
| 10 | 1 | 2 | 10 | 60 | 8 | 19 ± 1 |
| 11 | 1 | 2 | 15 | 45 | 8 | 17 ± 1 |
| 12 | 1 | 2 | 20 | 30 | 8 | 17.3 ± 0.5 |
| 13 | 1 | 3 | 15 | 45 | 15 | 17.4 ± 0.4 |
| 14 | 1 | 2 | 15 | 30 | 15 | 17.1 ± 0.8 |
| 15 | 1 | 2 | 15 | 45 | 8 | 17 ± 1 |
| 16 | 1 | 2 | 20 | 45 | 1 | 15 ± 1 |
| 17 | 1 | 1 | 15 | 60 | 8 | 19 ± 1 |
| 18 | 1 | 3 | 15 | 30 | 8 | 17.7 ± 0.8 |
| 19 | 1 | 3 | 15 | 60 | 8 | 18.0 ± 0.8 |
| 20 | 1 | 2 | 15 | 60 | 15 | 19.2 ± 0.3 |
| 21 | 1 | 2 | 10 | 45 | 1 | 16.4 ± 0.3 |
| 22 | 1 | 2 | 15 | 30 | 1 | 16 ± 1 |
| 23 | 1 | 1 | 15 | 30 | 8 | 17.7 ± 0.4 |
| 24 | 1 | 1 | 15 | 45 | 1 | 17 ± 1 |
| 25 | 1 | 1 | 10 | 45 | 8 | 18.0 ± 0.3 |
| 26 | 1 | 2 | 20 | 60 | 8 | 18 ± 1 |
| 27 | 1 | 2 | 20 | 45 | 15 | 17.8 ± 0.4 |
| 28 | 1 | 3 | 10 | 45 | 8 | 16.8 ± 0.9 |
| 29 | 1 | 1 | 15 | 45 | 15 | 17 ± 1 |

701 Table 5. Sequences and characteristics of peptides identified in hydrolysates from extracts obtained by PLE or using a DES with alcalase and
702 thermolysin enzymes using RP-HPLC- and HILIC-QTOF[a].

| | Alcalase enzyme | | | | Thermolysin enzyme | | | |
|---|---|---|---|---|---|---|---|---|
| | **Peptide sequence** | **RT (min)** | **Mass (Da)** | **Ip[b]** | **Peptide sequence** | **RT (min)** | **Mass (Da)** | **Ip[b]** |
| **Peptides obtained by PLE hydrolysates using RP-HPLC** | YYGK | 2.08 | 529.2537 | 9.33 | HVNR | 1.09 | 524.2819 | 10.59 |
| | NAGDY | 2.79 | 538.2023 | 0.74 | LVSE | 2.75 | 446.2376 | 1.00 |
| | STYPTN | 3.47 | 681.2969 | 3.43 | LLSS | 3.51 | 418.2427 | 3.72 |
| | NEGTL | 4.92 | 532.2493 | 0.92 | FADY | 19.41 | 514.2063 | 0.74 |
| | FLGGQ | 15.30 | 520.2645 | 3.45 | | | | |
| | YDTL | 16.88 | 510.2326 | 0.69 | | | | |
| | ADGAELEVF | 26.22 | 949.4392 | 0.65 | | | | |
| | WNNF | 26.41 | 579.2441 | 3.58 | | | | |
| | YVLV | 27.23 | 492.2948 | 3.37 | | | | |
| | VFDNL | 28.96 | 606.3013 | 0.69 | | | | |
| | AYVLV | 29.43 | 563.3318 | 3.65 | | | | |
| | FYDTL | 30.12 | 657.3010 | 0.69 | | | | |
| | TFYDTL | 32.57 | 758.3486 | 0.69 | | | | |
| | VAFDNV | 33.81 | 663.3228 | 0.72 | | | | |
| **Peptides obtained by HIFU hydrolysates with DES using RP-HPLC** | APPPGPH | 2.12 | 671.3391 | 7.88 | LYSK | 1.87 | 509.2849 | 9.74 |
| | DFGGH | 2.83 | 531.2078 | 4.87 | VVAAE | 2.35 | 487.2642 | 1.00 |
| | SLGGASGSTAFQQ | 3.47 | 1209.5625 | 3.43 | LLEK | 3.09 | 501.3162 | 6.85 |
| | KGTTF | 3.65 | 552.2908 | 9.91 | LLSS | 3.72 | 418.2427 | 3.72 |
| | KDLDLK | 5.24 | 730.4225 | 6.57 | WTSSTTAGK | 4.38 | 937.4505 | 10.01 |
| | HVGEL | 7.65 | 553.2860 | 5.10 | VTDYT | 4.59 | 597.2646 | 0.75 |

| | Alcalase enzyme | | | | Thermolysin enzyme | | | |
|---|---|---|---|---|---|---|---|---|
| | Peptide sequence | RT (min) | Mass (Da) | Ip[b] | Peptide sequence | RT (min) | Mass (Da) | Ip[b] |
| | ALYE | 13.06 | 494.2376 | 1.00 | LVSEADANN | 6.05 | 931.4247 | 0.71 |
| | FYDTT | 17.54 | 645.2646 | 0.75 | LENY | 8.29 | 537.2435 | 0.95 |
| | SKFYDTT | 18.45 | 860.3916 | 6.36 | LLYK | 9.89 | 535.3370 | 9.74 |
| | KVLL | 18.79 | 471.3420 | 9.91 | LTEDVDAH | 9.91 | 898.4032 | 3.54 |
| | VVDL | 21.32 | 444.2584 | 0.69 | LSYE | 10.38 | 510.2325 | 1.00 |
| | DLPGLK | 24.32 | 641.3748 | 6.66 | LVNYD | 12.26 | 622.2962 | 0.88 |
| | FEEL | 25.37 | 536.2482 | 0.76 | LLNEPT | 15.38 | 685.3646 | 0.97 |
| | NNFL | 25.77 | 506.2489 | 3.21 | YDTTY | 18.02 | 661.2595 | 0.74 |
| | WNNF | 26.47 | 579.2441 | 3.58 | LEGDL | 19.39 | 545.2697 | 0.63 |
| | EHPVLL | 26.80 | 706.4014 | 5.10 | VGAGGF | 19.55 | 506.2489 | 3.67 |
| | AVPLLAK | 30.19 | 710.4691 | 10.19 | FADY | 19.97 | 514.2063 | 0.74 |
| | FYDTL | 30.26 | 657.3010 | 0.69 | LYSKF | 22.76 | 656.3533 | 9.74 |
| | KDFPFPN | 30.76 | 863.4177 | 6.43 | HTMEEYSTT | 26.06 | 1097.4336 | 4.14 |
| | LLTF | 32.78 | 492.2948 | 3.70 | FADENF | 27.19 | 741.2969 | 0.70 |
| | TFYDTL | 32.83 | 758.3486 | 0.69 | | | | |
| | AVFDNV | 34.13 | 663.3228 | 0.72 | | | | |
| | NFADYL | 36.53 | 741.3333 | 0.69 | | | | |
| **Peptides obtained by HIFU hydrolysates with DES using HILIC-HPLC** | HPVLV[c] | 10.10 | 563.3431 | 7.56 | VTYDYYEL[c] | 31.55 | 1064.4702 | 0.63 |
| | | | | | LSGGPMVVAHE[c] | 31.69 | 1095.5383 | 5.10 |
| | | | | | MPVVAEH[c] | 32.11 | 781.3793 | 5.10 |
| | | | | | ARAR[c] | 34.83 | 472.2870 | 12.10 |

703 [a] All identified peptides showed ALC > 90%, at least in three replicates.

704 [b] Isoelectric point (Ip) was determined by Innovagen's peptides property calculator.

705 [c] Peptides obtained by HILIC column.

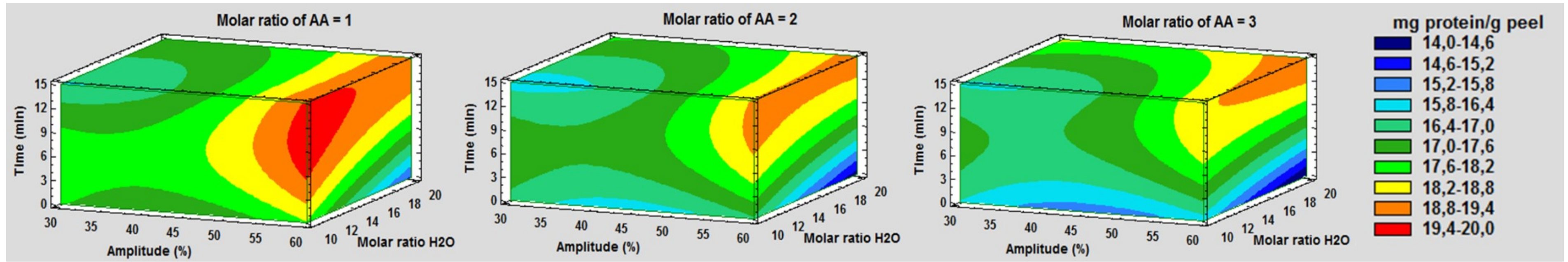
706

707 Table 6. Peptides in hydrolysates obtained from PLE and DES extracts assigned to proteins from *Punica granatum*.

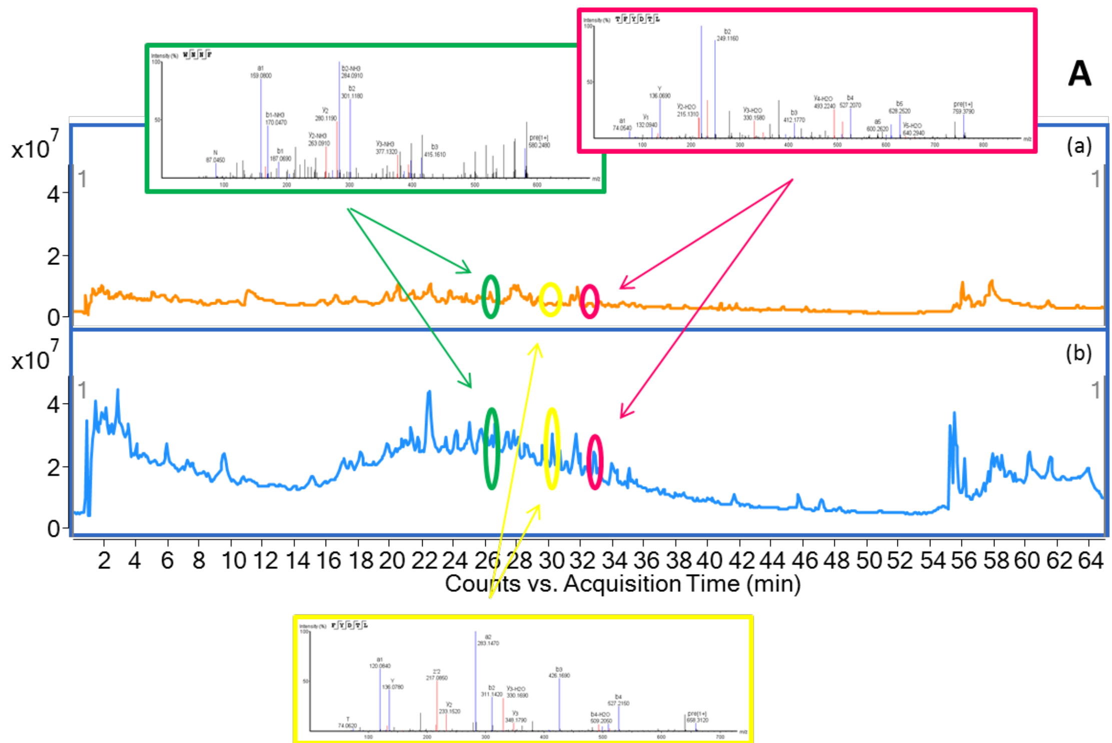
| Accesion number | Gene name | Protein name | Length | Mass (Da) | Number of peptides found in the protein sequence | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | PLE | | DES (RP-HPLC) | | DES (HILIC-HPLC) | |
| | | | | | A | T | A | T | A | T |
| G1UH28 | PSC | Acidic endochitinase Pun g 14, amyloplastic | 299 | 31,747 | 36 | 7 | 50 | 28 | 0 | 2 |
| Q84VT2 | FAD12 | Delta(12)-acyl-lipid-desaturase | 387 | 44,280 | 0 | 0 | 1 | 0 | 0 | 1 |
| Q84UB8 | FADX | Bifunctional fatty acid conjugase/Delta(12)-oleate desaturase | 395 | 45,828 | 0 | 0 | 1 | 0 | 0 | 0 |

708 A: Hydrolysates obtained with alcalase enzyme
709 T: Hydrolysates obtained with thermolysin enzyme

710




711 Fig.1

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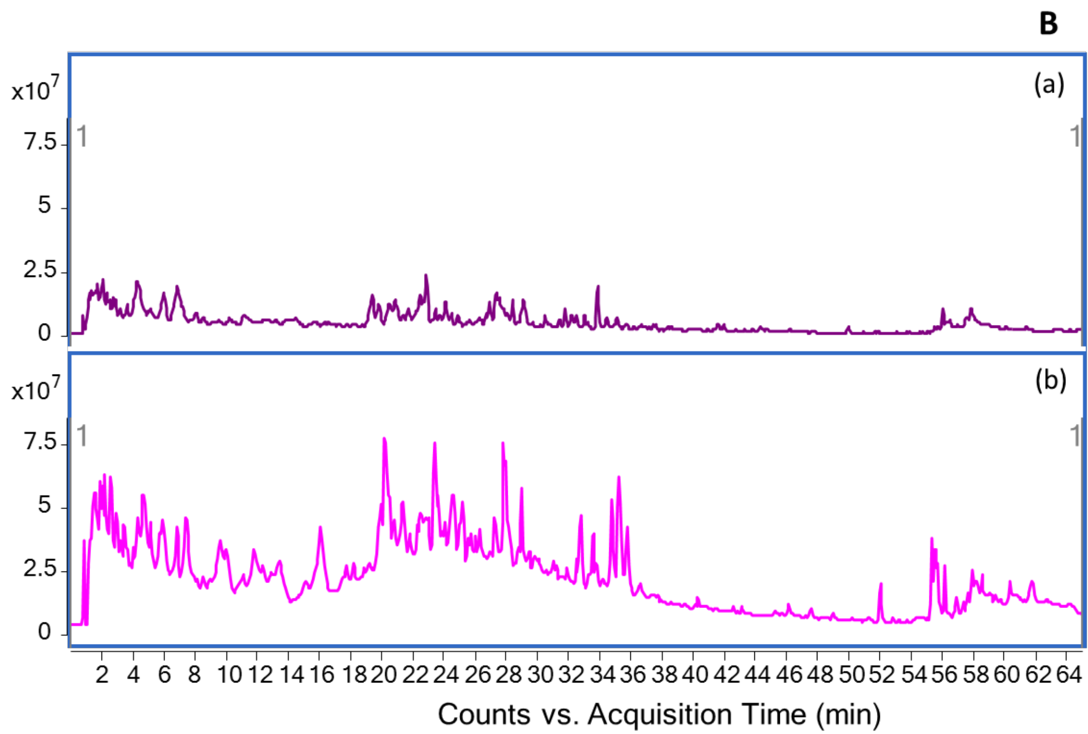
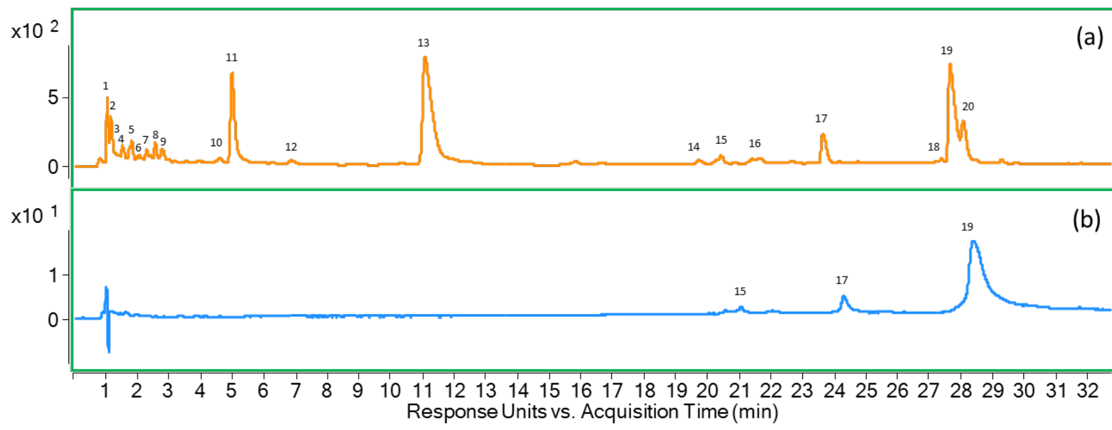



713

714 Fig.2

715

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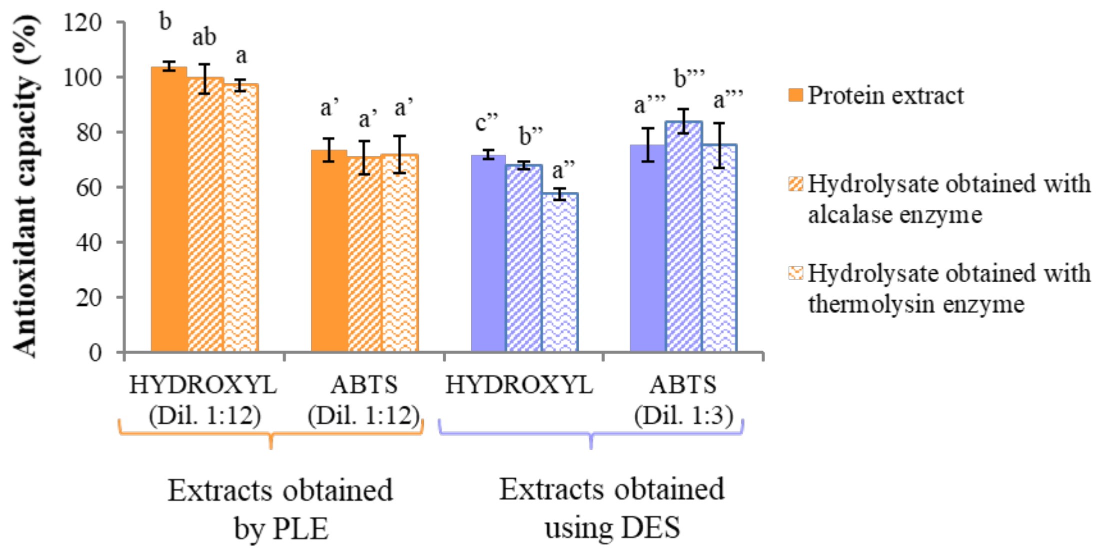
717 Fig.3

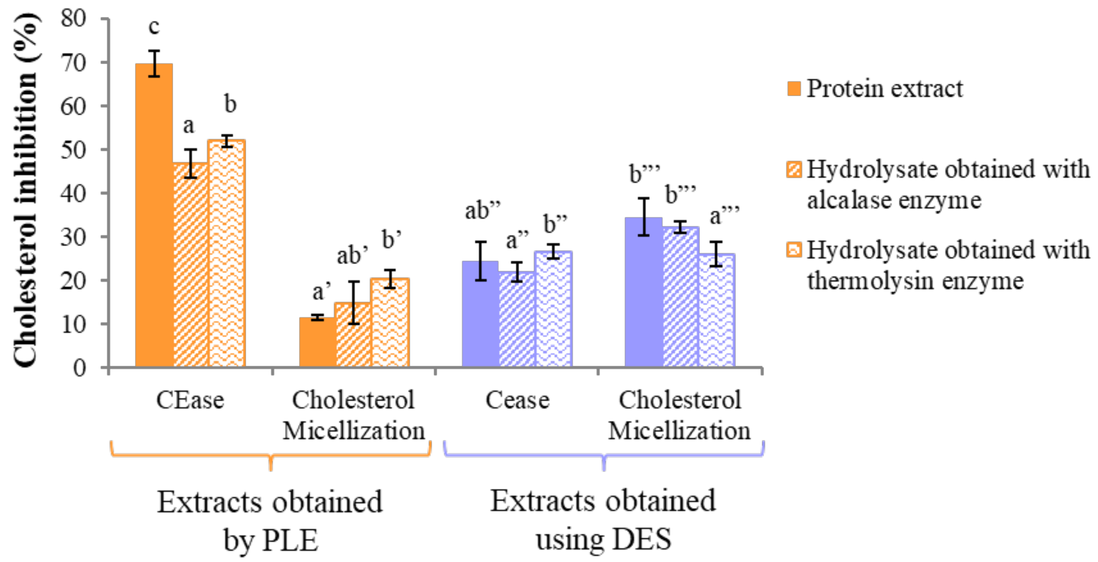
718

719



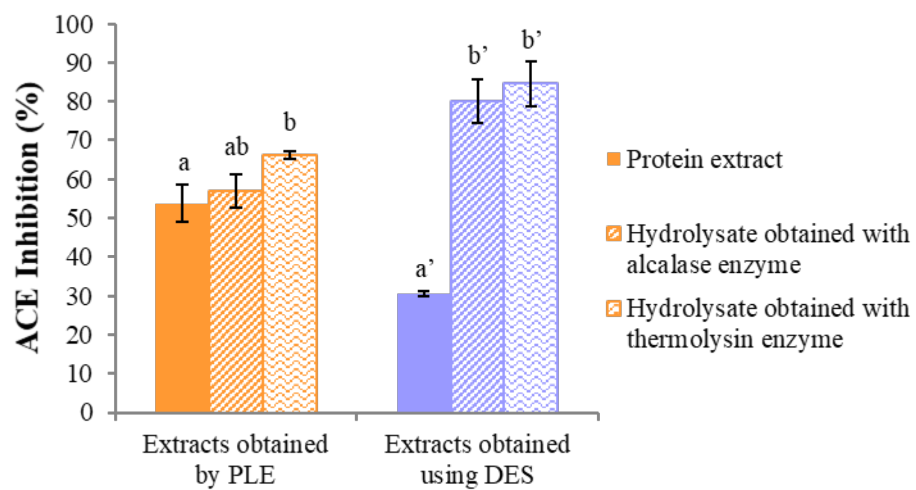

720

721 Fig.4




722

723 Fig.5




724

725 Fig.6

726