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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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ABSTRACT

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

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

1. Introduction

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

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

Pressurized Liquid Extraction (PLE) uses high pressures and temperatures enabling a reduced extraction time, less solvent consumption, high extraction yields, 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

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

2. Materials and methods

2.1 Chemicals and samples

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

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

2.2 Preparation of deep eutectic solvents (DES)

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

2.3 Extraction of proteins and bioactive compounds from pomegranate peels

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

2.3.1 Pressurized Liquid Extraction (PLE)

Extraction of proteins and bioactive substances was performed using an accelerated solvent extractor system (ASE 150, Dionex, Sunnyvale, CA, USA). Solvents were degassed in an ultrasound bath for 30 min. In every extraction, 2 g of dried pomegranate peels were mixed with 8 g sand and put into a 10 mL stainless steel extraction cell. A circular cellulose filter (2.5 cm, Whatman) was placed at the bottom of the extraction cell to prevent suspended particles from entering the collection bottles. Before extraction, the oven was preheated for 6 min. Optimal extraction conditions were: extraction pressure, 1500 psi; extraction solvent, 70% (v/v) EtOH; extraction 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 β_0 is the constant, β_i is the linear regression coefficient, β_{ii} is the quadratic
164 regression coefficient, and β_{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

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

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

2.4 Enzymatic digestion of proteins

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

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

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

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

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

2.5 Evaluation of the functionality of extracts and hydrolysates

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

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

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

Peptides and phenol compounds were identified using a 6530 series high sensitivity mass spectrometry Quadrupole-Time-of-Flight (Q-TOF) coupled to a High-Performance Liquid Chromatograph (HPLC), model 1100, both from Agilent Technologies. Extracts obtained by PLE were filtered using regenerated cellulose 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)*

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

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

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

3.1.2. Extraction using DES

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

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

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

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

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

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

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

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

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

Peptide content in the hydrolysates of the extract obtained by PLE was 0.3 ± 0.1 mg/mL, when using alcalase enzyme, and 0.58 ± 0.02 mg/mL, when using thermolysin, while the peptide content in the hydrolysates obtained from proteins extracted using the DES was 0.9 ± 0.1 mg/mL, in the case of alcalase enzyme, and 0.8 ± 0.1 mg/mL, in the case of thermolysin. These peptide concentrations are lower than the observed in the non-sustainable method (2.9 ± 0.1 mg/mL and 2.2 ± 0.1 mg/mL in the hydrolysates obtained with alcalase and thermolysin, respectively). In the case of the extract obtained by PLE, the lower peptide concentration can be attributed to the lower protein concentration of the PLE extract, while in DES hydrolysates, the lower peptide 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

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

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

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

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

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

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

3.4. Evaluation of the antioxidant capacity

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

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

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

3.5. Evaluation of hypocholesterolemic capacity

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

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

3.6. *Evaluation of antihypertensive capacity*

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

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

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

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

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

Conclusions

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

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

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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

Table 5. Sequences and characteristics of peptides identified in hydrolysates from extracts obtained by PLE or using a DES with alcalase and 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

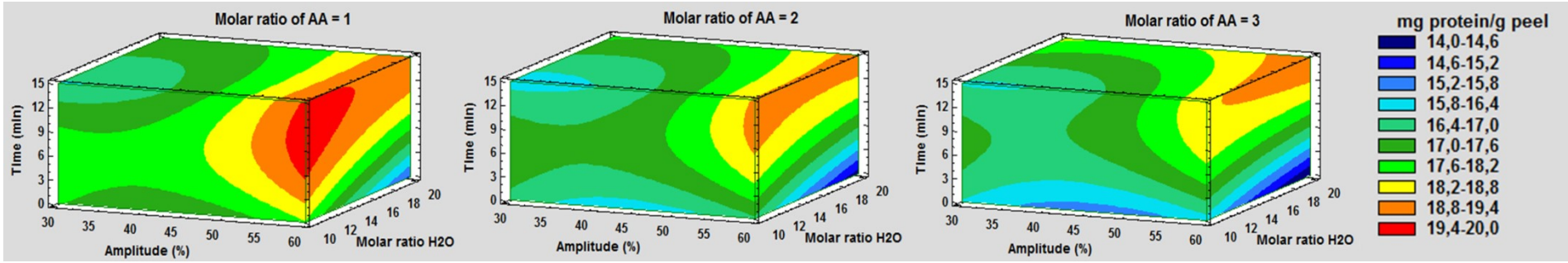
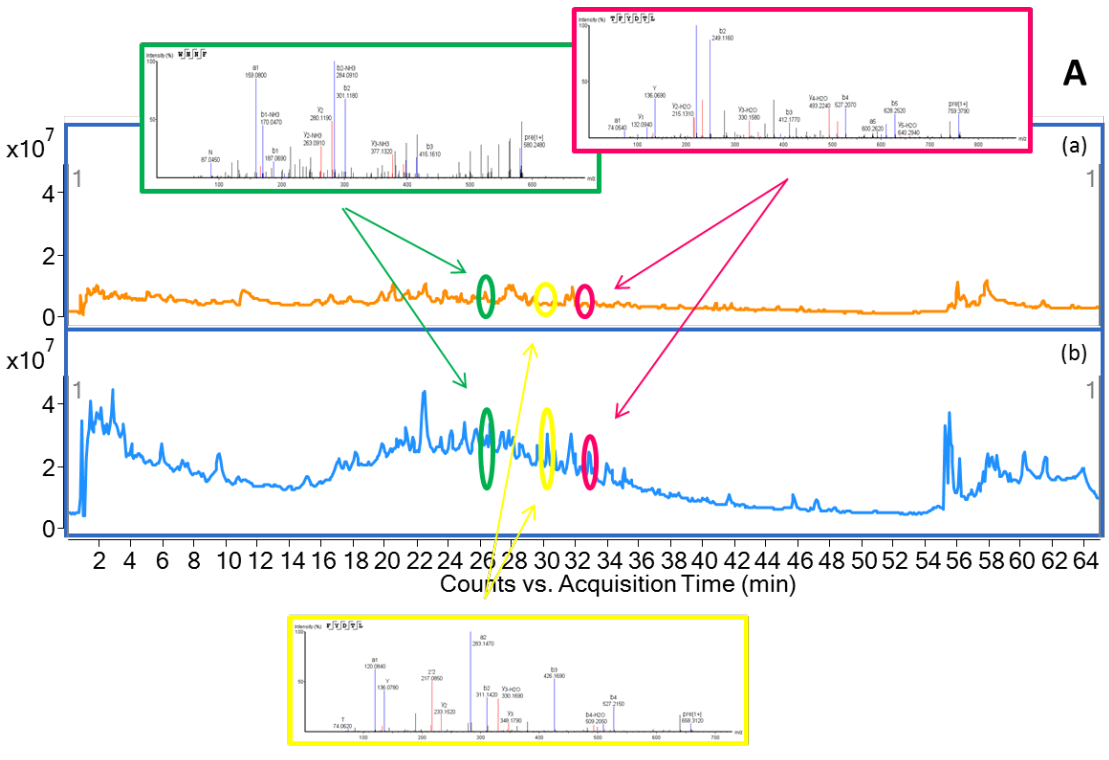
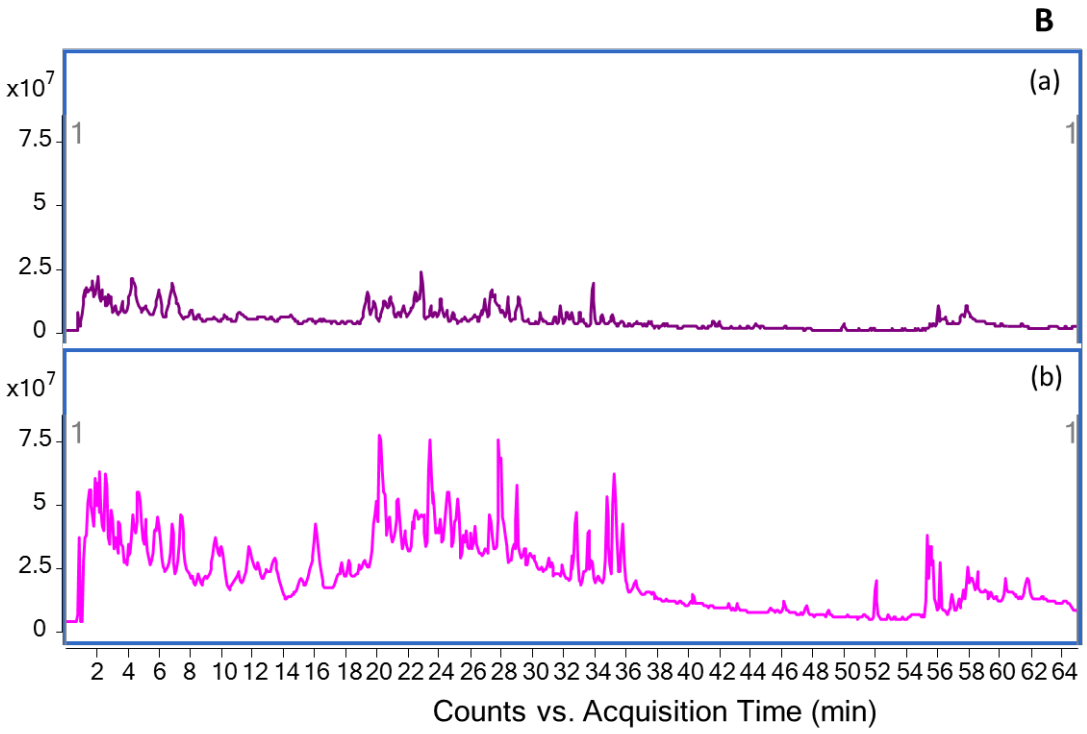

Molar ratio of AA = 1
Molar ratio of AA = 2
Molar ratio of AA = 3
Time (min)
Amplitude (%)
Molar ratio H2O
mg protein/g peel
14,0-14,6
14,6-15,2
15,2-15,8
15,8-16,4
16,4-17,0
17,0-17,6
17,6-18,2
18,2-18,8
18,8-19,4
19,4-20,0

Fig.1



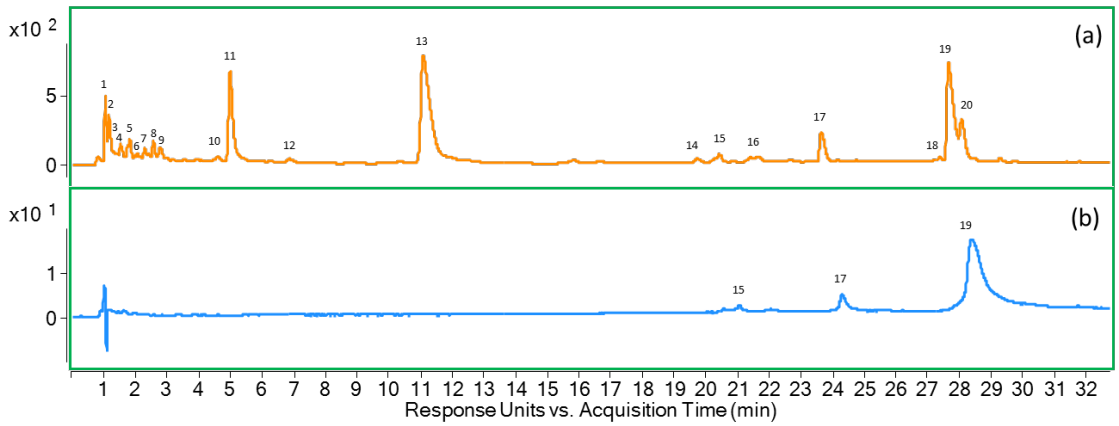
712



713

714 Fig.2

715

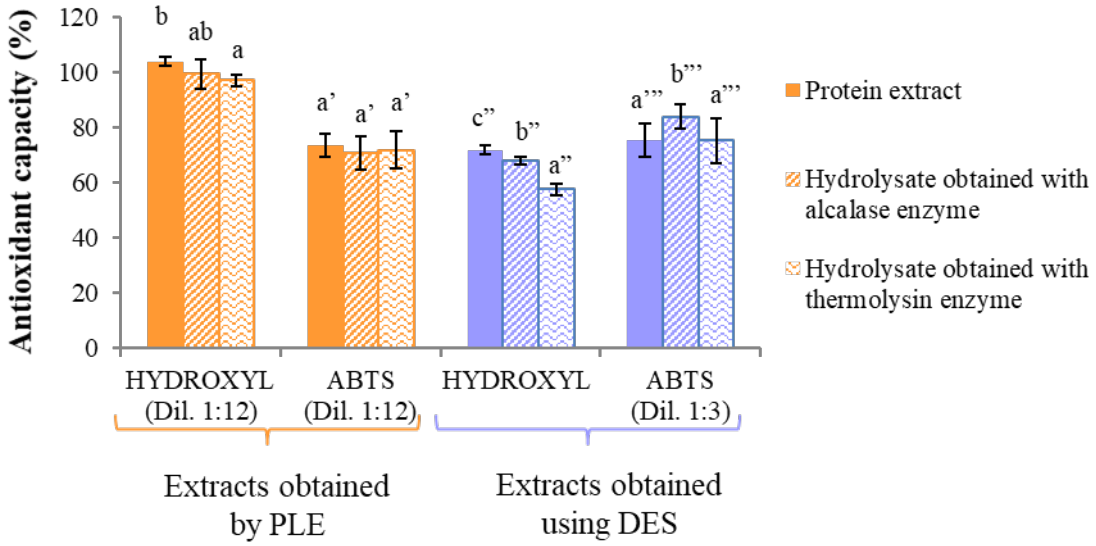


716

717 Fig.3

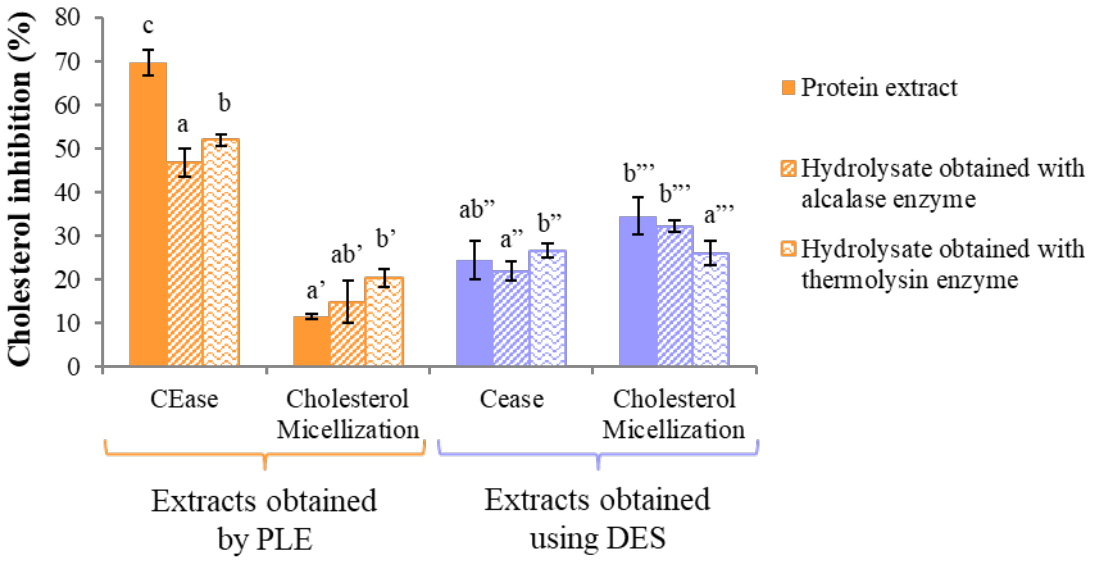
718

719


Antioxidant capacity (%)
120
100
80
60
40
20
0
b
ab
a
a'
a'
a'
c"
b"
a"
a'''
b'''
a'''
HYDROXYL (Dil. 1:12)
ABTS (Dil. 1:12)
HYDROXYL
ABTS (Dil. 1:3)
Extracts obtained by PLE
Extracts obtained using DES
Protein extract
Hydrolysate obtained with alcalase enzyme
Hydrolysate obtained with thermolysin enzyme

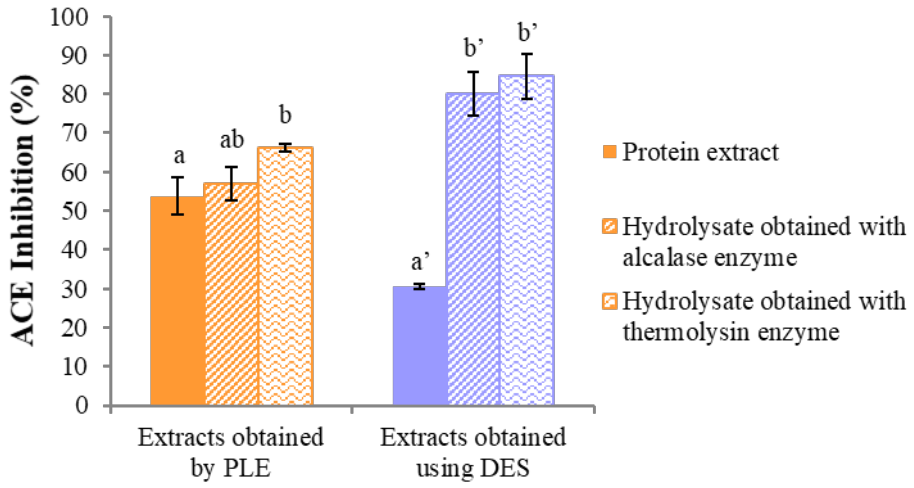
720

721 Fig.4


Cholesterol inhibition (%)
80
70
60
50
40
30
20
10
0
c
a
b
a'
ab'
b'
ab"
a"
b"
b"'
b"'
a"'
Protein extract
Hydrolysate obtained with alcalase enzyme
Hydrolysate obtained with thermolysin enzyme
CEase
Cholesterol Micellization
Cease
Cholesterol Micellization
Extracts obtained by PLE
Extracts obtained using DES

722

723 Fig.5



724

725 Fig.6

726