Proteins isolated from *Ganxet* common bean (*Phaseolus vulgaris* L.) landrace: Technofunctional and antioxidant properties

Ingrid Aguiló-Aguayo[1]*, Carlos Álvarez[2], Montse Saperas[3], Ana Rivera[4, 5], Maribel Abadias[1] and Tomás Lafarga[1,6]

[1] IRTA, Postharvest Programme, Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, Parc de Gardeny, 25003, Lleida, Catalonia, Spain.

[2] Department of Food Quality and Sensory Analysis, Teagasc Food Research Centre, Dublin 15, Ireland.

[3] Grup de Recerca en Cuina i Gastronomia, CETT-UB, Campus Turisme, Hoteleria i Gastronomia, Av. Can Marcet 36-38, 08035, Barcelone, Spain.

[4]Miquel Agustí Foundation, Campus Baix Llobregat, Esteve Terrades 8, 08860 Castelldefels, Spain

[5]Department of Agri-Food Engineering and Biotechnology, Universitat Politècnica de Catalunya, BarcelonaTech, Campus Baix Llobregat, Esteve Terrades 8, 08860 Castelldefels, Spain

[6]Department of chemical Engineering, University of Almeria, Almeria, Spain

***Corresponding authors**: Dr. Aguiló-Aguayo; email: Ingrid.Aguilo@irta.cat; Dr. Lafarga; lpt365@ual.es

Abstract

Proteins isolated from *Ganxet* common beans (GPI) were assessed for antioxidant and functional properties including emulsifying and foaming capacity. The protein content and a_w value of GPI were 91.08 ± 4.15% and 0.248 ± 0.008, respectively. The oil- and water-holding capacities of GPI were calculated as 2.76 ± 0.33 and 1.25 ± 0.11 g/g of GPI, respectively (p<0.05). Foaming and emulsifying properties were found to be pH-dependent (p<0.05). The highest foaming capacity values were observed at pH 8.0 and 10.0 and were calculated as 86.25 ± 5.30 and 78.75 ± 1.77%, respectively. In addition, the generated emulsions were found to be stable, especially at pH 8.0 and 10.0 with emulsion stability values of 94.1 ± 0.0 and 93.9 ± 0.1, respectively (p<0.05). Results obtained in the current study demonstrate the potential applications of *Ganxet*-derived proteins as techno-functional ingredients for the development of novel foods.

Keywords: functional properties, antioxidant activity, vegetable proteins, common beans, Ganxet beans

1. Introduction

Proteins are used in the food industry not only for their nutritional importance but also for their excellent techno-functional properties, which include emulsifying and foaming properties. There is an increasing demand for plant-derived proteins as a techno-functional ingredient and extensive research is devoted to consider legumes as alternative sources of protein. According to Cheng et al. (2019), lesser-known legumes with similar nutritional properties to soybean are still under exploration opening opportunities to different species from the Mediterranean-climate areas. Common beans (*Phaseolus vulgaris* L.) are excellent protein sources, which have between 2 and 3 times as much protein as cereals (Rivera et al,. 2015). Particularly, *Ganxet* bean is a landrace grown in Catalonia (in the northeastern area of the Iberian Peninsula). Its seeds are easily identified by their white colour and the markedly hooked shape, from which its name derives. *Ganxet* bean is characterized by a high content of protein and a large amount of uronic acids in the seed-coat (Casañas et al. 1999; 2006). Proteins derived from Ganxet beans showed good foaming and emulsifying properties previously, especially at acidic and alkaline conditions (Lafarga et al., 2018). However, Ganxet-derived proteins obtained in that study showed lower functionality at neutral pH values, probably because of the extraction methodology. The aim of the present study was to investigate the functional properties of proteins extracted from *Ganxet* beans using food-grade chemicals. Colour, pH, water activity (a_w), WHC, OHC, emulsifying and foaming properties of the extracted proteins were assessed. In addition, the antioxidant capacity and the molecular weight (MW) of the extracted proteins were also evaluated to assess the potential of proteins derived from this valuable bean for use in the food industry.

2. Materials and methods

2.1 Protein extraction and determination

Dried seeds of Ganxet beans were obtained from the Fundació Miquel Agustí (Barcelone, Spain) and milled with a MINIMOKA GR-020 grinder (Taurus Group, Barcelona, Spain) and passed through a sieve of 1 mm. Flours were suspended in distilled water at a sample:solvent ratio of 1:10 (w/v). The suspended samples were sonicated for 1 h using a JP Selecta ultrasonic bath (JP Selecta S.A., Barcelona, Spain) operating at 40 kHz and 250 W. The samples were left to stir overnight on a magnetic stirrer plate at 4 ºC and 350 rpm. After 24 h, the solution was centrifuged at 10,000 × *g* for 20 min and the supernatant decanted. The pellet was re-suspended in half the initial volume of distilled water and subjected to a second extraction as described above. Supernatants from both days were pooled together and saturated to 80% (w/v) with ammonium sulphate for 1 h at 4 ºC followed by centrifugation at 10,000 × *g* for 30 min using a Sigma 3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) to precipitate the protein. Protein precipitates were re-suspended in a minimum volume of water and were dialyzed using Thermo Scientific™ SnakeSkin™ 3.5 kDa MWCO tubing against ultrapure water at 4 ºC overnight. Dialyzed protein extracts were frozen and freeze-dried using a Crydos-50 freeze-dryer (Telstar, Barcelona, Spain). Freezing temperature was -50 ± 2 ºC and drying temperature was kept under 25 ± 1 ºC. Freeze-dried samples, labelled as GPI (*Ganxet* protein isolate), were vacuum-sealed and stored at -20 ºC until further analysis.

The protein content of *Ganxet* beans was determined using a Leco FP 628 Protein Analyser (Leco Corporation, MI, USA). The protein content of the GPI was determined using the Quick Start™ Bradford Protein assay kit (Bio-Rad Laboratories Inc., CA, USA) following the manufacturers' instructions. The protein yield of the process was calculated as g of GPI per 100 g of *Ganxet* bean on a dry weight (DW) basis.

2.2 *In vitro* and *in silico* enzymatic hydrolysis

Enzymatic hydrolysates of the isolated proteins were prepared in triplicate using pepsin and a CelliGen® 115 fermenter (New Brunswick Scientific Co., Cambridge, England) with agitation, temperature, and pH control. A substrate solution was prepared by re-suspending the freeze-dried *Ganxet* isolated proteins in distilled water at a concentration of 20 g/L at a total volume of 500 mL. Agitation, temperature, and pH conditions were adjusted to 350 rpm, 37 ºC, and 2.0, respectively. The enzyme was added once the optimum temperature and pH conditions were achieved in a substrate to enzyme ratio of 100:1 (w/w). After 60 min, the enzyme was heat-deactivated at 90 ºC for 5 min in a water bath. The generated hydrolysate was centrifuged at 10,000 × *g* for 10 min and the supernatant was frozen, freeze-dried, and stored at -20 ºC until further use. The *Ganxet* protein hydrolysate was labelled as GPH. The amino acid sequences of proteins previously reported from *Phaseolus vulgaris* L. were accessed from the UniProtKB database available at http://www.uniprot.org/. These proteins were hydrolysed *in silico* using pepsin and BIOEP-UWM data based was used (Minkiewich *et al*., 2019) (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). Peptides obtained after *in silico* hydrolysis were compared to bioactive peptides obtained in their database.

2.3 HPLC-SEC analysis

Size exclusion analysis was carried out in a Waters Alliance 2795 Chromatography Separations Module (Waters Corp., Milford, USA) coupled to a Waters 2996 PDA detector at a wavelength of 214 nm following a previously described methodology (Ojha *et al*,. 2016).

2.4 Colour evaluation, pH and water activity determination

Colour recordings were taken in triplicate using a Minolta CR-200 colorimeter (Minolta INC, Tokyo, Japan). Chroma (C^*_{ab}) and difference from the control (δE) were calculated as described by Wibowo *et al,.* (2015). Freeze-dried *Ganxet* bean proteins were re-suspended in distilled water at 1% (w/v) and the pH was measured using a Basic 20 pH meter (Crison Instruments S.A., Barcelona, Spain). The a_w was measured using an AquaLab meter (Decagon Devices Inc., Pullman, USA) at 22.0 ± 0.9 ºC.

2.5 Technofunctional properties

The water-(WHC) and oil-holding (OHC) capacities and foaming capacity (FC) of the *Ganxet* protein extracts were determined following the methodology previously described by Garcia-Vaquero *et al,.* (2017) using using a T-25 digital ULTRA-TURRAX® homogenizer (IKA, Staufen, Germany). WHC and OHC was expressed as g of water or sunflower oil per g of protein concentrate, respectively. FC was measured as the volume of foam generated as a percentage of the initial volume and foaming stability (FS) was expressed as the percentage of decrease of foam volume over time as described by Lafarga *et al,.* (2018). Emulsifying activity (EA) of the freeze-dried *Ganxet* proteins was determined as described by Lafarga *et al,.* (2018).

2.6 Assessment of antioxidant activity

Antioxidant capacity of the isolated proteins and of the pepsin hydrolysates was determined using the DPPH· scavenging activity following the methodology described by Bougatef *et al,.* (2010) using a GENESYS™ 10S-UV Vis spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.7 Statistical analysis

Determinations were carried out in triplicate for each sample. Results were expressed as mean ± standard deviation (S.D.). Differences between samples were analysed using analysis of variance (ANOVA) with JMP 13 (SAS Institute Inc., Cary, USA). Where significant differences were present, a Tukey pairwise comparison of the means was conducted to identify where the sample differences occurred. The criterion for statistical significance was $p<0.05$. To identify relationships between physicochemical parameters, bivariate Pearson's correlation analysis was carried out.

3. Results and discussion

Crude protein content of raw *Ganxet* beans was calculated as 22.7 ± 0.2%, which is comparable to that reported in previous studies (Rivera *et al,*. 2015, Lafarga *et al,*. 2018) or in line to other legumes such as pea and lupine, calculated as 21.9 and 35.1%, respectively (Pelgrom *et al,*. 2015). In addition, the protein content and protein yield of GPI were calculated as 91.08 ± 4.15% and 9.12 ± 0.85%, respectively, which was similar to other protein aisle determined in white or red cowpea (87.7 - 85.9%), several kidney bean (83.3-89.8%) or field pea varieties (90.8-94.7%) (Shevkani *et al,*. 2015a, 2015b). The protein content and yield obtained in the current study compared well with those obtained by Garcia-Vaquero *et al,*. (2017) seaweed-derived proteins, obtained using the same methodology and calculated as 63.3 and 6.5%, respectively. In a previous study, ultrasound-assisted isoelectric solubilisation-precipitation methodology was used achieving high *Ganxet* protein recoveries ranging between 45.6 and 78.7%, but lower purities in the protein isolates (Lafarga *et al,*. 2018).

3.1 Molecular weight distribution

Figure 1 represents the SEC chromatogram of the protein profile obtained for GPI. A first peak can be observed at a retention time of 5.40 min. Such peak might be composed by proteins larger than 150 kDa, which is the upper limit of resolution for the column employed. Vioque *et al*,. (2012) reported a peak of 226 kDa when analysing *Vicia faba*, which was attributed to trimmers of legumin, which has an isoelectric point close to the pH employed for extraction in this paper. Minor amounts were expected to be extracted following the protocol employed in this study, and this explains the relative low abundance of this large protein in the extract here studied. Two main peaks can be observed in Fig. 1 corresponding to molecular weights of 95 and 65 kDa, respectively and represent 29.48% and 34.03% of the total protein detected. Those peaks can correspond to convicilin, as reported in a previous study where such proteins were extracted using alkaline and acid solubilization (Lafarga *et al*,. 2018). However, due to the enormous variation on the SEC profile observed for the different varieties of *Vicia faba*, it is very hard to identify which protein corresponds to each one of the peaks observed in the present study (Mirali *et al*,. 2007, Nikolić *et al*,. 2012). Next peaks in relevance are those that correspond to molecular weights of 20 and 15 kDa, which could correspond to α- and β-legumin. Their areas represent 7.11 and 13.99% of total proteins detected, respectively. Finally, two peaks corresponding to very low molecular weight compounds were also detected. These correspond to 1.3 and 0.2 kDa and represent 4.04 and 1.57% of the total protein identified, respectively, which can be either peptides or free amino acids extracted along the main proteins.

3.2 Colour, pH and water activity

*L**, *a**, and *b** values of GPI were 76.72 ± 0.70, 0.72 ± 0.11, and 17.17 ± 0.97, respectively. The *L** parameter was significantly lower than that of *Ganxet* proteins obtained by isoelectric solubilisation-precipitation, which was 91.40 ± 1.63 (Lafarga *et al*,. 2018). However, similar $L*$ values were reported for kidney bean and amaranth protein isolates, which were reported as 79.6 ± 0.1 and 78.0 ± 0.8, respectively (Shevkani *et al*,. 2015b). No major differences were observed between the $a*$ value reported herein and those reported for other proteins derived from pulses (Hadnađev *et al*,. 2018, Lafarga *et al*,. 2018). The $b*$ value of GPI was higher when compared to that of proteins derived from soybean, pigeon pea, or cowpea (Garcia-Vaquero *et al*,. 2017). $C*_{ab}$ represents the degree of departure from grey towards pure chromatic colour and is a quantitative indicator of colourfulness. The $C*_{ab}$ of the GPI obtained in the current study was calculated as 17.19 ± 0.57. The δE combines the change in $L*$, $a*$, and $b*$ values to quantify the colour deviation from a standard reference sample. The δE was higher than 3, meaning that colour deviations were visible to the human eye (Wibowo *et al*,. 2015), when compared GPI with proteins derived from soybean, pigeon pea, cowpea, kidney bean, and field pea (Garcia-Vaquero *et al*,. 2017, Shevkani *et al*,. 2015a). Therefore, the colour of GPI was perceptually different to that of other vegetables derived proteins, including a *Ganxet* protein concentrate obtained by isoelectric precipitation (Lafarga *et al*,. 2018).

The pH and a_w values of GPI were 4.65 ± 0.11 and 0.248 ± 0.008, respectively. The a_w value was lower than that of the a_w *Ganxet* protein concentrate obtained by isoelectric precipitation, which was reported as 0.180 ± 0.002 (Lafarga *et al*,. 2018), and than those previously reported for proteins isolated from different food sources (Lafarga *et al*,. 2016a, Garcia-Vaquero *et al*,. 2017, Tontul *et al*,. 2018). The low a_w value suggested a stable product during storage as a_w values in the range 0.1 – 0.3 usually do not enable microbial growth.

3.3 Technofunctional properties

The WHC and OHC of GPI were 1.25 ± 0.11 and 2.76 ± 0.24 g/g of GPI, respectively. Similar WHC values were obtained for *Ganxet* bean (Lafarga *et al*,. 2018) and cowpea (Ragab *et al*,. 2004) proteins. The ability of proteins to hold water without dissolving is desirable mainly in viscous foods such as sausages or custards. High WHC values help to maintain freshness and moist mouth feel of foods. However, WHC values observed in the current study were low when compared to those reported for other plant-derived proteins such as for kidney bean proteins (5.34- 5.85 g/g) (Wani *et al*., 2015). Differences can be attributed mainly to the different extraction methods used, as proteins studied herein are water soluble and those studied by Wani *et al*,. (2015) were obtained by isoelectric solubilisation/precipitation.

Proteins with high OHC can be used in oily foods such as sausages or salad dressings (Tontul *et al*,. 2018), providing flavour retention and palatability and promoting longer shelf-life (Zhao *et al*,. 2013). The OHC of the GPI was also low when compared to that obtained previously for kidney beans, which ranged from 5.8 to 6.9 g/ g (Wani *et al*,. 2015), but were comparable to those reported for proteins chickpea- (Tontul *et al*,. 2018), mung bean- (Li *et al*,. 2010), and *Ganxet* bean- (Lafarga *et al*,. 2018) derived proteins.

Foaming properties are also of key importance for the development of certain foods such as meringues or mousses, which are generally made using egg white proteins. However, the increased demand for vegan proteins and foods has led to an increased interest in plant-derived proteins with the ability to form foams. FC and FS values are shown in Figure 2. A positive correlation was revealed between pH and FC (r^2 = 0.900). Higher FC values were observed at pH 8.0 and 10.0 and were calculated as 86.25 ± 5.30 and 78.75 ± 1.77%, respectively. These values were significantly higher than those obtained at lower pH values (p<0.05). Higher FC of proteins at high pH values can be attributed to increased net charges on the protein, which weaken the hydrophobic interactions but increase the flexibility of the protein (Ragab *et al,*. 2004). Results were in line with those obtained for other proteins derived from *Kappaphycus alvarezii* (Kumar *et al,*. 2014) and cowpea (Ragab *et al,*. 2004). FC values obtained herein were higher to those obtained by isoelectric precipitation of proteins from *Ganxet* beans, which were higher at pH 2.0 - FC was approximately 65% at this pH (Lafarga *et al,*. 2018). These results demonstrated the importance of selecting a suitable extraction protocol depending on the desired functionality. FS was significantly affected by time (p<0.001), pH (p<0.001), and the interaction between both factors (p<0.001). Both FC and FS were higher than those obtained previously for chickpea proteins, which ranged between 3.7-37.0% and 0.0-11.7%, respectively (Tontul *et al,*. 2018). GPI showed lower FS at pH 6.0 and pH 8.0, being statistically different to the rest of the groups during the first 90 min - except for the FS assessed at pH 10.0 after 90 min. Similar results were reported for previously (Garcia-Vaquero *et al,*. 2017, Ragab *et al,*. 2004, Khalid *et al,*. 2003).

Figure 3 shows the EA and ES of GPI. EA was found to be pH-dependent (p<0.05). The highest EA was observed at pH 6.0 and was calculated as 71.0 ± 1.4% (p<0.05). No significant differences were observed between the EA when assessed at pH 2.0, 4.0, 8.0, and 10.0. The EA of GPI was similar to that obtained for seaweed-derived proteins which showed EA values ranging from 70-95% when assessed using sunflower oil (Garcia-Vaquero *et al,*. 2017). Similar EA values were reported previously for *Ganxet* proteins (Lafarga *et al,*. 2018). However, because of the differences in the extraction protocols, the optimum EA values in that study were observed at higher pH values (pH 8.0). ES was found to be pH-dependent (p<0.05). The generated emulsions were found to be stable, especially at pH 6.0, 8.0, and 10.0 (p<0.05). A significant decrease in ES was observed at pH 4.0 in comparison with pH 2.0 (p<0.05). Dependence of EA and ES on pH was observed previously and it was suggested to be caused because the emulsifying capacity of proteins depend on the hydrophilic-lipophilic balance, which is affected by the pH (Ragab *et al,*. 2004).

3.4 Antioxidant activity

Figure S1 shows the antioxidant capacity of GPI and the enzymatic hydrolysate generated thereof. As expected, both samples showed a concentration dependency and their ability to scavenge radicals was higher with increase in concentration. Overall, the antioxidant capacity was higher after enzymatic hydrolysis (p<0.05). According to Matemu *et al,*. (2021) the health implications of legume-derive antioxidant peptides are linked to their potent action against oxidation. Despite nutritional quality of plant-based proteins could be lower than that animal-based due to the possible unbalanced essential amino acids, legumes are good sources of high-quality proteins. In this way, lentil and mung bean proteins have a good balance of other amino acids that exhibit high antioxidant activity (Young and Pellet, 1994). Results obtained in this study were comparable to those obtained for cod-derived proteins and hydrolysates (Sabeena Farvin *et al,*. 2014). In addition, the EC_{50} value, which is defined as the concentration of sample needed to inhibit DPPH· activity by 50%was calculated as 1.21 ± 0.06 and 1.04 ± 0.02 mg/mL for GPI and GPH, respectively, showing significant differences (p<0.05). The EC_{50} value of GPH was comparable to that of egg protein (Chalamaiah *et al,*. 2013) and sardine or mackerel (García-Moreno *et al,*. 2014) hydrolysates. Reported peptide fractions obtained from chickpea proteins hydrolysates showed DPPH radical scavenging activities of 57% at concentrations of 1 mg/ml (Kou *et al*., 2013). Segura Campos et al. (2010) reported IC_{50} values ranging 44.7-112 μg/mL of cowpea hydrolysates with pepsin-pancreatin. Xie et al. (2019) reported DPPH values of 74.23% at concentrations of protein hydrolysates from mung bean of 2.6 mg/mL at low molecular fractions of <3 kDa. - Functional properties of antioxidative peptides are highly influenced by molecular mass. Different studies in peptides obtained from legume protein hydrolysates indicated that molecular mass less than 1 kDa contained high proportion of antioxidant peptides (Li et al., 2008; Zhang et al., 2011; Kou et al., 2013; (Segura Campos et al., 2010; Sonklin et al., 2018).

In silico analysis was carried out to predict antioxidant peptides formed after hydrolysis of proteins found in common beans using pepsin. This strategy can also be used to predict which protease could be used to obtain hydrolysates with optimal bioactivity or to predict properties such as potential allergenicity and toxicity (Lafarga *et al*,. 2016b). Available reported proteins from *Phaseolus vulgaris* L. were obtained from Luna-Vital *et al*,. (2015) and included α- and β-phaseolin which belong to the 7S seed storage protein family. Antioxidant peptides identified included de di-peptide VY which corresponded to *f*(435-436) and *f*(420-421) of α- and β-phaseolin, respectively. The peptide VY was characterized by Cheng *et al*,. (2010) and was reported to inhibit lipid oxidation in soybean oil-in-water emulsions. In addition, the di-peptide EL, which corresponded to *f*(159-160) of RNA polymerase subunit beta, was previously obtained from casein using pepsin and reported to possess antioxidant properties. Not only antioxidant peptides were obtained after *in silico* hydrolysis of common bean proteins. Several renin (EC 3.4.23.15), angiotensin-I-converting enzyme (ACE-I; EC 3.4.15.1), and dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5) inhibitory peptides were also predicted to be released. Inhibition of these enzymes is one of the strategies followed to treat and prevent diseases related with metabolic syndrome such as hypertension and type-2 diabetes.

Conclusions

Functional properties of proteins depend largely on the extraction method used. Water soluble proteins extracted from *Ganxet* beans showed low WHC and OHC values when compared to other plant-derived proteins. However, high FC and EA values were observed. Enzymatic hydrolysis using pepsin resulted in increased antioxidant capacity. *In silico* analysis results suggested that the observed increase in the antioxidant activity could be caused by the release of peptides with antioxidant activity. Although further studies would be needed, the enzymatic hydrolysates of *Ganxet* bean proteins showed potential for being used as novel sources for peptides with varied health-promoting bioactivities.

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Legends to Figure

Figure 1. Chromatogram of proteins extracted from *Ganxet* common beans. The molecular weight of the main peaks is pointed with an arrow.

Figure 2. (A) Foaming capacity and (B) foam stability of *Ganxet* bean proteins

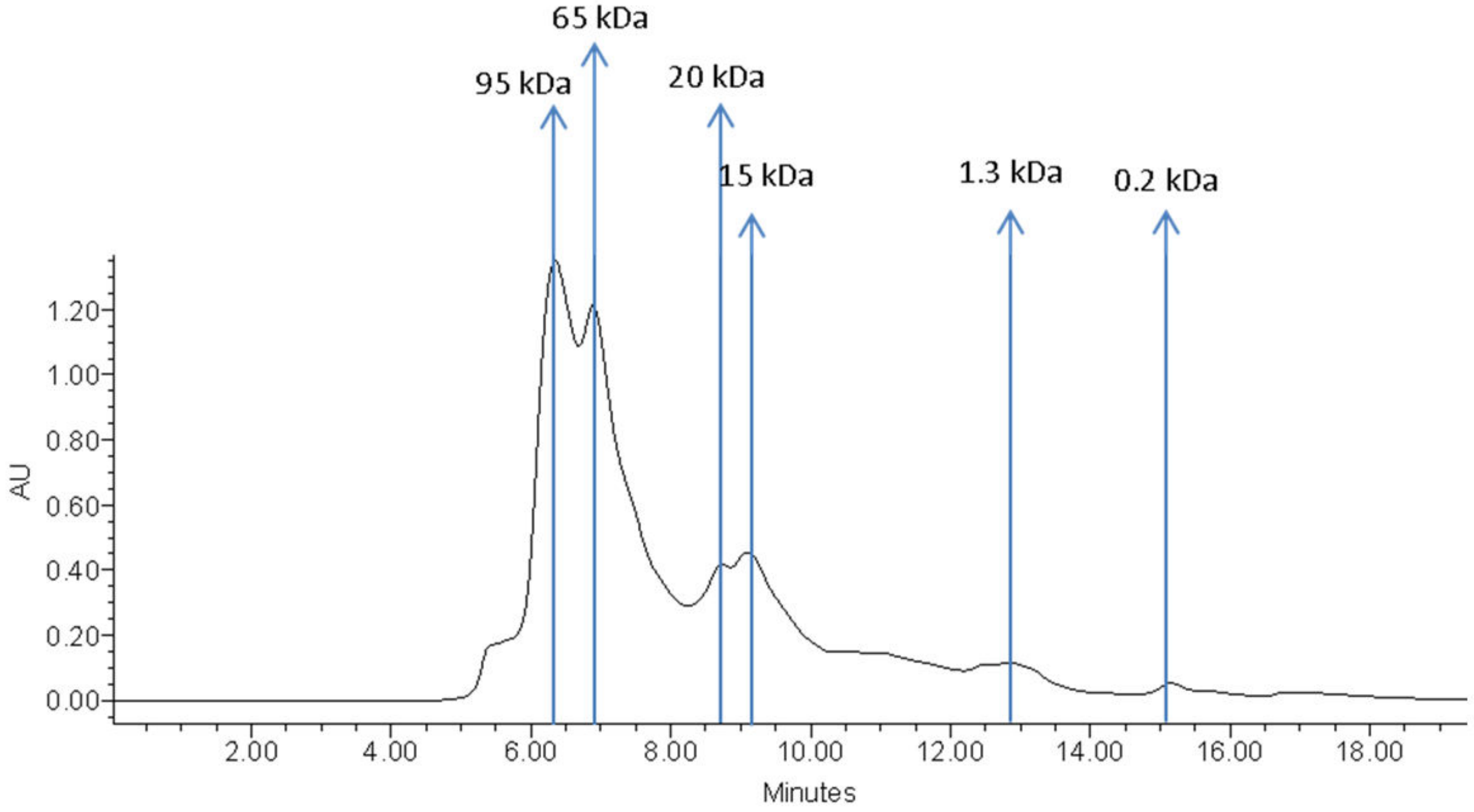
Values represent the mean of three independent experiments ± S.D. Different letters indicate significant differences. The criterion for statistical significance was $p<0.05$. Foam stability was significantly affected by time ($p<0.001$), pH ($p<0.001$), and the interaction between both factors time*pH ($p<0.001$).

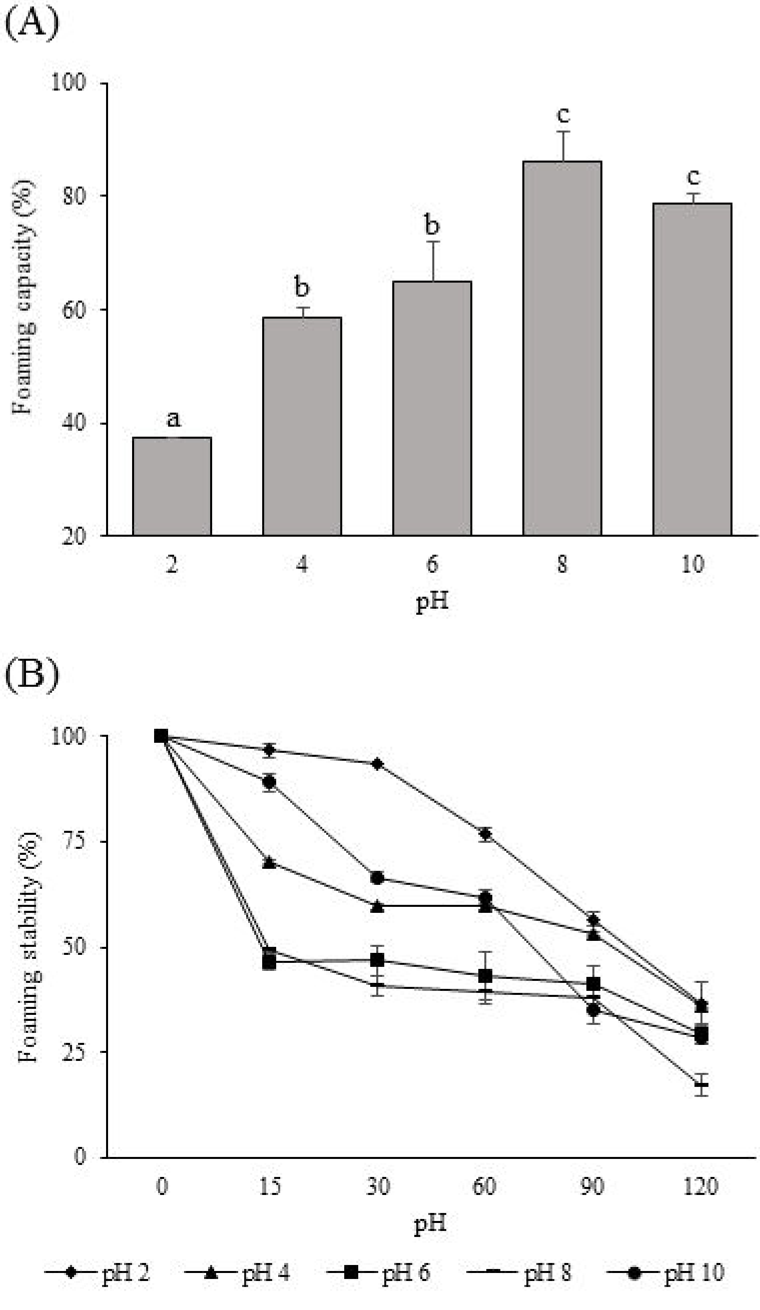
Figure 3. (A) Emulsifying activity and (B) stability of *Ganxet* bean proteins

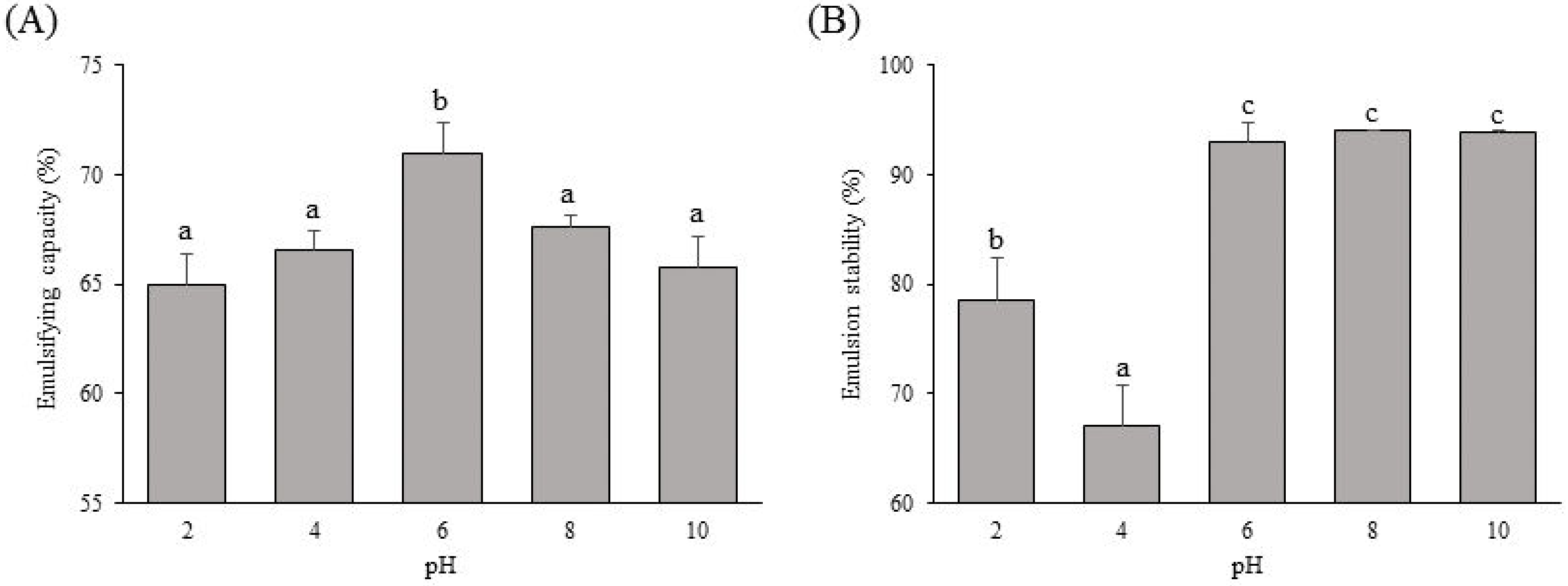
Values represent the mean of three independent experiments ± S.D. Different letters indicate significant differences. The criterion for statistical significance was $p<0.05$.

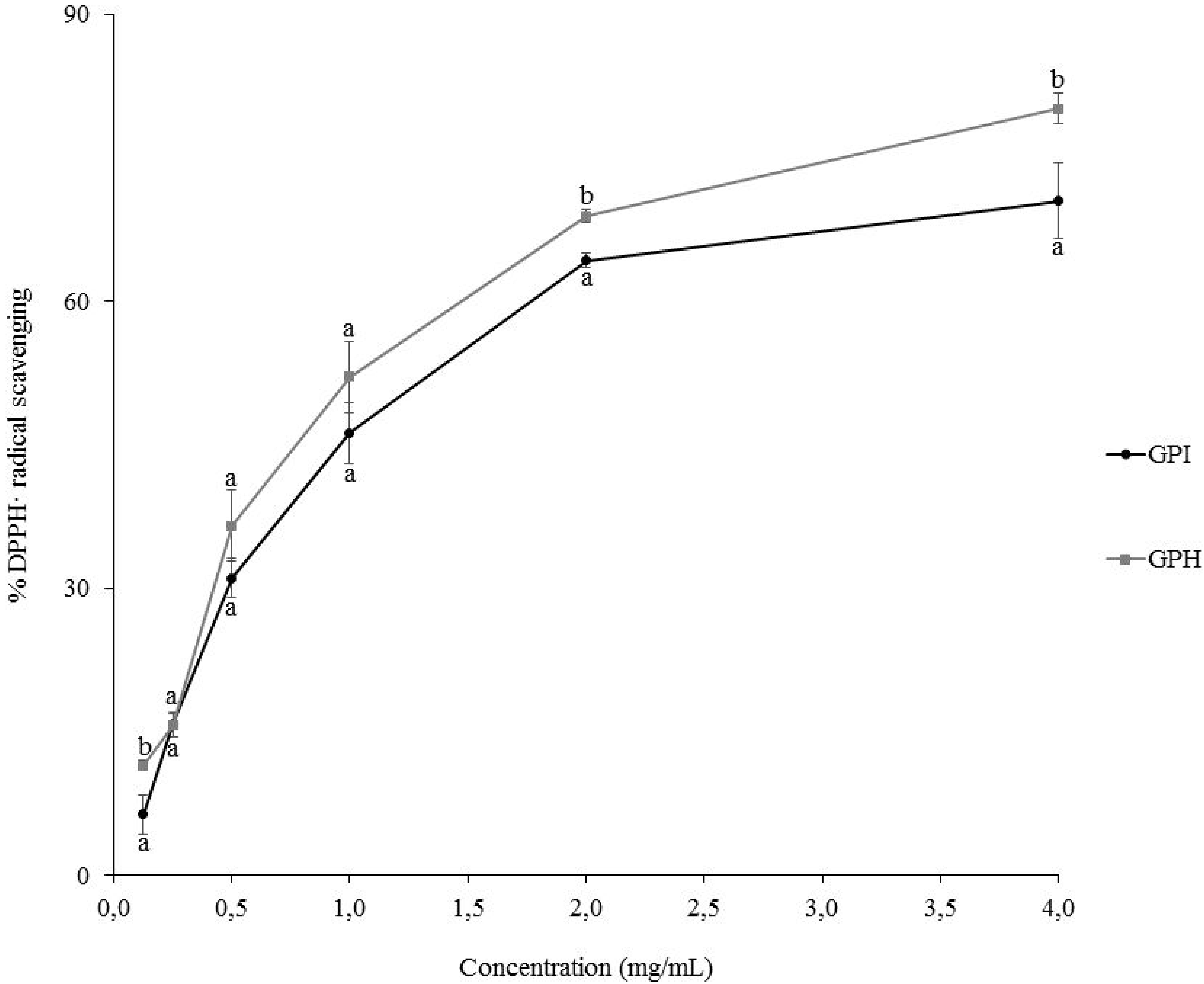
Supplementary items

Figure S1. Antioxidant activity of native and hydrolysed *Ganxet* bean proteins assessed using the DPPH· scavenging activity assay


65 kDa
95 kDa
20 kDa
15 kDa
1.3 kDa
0.2 kDa
1.20
1.00
0.80
0.60
0.40
0.20
0.00
AU
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
Minutes


(A)
100
80
60
40
20
Foaming capacity (%)
a
b
b
c
c
2
4
6
8
10
pH
(B)
100
75
50
25
0
Foaming stability (%)
0
15
30
60
90
120
pH
pH 2
pH 4
pH 6
pH 8
pH 10


(A)
75
70
65
60
55
Emulsifying capacity (%)
a
a
b
a
a
2
4
6
8
10
pH
(B)
100
90
80
70
60
Emulsion stability (%)
b
a
c
c
c
2
4
6
8
10
pH



90
60
30
0
% DPPH· radical scavenging
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
Concentration (mg/mL)
GPI
GPH
a
b


Phaseolus vulgaris L. var. Ganxet
PROTEIN EXTRACTION
TECHNOFUNCTIONAL PROPERTIES
ANTIOXIDANT PROPERTIES
95 kDa
65 kDa
20 kDa
15 kDa
1.3 kDa
0.2 kDa
AU
Minutes
HPLC-SEC
Foaming capacity (%)
Foaming stability (%)
Emulsifying capacity (%)
Emulsion stability (%)
pH
pH 2
pH 4
pH 6
pH 8
pH 10
% DPPH· radical scavenging
Concentration (mg/mL)
GPI
GPH