Changes of structural and techno-functional properties of high hydrostatic pressure (HHP) treated whey protein isolate over refrigerated storage

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Abstract

This work assessed the effect of storage time on the structure and functionality of HHP-treated whey protein isolate (WPI). Different pressures (100–600 MPa) and treatment times (15–30 min) were applied to aqueous WPI dispersions (5% w/v). The induced degree of unfolding was evaluated to select optimal HHP treatment conditions of WPI before storage at 4 °C. Conformational and techno-functional properties of untreated and optimally HHP-treated WPI samples were determined by UV-Vis and IR spectroscopy, foaming capacity, and interfacial tension measurements, respectively. Further tests of HHP-assisted hydrolysis of WPI were performed by α-chymotrypsin, bromelain, or their mixture (1:1 w/w), with the degree of hydrolysis (DH%) and electrophoretic patterns analyzed. The maximum unfolding degree was detected after a treatment of 400 MPa and 15 min and, at these processing conditions, no aggregation occurred. However, the structural changes achieved upon HHP were gradually lost during storage through a first-order refolding process ($k_{REF.}$ = 0.031 h^{-1}), with restoring of native functionality.

Hydrolysis performances of selected enzymes towards WPI were significantly promoted by high-pressure. Interestingly, a clear synergistic effect of α-chymotrypsin and bromelain combination on the WPI hydrolysis yield was detected, which resulted in the highest protein rupture (DH = 17%).

Keywords – High hydrostatic pressure (HHP); whey protein isolate (WPI); refolding; techno-functional properties; denaturing gel electrophoresis (SDS-PAGE).

1. Introduction

In the last decades, whey proteins have been gaining increasing attention at the industrial level, since they are used for the formulation/development of functional foods or as ingredients for cosmetics and pharmaceuticals, due to their well-recognized techno-functional properties such as solubility,

foamability, thickening and emulsifying capacity (Ambrosi, Polenta, Gonzales, Ferrari, & Maresca, 2016; De Maria, Ferrari, & Maresca, 2016; Rodiles-Lopez et al., 2008).

However, several authors have highlighted that the change in protein functional properties, being structure-dependent parameters, could occur under a physical/chemical stress induced to the protein system (Queirós, Saraiva, & Lopes da Silva, 2018). Consequently, significant arrangements at structural level via polypeptide chain breakage or unfolding/aggregation phenomena could be produced (Bouaouina, Desrumaux, Loisel, & Legrand, 2006).

Recently, innovative approaches for structural modifications of food proteins, in replacement to conventional thermal processing methods, were proposed, such as those based on the use of pulsed electric fields, high-intensity pulsed light, high hydrostatic pressure, and high-pressure homogenization treatments (Carullo, Donsì, & Ferrari, 2020; De Maria et al., 2016; Siddique, Maresca, Pataro, & Ferrari, 2016, 2017; Xiang, Ngadi, Ochoa-Martinez, & Simpson, 2011).

High hydrostatic pressure (HHP) is the most utilized nonthermal technology at the industrial scale, especially for microbial inactivation processes due to its minimal impact on organoleptic, nutritional, and sensorial properties of foods (De Maria et al., 2016; Larrea-Wachtendorff, Tabilo-Munizaga, & Ferrari, 2019). However, HHP treatments may also trigger the conformational disordering of food proteins, as well as affect their main techno-functional properties, depending on pressure, time and temperature applied (Ambrosi et al., 2016; Rodiles-Lopez et al., 2008).

According to Le Chatelier's principle, increasing hydrostatic pressure shifts the equilibrium towards a minimization of protein specific volume (Espinosa, Caffarena, & Grigera, 2019). This is due to the penetration of water molecules surrounding proteins into the polypeptide core, which then gradually fill and disassemble cavities initially solvent-free, thus destabilizing the tertiary/quaternary structures and, eventually, leading to unfolding (de Oliveira & Silva, 2015, 2017). Moreover, under these conditions, a greater exposure of free sulfhydryl groups occurs, with subsequent increase in hydrophobic interactions (De Maria et al., 2016; Khan, Mu, Sun, Zhang, & Chen, 2015).

As far as whey proteins are concerned, several works showed that HHP-assisted unfolding could enhance both their techno-functional properties (Lim, Swanson, & Clark, 2007; Lopez-Fandino, 2006; Padiernos, Lim, Swanson, Ross, & Clark, 2009) and the yields of enzymatic hydrolysis, with the latter generally performed to obtain peptides with higher bioactivity and lower allergenicity than native proteins (Ambrosi et al., 2016; Blayo, Vidcoq, Lazennec, & Dumay, 2016).

For instance, Lim et al. (2007) demonstrated that the application of HHP treatments at 300 MPa for 15 min significantly promoted the foam stability of whey proteins concentrate (WPC) with respect to untreated samples, thus suggesting the potential utilization of WPC as fat-replacers in the formulation of ice creams and whipping creams. Ambrosi et al. (2016) concluded that the great unfolding degree of WPC achieved at 400 MPa caused the unmasking of previously inaccessible hydrolytic sites, which promoted both rates and yields of reaction over samples hydrolyzed at ambient pressure, independently of the utilized proteolytic enzyme (α-chymotrypsin, bromelain). However, the authors did not investigate the effect of a combination of enzymes on the performances of HHP-assisted hydrolysis of whey proteins, whose concomitant action may potentially lead to an increase in the degree of protein breakage over single enzymes. This aspect is of utmost importance in order to furtherly widen their range of application, thus expanding market opportunities (Segat et al., 2014).

To the best of our knowledge, only a few works studied the reversibility of HHP-induced unfolding of proteins derived from whey (e.g. β-Lactoglobulin) as a function of temperature and pH during short storage time ($t < 2$ days) (Belloque, Chicon, & Lopez-Fandino, 2007; Ikeuchi et al., 2001; Møller, Stapelfeldt, & Skibsted, 1998). Moreover, none of them were addressed to demonstrate whether the potential occurrence of refolding after pressure release would have an influence on whey proteins functionality, which might have allowed to define the proper storage conditions for their potential industrial exploitation.

Therefore, this work aimed to investigate the impact of storage time on structural and techno-functional properties of whey proteins previously modified by HHP treatments, as well as to assess the effectiveness of using a combination of enzymes to further enhance the hydrolysis yields under

pressure. In particular, the objectives of this study included: (i) the analysis of the effect of pressure level and processing time on the extent of proteins unfolding; (ii) the evaluation of the stability over time of structural and techno-functional properties of untreated and HHP-treated whey proteins; and (iii) the yield determination of the hydrolysis reaction, carried out at high pressure (HHP, 400 MPa) or ambient (0.1 MPa) pressure, with single proteolytic enzymes or their combination.

2. Materials and Methods

2.1. Sample preparation and chemicals

Lyophilized whey protein isolate (WPI, UltraWhey 90 instant), derived from sweet cheese whey and purchased from Volac International Ltd. (Orwell, UK), was used in this work and stored under refrigerated conditions (T = 4 °C) until processing. The weight composition of WPI was as follows: 90% proteins, 1.0% fat, 2.5% lactose, 2.0% ash, and 4.5% moisture. The protein fraction included β-Lactoglobulin (β-Lg, 50–60%), Glycomacropeptide (GMP, 15-20 %), α-Lactalbumin (α-La, 15–20%), Bovine Serum Albumin (BSA, 1–2%), Immunoglobulin G (IgG, 1–2%), Immunoglobulin A (IgG, < 1%), and Lactoferrin (Lf, < 1%). All chemicals and enzymes used in this study were purchased from Sigma Aldrich (Milan, Italy) unless otherwise specified. MilliQ water was used to dilute samples and prepare all reagents and buffers. WPI samples were obtained by dissolving the protein at a constant concentration (5% w/v) in a sodium phosphate buffer (50 mmol/L, pH = 7.5) and kept under gentle stirring in an ice-water bath until complete solubilization. The pH of the dispersions, which were stored under refrigerated conditions before use, was determined by pH-meter (S400 Seven Excellence, Mettler Toledo International Inc., Milan, Italy). Enzymatic solutions for hydrolysis were prepared by dissolving α-chymotrypsin, bromelain or their mixture (1:1 w/w) in a sodium phosphate buffer (50 mmol/L, pH = 7.5) at a constant concentration (2% w/v) and stored under refrigerated conditions (T = 4 °C) before usage.

2.2. High hydrostatic pressure processing

111 HHP treatments of WPI dispersions were performed in a U111 high-pressure multivessel system
112 (UNIPRESS-Polish Academy of Sciences, Warsaw, Poland), as previously described in detail in the
113 work of Larrea-Wachtendorff et al. (2019), coupled to a thermostatic bath for temperature control
114 (Huber CC245 wl, Offenburg, Germany). The HHP unit can be operated at pressures up to 700 MPa
115 in the temperature range of -40 to 100 °C. A silicon oil (Huber thermofluid M40.165/220.10,
116 Offenburg, Germany) was used as pressurizing medium and as the main fluid in the thermostatic bath.
117 Preliminary tests allowed to assess the effect of HHP treatments on structural modifications induced
118 on WPI, with the aim to identify the optimal processing conditions, as those granting the highest
119 unfolding degree, which were then applied on samples for stability evaluation. To this purpose,
120 samples of 2.5 mL of whey protein dispersions were packed into flexible pouches made of a
121 polymer/aluminum/polymer film (OPP30-A19-LDPE70), avoiding headspace, and immediately
122 processed at different pressures (P = 100, 200, 300, 400, 500, and 600 MPa) and treatment time (t =
123 15, and 30 min). All the experiments were executed at a temperature of 25°C, while the compression
124 and decompression rates were set at 8 MPa/s. Although the depressurization rate is known to affect
125 the structural features of proteins (Fertsch, Müller, & Hinrichs, 2003), the U-111 system could not be
126 set at variable pressure release rate, due to equipment limitations. At the end of each treatment,
127 samples were collected in plastic tubes and immediately placed in an ice-water bath before analyses.
128 Untreated samples (controls) were also collected and used as a reference for further characterization.
129 For the stability tests, the structural and techno-functional properties of untreated and optimally HHP
130 treated WPI dispersions were observed by performing analyses immediately after treatment and after
131 1, 2, 3, 4, 7, and 10 days of refrigerated storage (T = 4°C). Longer storage times were not considered
132 due to microbial proliferation in both untreated and HHP pre-treated samples (data not shown).
133 HHP-assisted hydrolysis experiments were carried out adding the proteolytic enzymes (α-
134 chymotrypsin, bromelain, or their mixture) to WPI samples at a constant enzyme/substrate ratio (1:10,
135 w/w), and then subjecting the dispersions to HHP treatments ($P_{OPT.}$, $t_{OPT.}$) at the optimal activation
136 temperature of the proteolytic enzyme utilized (37 °C for α-chymotrypsin; 45 °C for bromelain and

enzymatic mixture). For the sake of comparison, the hydrolysis of untreated samples was performed also at ambient pressure (0.1 MPa). The hydrolysis reaction was stopped via enzymatic inactivation, carried out by heating all the samples at 100°C for at least 5 min. The hydrolysates were stored in an ice-water bath for further analyses.

2.3. Stability tests: analysis of WPI dispersions

2.3.1. Free sulfhydryl (-SH) groups

The estimation of free sulfhydryl groups (-SH) has been successfully used to study conformation variations induced upon HHP treatments of proteins from different sources, such as milk whey (Ambrosi et al., 2016), egg white (Quirós, Chichón, Recio, & López-Fandiño, 2007), soybean (Li, Zhu, Zhou, & Peng, 2012) and squid (Jin et al., 2015).

In this work, the content of free -SH groups of both untreated and HHP treated WPI dispersions was analyzed according to the method of Ellman (1959), with slight modifications. The protein dispersions were diluted to a final concentration of 2 g/L with a 50 mmol/L Tris-HCl buffer (pH = 7.0) in 15 mL plastic tubes. Subsequently, 2.75 mL of diluted protein were mixed with 0.25 mL of a 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) solution (1 g/L) in 50 mmol/L Tris-HCl buffer. A spectrophotometer V-650 (Jasco Inc. Easton, MD, USA) was used to measure the absorbance at 412 nm against a blank (2.75 mL of 50 mmol/L Tris-HCl buffer + 0.25 mL of DTNB reactant), after an incubation time of 30 min. The concentration of free -SH groups was calculated as reported by Siddique et al. (2016), and the results were expressed in μmol free -SH groups/g protein.

2.3.2. FTIR measurements

The evolution of secondary structure components of untreated and HHP treated WPI dispersions was monitored utilizing an IR spectrophotometer (FTIR-4100, Jasco Europe Srl, Italy), with the spectra collected at a constant resolution (2 cm^{-1}) and a fixed number of scans (64) in the Amide I region

(1700 - 1600 cm^{-1}). Background spectra were also collected and used to remove spectral signals that originated from air, moisture, and coating materials on reflecting mirrors along the IR radiation path. The resulting averaged spectra, each deriving from 9 repetitions per sample, were smoothed with an eleven-point under adaptive-smoothing function to remove any eventual noise. Finally, the baseline modification was applied for each spectrum.

2.3.3. Foamability (FA) and foam stability (FS)

The foaming capacity of WPI samples during refrigerated storage was determined according to the method of Segat et al. (2014), with some slight modifications. 10 mL of WPI dispersion were placed in a 50 mL graduated tube and homogenized at 10000 rpm for 2 min using an Ultra Turrax T25 disperser (IKA Werke GmbH & Co., DE). At the end of each homogenization cycle, the rotor-stator head was gently lifted to avoid affecting the volume of the formed foam. Foamability (FA) and foam stability (FS) were calculated according to Eq. (1) and Eq. (2):

$$FA\ (\%) = \frac{V_{F,0}}{V_L} \cdot 100 \quad (1)$$

$$FS\ (\%) = \frac{V_{F,30\ min}}{V_{F,0}} \cdot 100 \quad (2)$$

where V_L, $V_{F,0}$, and $V_{F,30min}$ represent the initial volume of WPI dispersion and the volumes of the foam formed immediately after whipping (time 0) or after standing at room temperature for 30 min, respectively.

2.3.4. Air-water interfacial tension

Interfacial tension at the air-water interface was measured by using the method of the pendant drop, as thoroughly described by Donsì, Sessa, & Ferrari (2012). Drops (5 – 10 μL in volume) of WPI

184 dispersions, prepared at a constant concentration (1 g/L), were produced using a micrometric syringe
185 (D = 0.71 mm), with the needle put in an empty transparent cuvette. Data of air-water interfacial
186 tension were collected via CAM200 tensiometer (KSV Instruments, Finland), consisting of an
187 experimental cell, an illuminating and viewing system, and a data acquisition system. After the
188 digitalization of captured drop frames, the interfacial tension was calculated as a function of the
189 curvature radius at the apex. The shape of the captured drop images was fitted to the Young-Laplace
190 equation (Eq. 3) which correlates the interfacial tension to the shape of the drop:

191 $$\frac{d\emptyset}{ds} = 2 + \beta Z - \frac{\sin \emptyset}{X} \quad (3)$$

192 $$\beta = -\frac{g\sigma q^2}{\rho_L} \quad (4)$$

193 where ϕ is the angle of the tangent at the point (X, Z), s is the linear distance along the drop profile,
194 dϕ/ds corresponds to the radius of curvature at the point (X, Z) and β is the shape parameter, given
195 by the Eq. (4), where g is the gravitational constant, ρ_L is the effective density of the liquid drop, σ is
196 the surface or interfacial tension, expressed in mN/m, and q is the radius of curvature at the origin.
197 For all the samples, the interfacial tension was monitored up to 1.5 10^3 s from the instant of drop
198 formation by sequential acquisition of drop frames at specified time intervals (5 s). Higher
199 observation times were not tested due to the detachment of the pendant drop from the syringe needle.
200

201 *2.4. Degree of hydrolysis*

202 The degree of enzymatic hydrolysis (DH, %) of untreated and HHP treated samples was evaluated
203 by measuring the o-phthaldialdehyde (OPA) reaction (Nielsen, Petersen & Dambmann, 2001). OPA
204 reagent was prepared by dissolving sodium tetraborate decahydrate, sodium dodecyl sulfate (SDS),
205 o-phthaldialdehyde 97% (OPA), and dithiothreitol 99% (DTT) in a deionized water solution. A serine
206 solution (0.1 g/L) in deionized water was used as standard. For each measurement, 3 mL of OPA

reagent were added to 400 µL of deionized water (blank), serine solution (standard), or WPI samples (untreated, HHP treated). The degree of hydrolysis was spectrophotometrically measured at 340 nm after 2 min of reaction, as previously described by Hardt, van der Goot & Boom (2013).

2.5. Reducing SDS-PAGE

SDS-PAGE electrophoresis was carried out under reducing conditions as described by O'Loughlin, Murray, Kelly, Fitz Gerald, & Brodkorb (2012), with slight modifications. To this purpose, A TV100Y twin-plate mini-gel unit equipped with an Apelex power supply (APELEX-Massy, France) was used. Briefly, untreated and hydrolyzed at ambient and high pressure WPI samples were diluted in a Tris-HCl buffer (0.125 mol/L, pH = 6.8) containing SDS (2% w/w), glycerol (10% w/w), bromophenol blue (0.02% w/w) and β-mercaptoethanol (5% w/w) as a reducing agent. Separating (12%) and stacking (6%) polyacrylamide gels were added with 50 µL of ammonium persulfate solution (10% w/v) and 5 µL of N, N, N', N'-tetra methylethylenediamine (TEMED), right before being poured in the electrophoretic system. 5 µL of all samples were loaded into the prepared gels, together with a pre-stained Protein Marker (peqGOLD, 10 – 260 kDa), and run at constant voltage (100 V) for 1h. Afterward, gels were recovered from the equipment and stained with a staining solution (0.1% Coomassie Brilliant Blue R 250, 10% acetic acid, 20% isopropanol) overnight. A de-staining solution (30% methanol, 10% acetic acid) was used until the background became clear.

2.6. Statistical analysis

All treatments and analyses were repeated three times unless otherwise specified. The mean values and standard deviations (SD) of the experimental data were calculated. Statistically significant differences ($p \leq 0.05$) among the averages were evaluated using a one-way analysis of variance (ANOVA) and Tukey's test ($p \leq 0.05$). Statistical analysis was carried out using IBM SPSS Statistics 20 software (IBM Corp., Armonk, New York, USA). The Pearson product-moment correlation

232 coefficient was used to measure the strength of the linear relationship between each couple of the
233 investigated variables.

234

235 ## 3. Results and discussion

236 ### *3.1. Impact of HHP treatments on WPI unfolding*

237 Figure 1 shows the content of free -SH groups in untreated and HHP treated WPI dispersions, as a
238 function of pressure intensity (100 – 600 MPa) and processing time (15 - 30 min). Regardless of the
239 duration of the treatment, the application of a pressure level of 100 MPa was insufficient to
240 significantly ($p > 0.05$) modify the conformational structure of WPI, being the free -SH groups
241 content similar to that detected in untreated samples. Instead, more severe HHP treatment conditions
242 (P = 200 – 400 MPa) contributed to trigger partial unfolding of WPI samples due to unmasking of
243 previously buried thiol groups which linearly increased ($p \leq 0.05$) with the pressure intensity. The
244 highest concentration of thiol groups (12.43 ± 0.21 μmol -SH/$g_{PROT.}$) was detected at 400 MPa for 15
245 min. In these conditions, neither aggregation nor polypeptides size-reduction was detected through
246 particle size distribution (PSD) measurements, being the PSD curves of untreated and HHP treated
247 samples almost overlapped (data not shown). Further increases of pressure up to 600 MPa did not
248 significantly ($p > 0.05$) intensify the WPI unfolding, independently of treatment time. The observed
249 behavior could be attributed to the likely concomitant occurrence of protein aggregation through
250 inter-/intramolecular SH/SS exchange reactions, promoted at P > 400 MPa, as already reported by
251 De Maria et al. (2016). Similar trends were observed by Li et al. (2012) in a study on the influence of
252 HHP treatments on structural properties of soy protein isolate (SPI). The authors found that a
253 significant increase in free -SH content of SPI was induced in the pressure range of 200 - 300 MPa
254 while, at higher pressure, it progressively decreased due to the predominance of aggregation over
255 unfolding.

Based on the results shown so far, additional investigations on the influence of HHP pre-treatments on structure and functionality of WPI during refrigerated storage, in comparison with untreated samples, were performed setting HHP parameters at 400 MPa and 15 min.

3.2. Effect of storage time on WPI structural properties

The concentration of free -SH groups in untreated and HHP pre-treated WPI dispersions was monitored over time to gain insight on the kinetics of polypeptide chains rearrangement after pressure release (Figure 2). Untreated WPI showed no appreciable changes in thiol reactivity with storage time, thus indicating a certain degree of conformational stability under refrigerated conditions, as previously observed by Ambrosi et al. (2016) and Siddique et al. (2017) within the range 60 - 120 min. Conversely, pressurized WPI samples exhibited a significant ($p \leq 0.05$) reduction in free -SH content during storage, until an equilibrium condition was reached. In particular, as shown in Figure 2, almost 80% of free thiol groups of WPI, previously made accessible by HHP processing, was lost approximately during the first 3 days of storage. Then, the free -SH groups slowly reduced with time to attain concentrations similar to those of untreated samples ($p > 0.05$).

In agreement with previous studies (Ambrosi et al., 2016; Belloque et al., 2007, Møller et al., 1998), the gradual loss in thiol reactivity detected in HHP pre-treated WPI could be ascribed to the occurrence of refolding, taking place during storage, through which proteins regain the original structure, reaching a thermodynamically stable configuration. Møller et al. (1998) found that the exposure of thiol groups in β-Lg (0.2 – 0.5% w/v in water) processed at 100 - 250 MPa for 30 min decreased over time after pressure release, by following an exponential decay kinetics with a refolding rate ($k_{REF.}$) of 0.104 h^{-1} (T = 5 °C). Additionally, based on the transition state theory, the authors reported a negative activation entropy (ΔS = -247 kJ/kmol·K), corroborating the hypothesis of proteins refolding due to the evolution of the system towards a more ordered state.

280 It is worth mentioning that β-Lg, the most abundant protein in whey, might be responsible for the
281 overall functionality of WPI (Bouaouina et al., 2006). Hence, taking this into account, the predictive
282 exponential-decay model of Møller et al. (1998) could be used to fit the data of Figure 2. The model
283 described quite well (R^2 = 0.976) the data, with a refolding rate ($k_{REF.}$) of 0.031 h^{-1} at 4 °C. The
284 significantly lower refolding rate observed in this work could be due to the processing conditions and
285 protein concentration utilized. More severe HHP treatment intensities applied to WPI samples (P =
286 400 MPa) caused greater structural modifications, thus longer storage times were required for protein
287 refolding and recovering of the initial state. Moreover, protein-protein interactions generally depend
288 on their concentration in the solubilizing medium (Wang, & Roberts, 2018). Therefore, the more
289 intense "crowding" effect achieved at the WPI concentration tested in this work might have decreased
290 the average inter-protein distance, as well as reduced the fluctuation of hydrophobic residues, thus
291 significantly slowing down the refolding rate. Interestingly, data not shown revealed that the acidity
292 of untreated and HHP treated WPI dispersions, evaluated by means of pH measurements, did not
293 appreciably change over the investigated duration of the storage phase.
294 The reversible nature of pressure-induced unfolding of WPI at 400 MPa for 15 min was confirmed
295 by the results of FTIR measurements. Figure 3 shows a typical FTIR spectrum of untreated whey
296 proteins in the Amide I region (1700 – 1600 cm^{-1}). The identification of peaks corresponding to
297 different secondary structure components of WPI was performed based on the assignments by Barth
298 (2007). The secondary structure of whey proteins showed two major peaks at 1636 cm^{-1} and 1651
299 cm^{-1}, due to the greater contribution of intramolecular β-sheet and α-helix components, and two
300 smaller peaks characteristics of turn (1667 cm^{-1}) and intermolecular β-sheet (1691 cm^{-1}) structures.
301 In this work, the changes in WPI conformational structure during refrigerated storage were
302 highlighted by plotting the absorbance of these peaks over time (Figure 4), for both untreated (a) and
303 optimally HHP pre-treated (b) WPI samples. In agreement with the results of Figure 2, untreated WPI
304 samples did not show any variation in terms of secondary structure components, which then remained

305 constant during the entire storage period. Pressure treatments at 400 MPa for 15 min induced an
306 evident alteration of WPI conformation, with increments in both α-helix and intermolecular β-sheet
307 structures over untreated samples, counterbalanced by a reduction in intramolecular β-sheet and turn
308 components. However, such induced conformational modifications by HHP were completely restored
309 already after 2 days of refrigerated storage, above which any difference in characteristic absorbances
310 of untreated and HHP treated WPI samples could be hardly evidenced. This trend is probably due to
311 the elastic behavior of polypeptide chains under pressure, in good agreement with the previous
312 findings of Larrea-Wachtendorff, Tabilo-Munizaga, Moreno-Osorio, Villalobos-Carvajal, & Perez-
313 Won (2015) who detected a full reversibility of pressure-induced secondary structure variation in
314 palm ruff muscles during a refrigerated storage of 35 days.
315
316 *3.3. Effect of storage time on WPI functionality*
317 The foaming capacity of untreated and HHP pre-treated (P = 400 MPa, t = 15 min) WPI dispersions
318 was assessed measuring foamability (FA, %) and foam stability (FS, %), whose time dependence
319 during refrigerated storage is reported in Figure 5a and Figure 5b, respectively.
320 Untreated WPI samples were characterized by poor foaming properties (FA = 19%; FS = 10%), due
321 to their compact structures which prevented the exposure of hydrophobic groups towards the aqueous
322 medium. However, in the absence of any physical stress, FA and FS values did not appreciably change
323 during storage, thereby confirming the stability of untreated WPI at 4 °C.
324 HHP treatments drastically enhanced the foaming capacity of whey proteins, which yielded
325 significantly ($p \leq 0.05$) higher values of foamability (98%) and foam stability (49%), compared to
326 untreated samples. Remarkably, a strong positive correlation was observed between the concentration
327 of free -SH groups and the foaming capacity values, as reported in Table 1. This can be explained by
328 the increased amount of hydrophobic residues upon unfolding which improved the protein capacity

329 to absorb air bubbles at the air/water interface, in agreement with previous literature findings (De
330 Maria et al., 2016; Rodiles-Lopez et al., 2008).
331 Figure 5 also highlights that the foaming capacity of HHP treated samples was not maintained after
332 pressure release since its values linearly decreased with storage time in the first 4 days until reaching
333 similar values observed in untreated WPI samples. These data are consistent with the previously
334 detected proteins refolding over storage (Figure 2).
335 Additional information on WPI surface properties and, hence, on their ability to produce/stabilize
336 foams were obtained from air/water interfacial tension measurements. Figure 6 shows the dynamics
337 of interfacial tension of untreated and HHP treated WPI dispersions (measured immediately after
338 treatment (a) and after 1 (b), 4 (c) and 7 (d) days of refrigerated storage). Regardless of the storage
339 time, the interfacial tension of the air/WPI dispersion system was about 52 mN/m at t=0 s. Moreover,
340 the curves of surface tension from both untreated and HHP treated samples decreased with the time
341 elapsing from the pendant drop formation (0 s) and the last acquired frame (1500 s). This was likely
342 due to the dynamic nature of proteins adsorption step at the gas/liquid interface, being driven by the
343 concentration gradient between the bulk and boundary layer (Donsì et al., 2012). However, the
344 observed trend was more pronounced for pressurized samples due to a greater surface hydrophobicity
345 and enhanced mobility of polypeptide chains in the bulk phase upon partial unfolding. Similarly,
346 Bouaouina et al. (2006) observed a more rapid surface tension reduction at the air/water interface for
347 dynamic high-pressure (DHP) treated (P = 300 MPa) WPI dispersion with respect to control samples,
348 caused by the improved unmasking of hydrophobic groups which enhanced the adsorption kinetics.
349 A significant correlation among time-averaged interfacial tension (Figure 6), foam capacity (Figure
350 5), and free -SH groups content (Figure 2) of HHP treated WPI dispersions was found (Table 1). This
351 confirmed the dependence of physicochemical properties governing the overall WPI functionality on
352 the structural rearrangement occurring upon processing (unfolding) and storage (refolding).
353
354 *3.4. Impact of HHP treatments and enzyme composition on WPI hydrolysis*

355 The nature of proteolytic enzyme and its interaction with the substrate under pressure may strongly
356 influence the extent of polypeptide chains rupture, as well as the molecular weights distribution of
357 the generated peptides. Therefore, we also investigated the effect of an enzymatic cocktail, used in
358 combination with HHP treatments, on the achieved degree of WPI hydrolysis, as compared with the
359 utilization of single enzymes. Figure 7 shows the hydrolysis degree of HHP-assisted (P = 400 MPa, t
360 = 15 min) enzymatic reaction of WPI with α-chymotrypsin, bromelain, or their mixture (1:1 w/w).
361 For the sake of comparison, the results obtained by hydrolyzing with the same enzymes at ambient
362 pressure (t = 15 min) untreated and HHP pre-treated WPI (P = 400 MPa) are reported.
363 Results demonstrated that the hydrolysis of control samples is almost independent on the type of
364 enzyme utilized, being the values of DH% statistically similar ($p > 0.05$) among them. This can be
365 explained by considering that untreated whey proteins are characterized by a highly folded structure
366 with a reduced accessibility to active sites for the enzymatic attack (Ambrosi et al., 2016). Instead,
367 WPI unfolding occurring during HHP treatments positively affected the extent of proteins rupture,
368 due to the significant ($p \leq 0.05$) increase in DH% values over control samples, independently on the
369 type of enzyme. Nonetheless, higher hydrolysis yields were observed when the enzymatic reaction
370 was performed under high pressure rather than at ambient conditions (Figure 7). The continuous
371 conformational change of WPI upon HHP treatments likely increased the number of sites susceptible
372 to enzymatic attack and the enzyme/substrate interaction, in good agreement with previous findings
373 (Ambrosi et al., 2016; Belloque et al., 2007). It could be also hypothesized that the catalytic activity
374 of α-chymotrypsin, bromelain, and their mixture (1:1 w/w) improved at 400 MPa, thus reducing the
375 reaction time to achieve a given degree of protein rupture, in comparison with the hydrolysis
376 performed at atmospheric pressure. The increase in α-chymotrypsin activity due to HHP treatments
377 is supported by literature (Mozhaev, Kudryashova, & Bec, 1996). However, additional research is
378 required to better elucidate the effect of pressure and time on the activity of both bromelain and the
379 enzymatic mixture used in this work.

380 Interestingly, a clear synergistic effect by combining the two enzymes was detected when WPI
381 hydrolysis was assisted by HHP treatments (Figure 7), leading to a significant ($p \leq 0.05$) higher yield
382 (DH = 17%) with respect to those obtained using α-chymotrypsin (DH = 12.3%) or bromelain (DH
383 = 14.3%) alone. These results could be due to the different substrate specificity of the investigated
384 proteolytic enzymes. In particular, α-chymotrypsin cleaves preferably peptide bonds on the C-
385 terminal side of phenylalanine, tyrosine, tryptophan, and leucine (De Maria, Ferrari & Maresca,
386 2017). Instead, bromelain catalyzes the selective rupture of protein sites with arginine, lysine,
387 glutamic acid, glycine, ornithine, methionine sulfoxide, and alanine (Ee, Khoo, Ng, Wong, & Chai,
388 2019). Therefore, it could be speculated that the transient unfolding phenomena occurring upon HHP
389 treatments caused a cumulative effect between both enzymes' specificity, thus further enhancing the
390 hydrolysis performance.
391 The reducing SDS-PAGE profile of hydrolysates from untreated and high-pressure treated (400 MPa)
392 WPI dispersions (Figure 8), corroborated the data of Figure 7. Untreated WPI was dominated by two
393 major bands, being β-Lg (MW ≈ 17 kDa) and α-La (10 kDa < MW < 17 kDa), together with a lighter
394 band, attributable to BSA, detected between 52 kDa and 72 kDa. A clear reduction in the intensity of
395 the β-Lg band and the complete disappearance of α-La were observed in samples hydrolyzed by α-
396 chymotrypsin at ambient pressure. These results are in good agreement with the findings of Kim et
397 al. (2007), who highlighted the lower susceptibility of β-Lg to tryptic hydrolysis. Despite the slight
398 but insignificant ($p > 0.05$) increase in DH% observed over α-chymotrypsin (Figure 7), the different
399 substrate specificity of bromelain or enzymatic mixture yielded different electrophoretic patterns of
400 WPI (Figure 8), which showed only a low-intensity band between 10 kDa and 17 kDa. Conversely,
401 due to the increased effectiveness of HHP-assisted hydrolysis (Figure 7), all WPI characteristic bands
402 completely vanished on hydrolysates obtained under pressure, whatever was the utilized enzyme. The
403 complete hydrolysis of WPI determined the formation of very small peptides that were not visible in
404 the electrophoretogram, being presumably characterized by MW < 10 kDa. Overall, these data are

405 consistent with the results previously obtained by other scientists, who demonstrated the key role
406 played by high-pressure in generating structural modification of proteins, thus intensifying the
407 enzymatic hydrolysis process (Ambrosi et al., 2016; De Maria et al., 2017; Jin et al., 2015; Quirós et
408 al., 2007; Rodiles-Lopez et al., 2008).
409
410 **4. Conclusions**
411 This work investigated the impact of storage, in refrigerated conditions, on the structural and techno-
412 functional properties of WPI, previously subjected to HHP treatments. Measurements of free -SH
413 groups revealed that the changes in WPI conformation depended on both pressurization intensity and
414 time, with the highest extent of unfolding detected at 400 MPa for 15 min. Under these conditions, a
415 strong positive correlation between the conformational changes and the improved interfacial
416 properties of WPI was found. However, after pressure release, proteins underwent a first-order
417 refolding process which restored the secondary, tertiary, and quaternary structure of untreated
418 samples, as well as their original functionality, mostly within three days of storage. As far as HHP-
419 assisted enzymatic hydrolysis is concerned, the α-chymotrypsin/bromelain mixture remarkably
420 improved the degree of protein breakage as compared to single enzymes, showing a clear synergistic
421 effect due to an increase in the overall substrate specificity.
422 In conclusion, the results of this work provided useful additional insight into the utilization of HHP
423 technology at mild conditions to modify the structural features of food proteins, whose time-
424 dependent behavior may allow defining their processability at the industrial scale.
425
426 **Acknowledgments**
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Figure captions

Figure 1. Concentration of free sulfhydryl groups (μmol/$g_{protein}$) from untreated and HHP treated WPI dispersions, as a function of pressure level (100 - 600 MPa) and treatment time (black bar: 15 min, light grey bar: 30 min). Different letters above the bars indicate significant differences among the mean values ($p \leq 0.05$).

Figure 2. Changes of free sulfhydryl groups concentration (μmol/$g_{protein}$) from untreated (black circle) and HHP (white circle) treated (400 MPa, 15 min) WPI dispersions, as a function of the storage time (0 – 10 days). Standard deviations were used as error bars ($p \leq 0.05$).

Figure 3. FTIR spectrum of untreated WPI sample plotted as a function of the wavenumber (cm^{-1}).

Figure 4. Changes of the secondary structure main components, namely α-helix (black circle), intramolecular β-sheet (white triangle), turn (black square) and intermolecular β-sheet (white rhombus), of untreated (a) and HHP treated at 400 MPa for 15 min (b) WPI dispersions, as a function of the storage time (0 – 10 days).

Figure 5. Changes of foamability (a) and foam stability (b) from untreated (black circle) and HHP (white circle) treated (400 MPa, 15 min) WPI dispersions, as a function of the storage time (0 – 10 days). Standard deviations were used as error bars ($p \leq 0.05$).

Figure 6. Air-water superficial tension as a function of time from untreated (black circle) and HHP (white circle) treated (400 MPa, 15 min) WPI dispersions, observed at day 0 (a), day 1 (b), day 4 (c) and day 7 (d) of storage. Standard deviations were used as error bars ($p \leq 0.05$).

Figure 7. Hydrolysis degree (%) of untreated (black bar) and HHP (light grey bar) treated (400 MPa) WPI samples in the presence of α-chymotrypsin, bromelain, and their mixture (1:1 w/w). The reaction time was set at 15 min. For comparison purposes, reaction yields of samples hydrolyzed at 0.1 MPa

after HHP (dark grey bar) treatment (400 MPa, 15 min) are also reported. Different letters above the bars indicate statistical differences among the samples ($p \leq 0.05$).

Figure 8. Reducing SDS-PAGE patterns of untreated and HHP treated (400 MPa) WPI samples in the presence of α-chymotrypsin, bromelain, and their mixture (1:1 w/w). The reaction time was set at 15 min. STD – protein marker, WPI – untreated sample, C - sample hydrolyzed at ambient pressure (control), T – sample hydrolyzed during HHP processing.

Figure 1

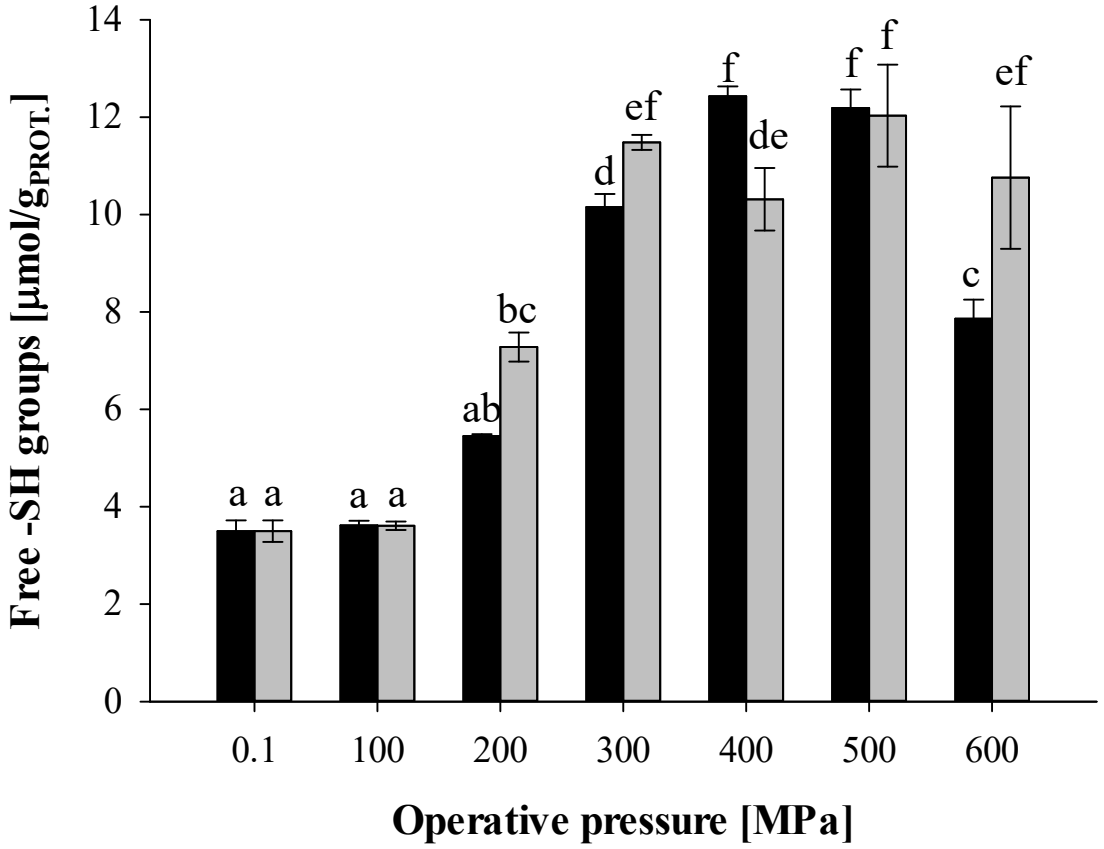


Figure 2

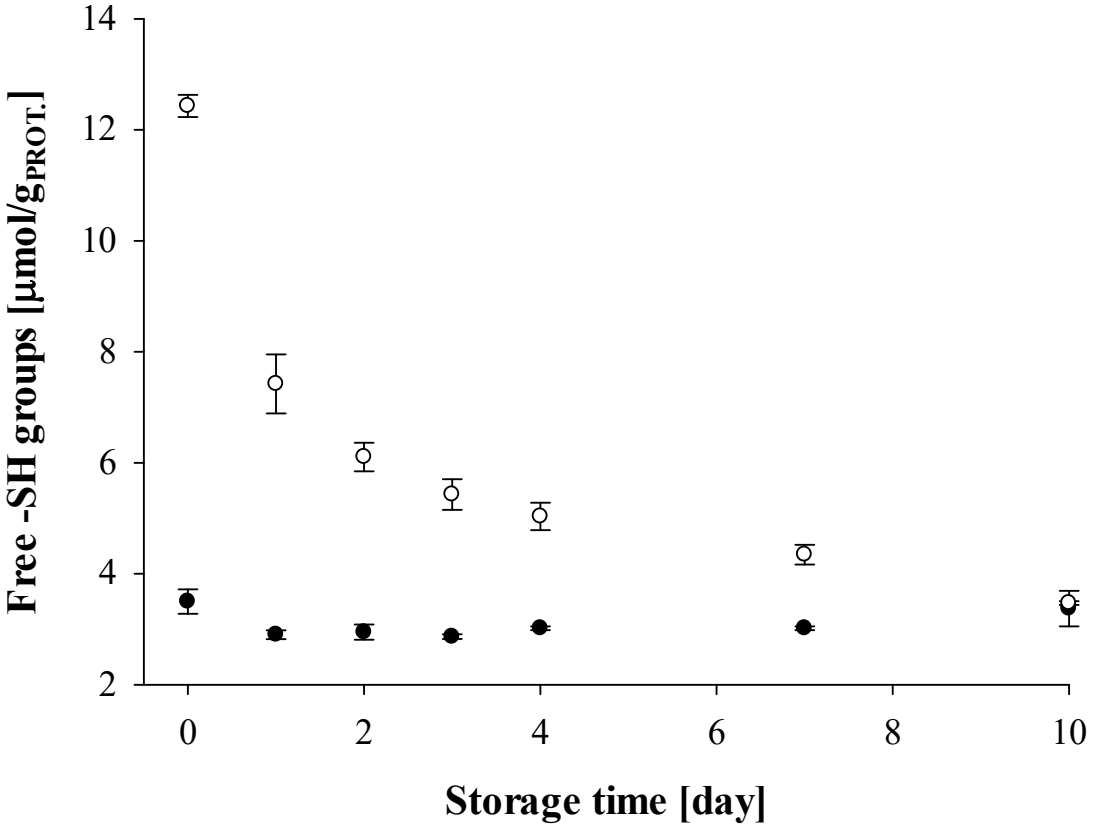


Figure 3

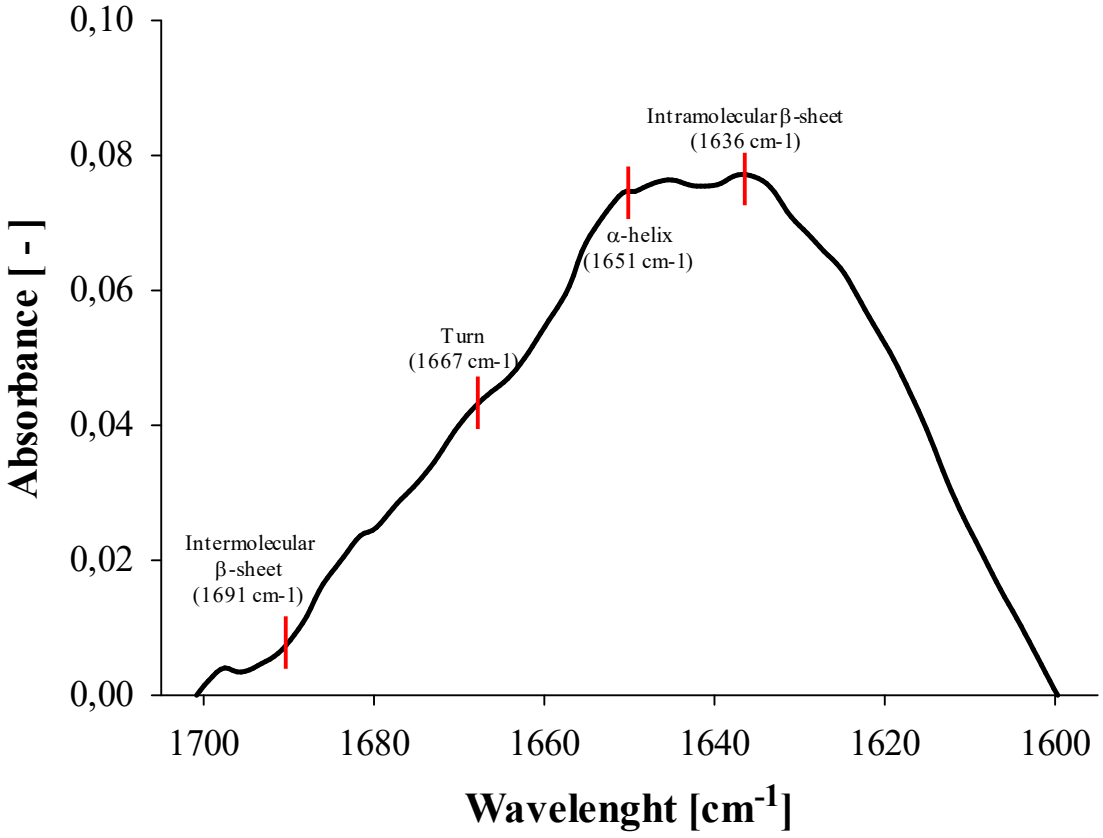
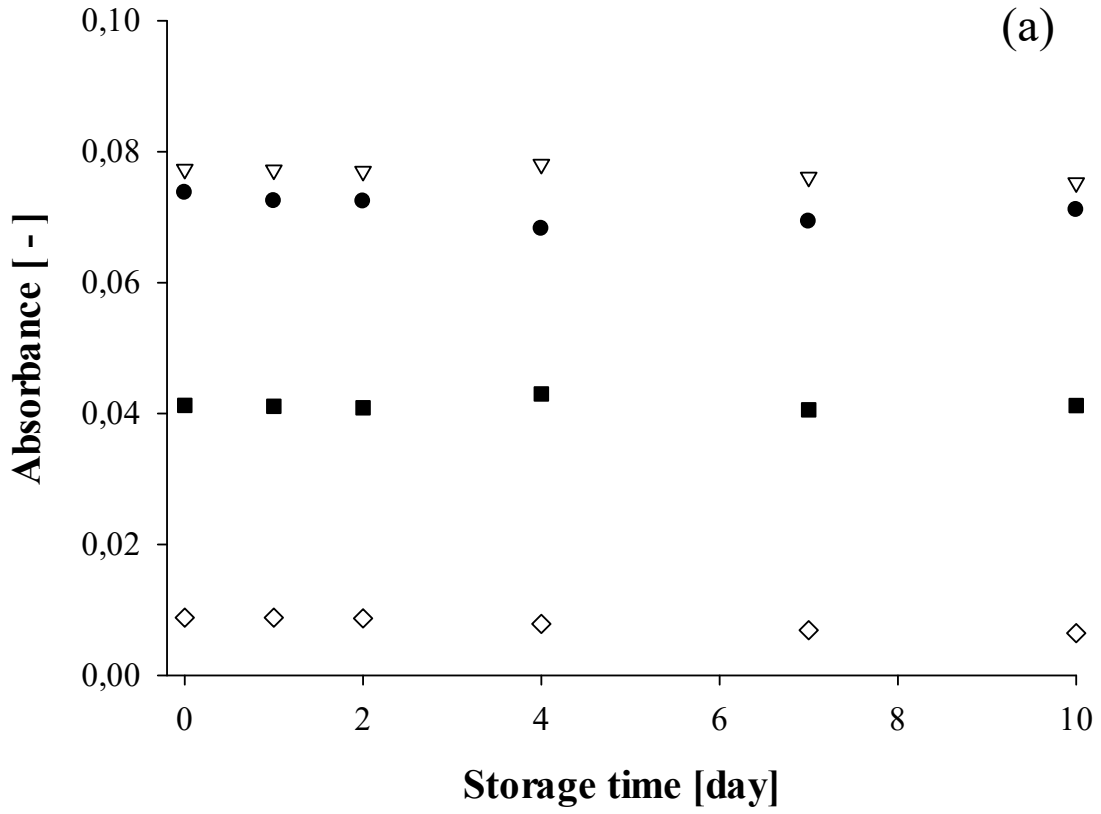

0,10
0,08
0,06
0,04
0,02
0,00
Absorbance [-]
Intramolecular β-sheet
(1636 cm-1)
α-helix
(1651 cm-1)
Turn
(1667 cm-1)
Intermolecular
β-sheet
(1691 cm-1)
1700
1680
1660
1640
1620
1600
Wavelenght [cm-1]

Figure 4


(a)
0,10
0,08
0,06
0,04
0,02
0,00
Absorbance [-]
0
2
4
6
8
10
Storage time [day]

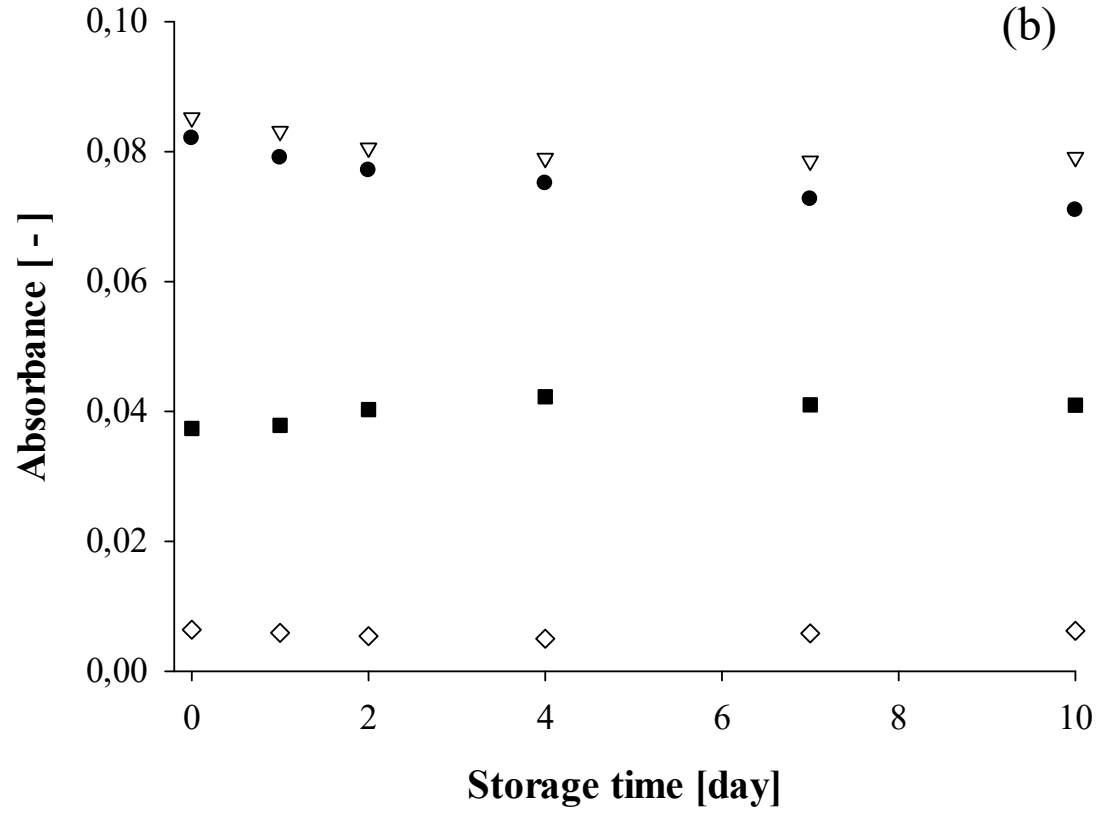
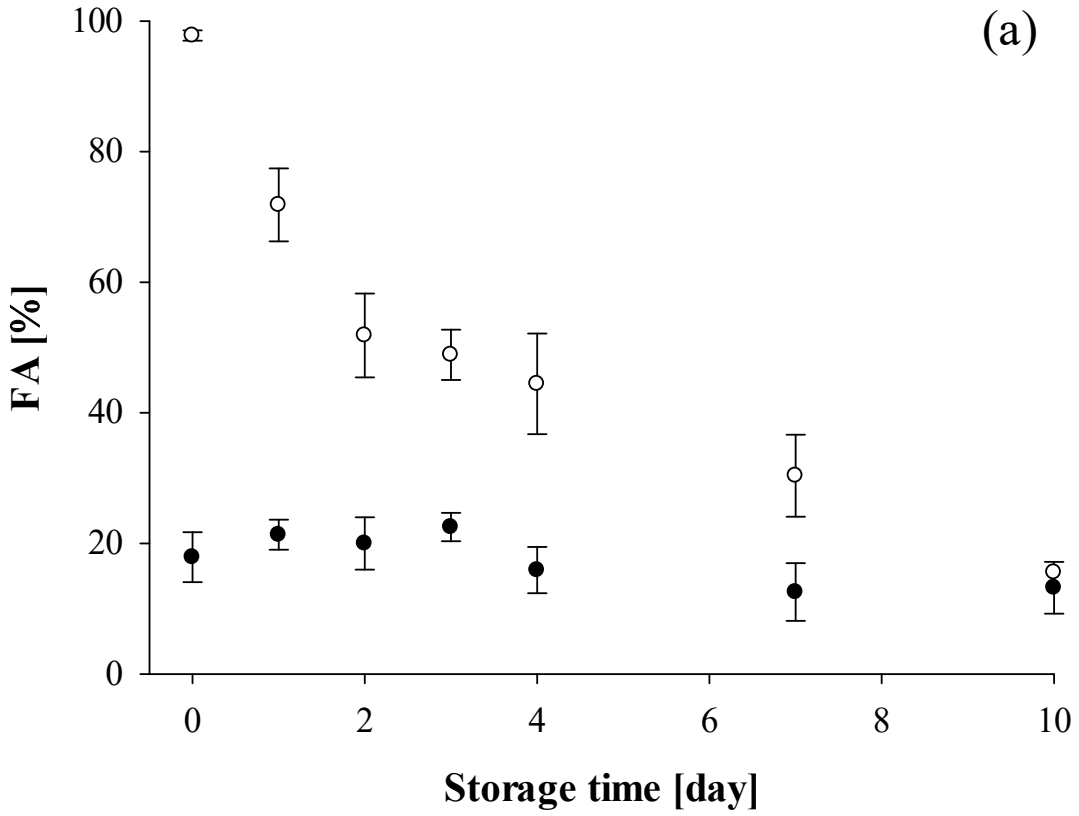

(b)
0,10
0,08
0,06
0,04
0,02
0,00
Absorbance [-]
0
2
4
6
8
10
Storage time [day]

Figure 5


(a)
FA [%]
100
80
60
40
20
0
0
2
4
6
8
10
Storage time [day]

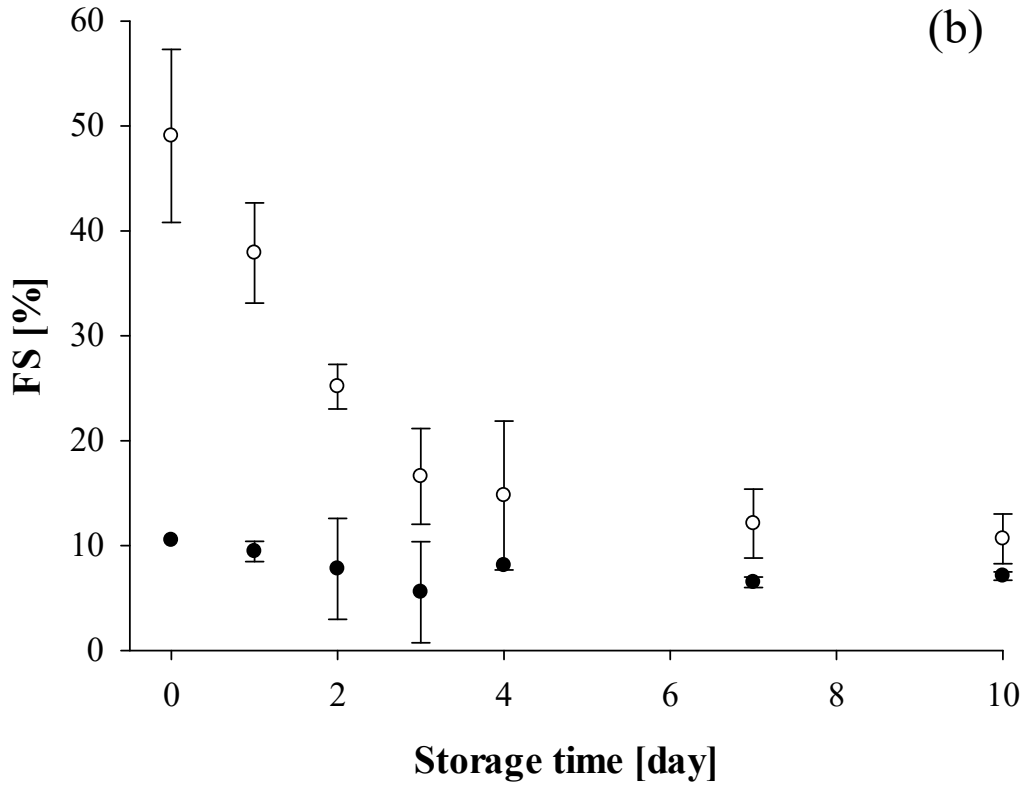
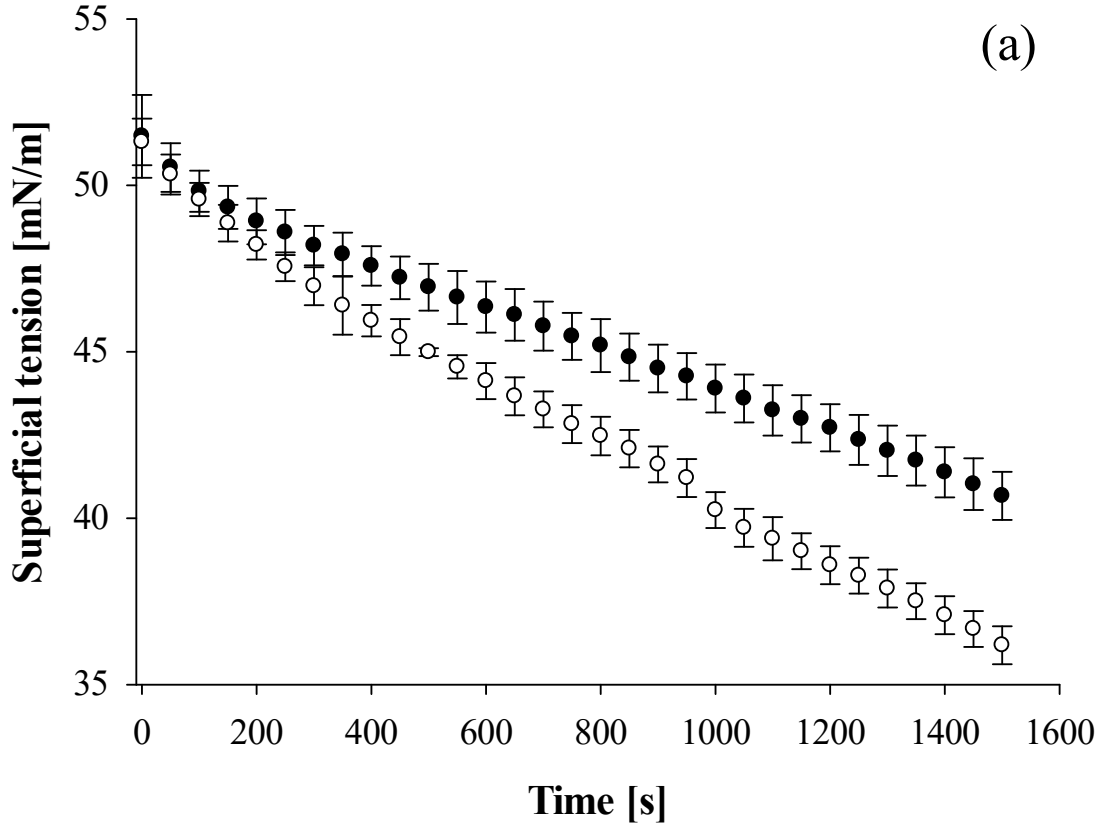
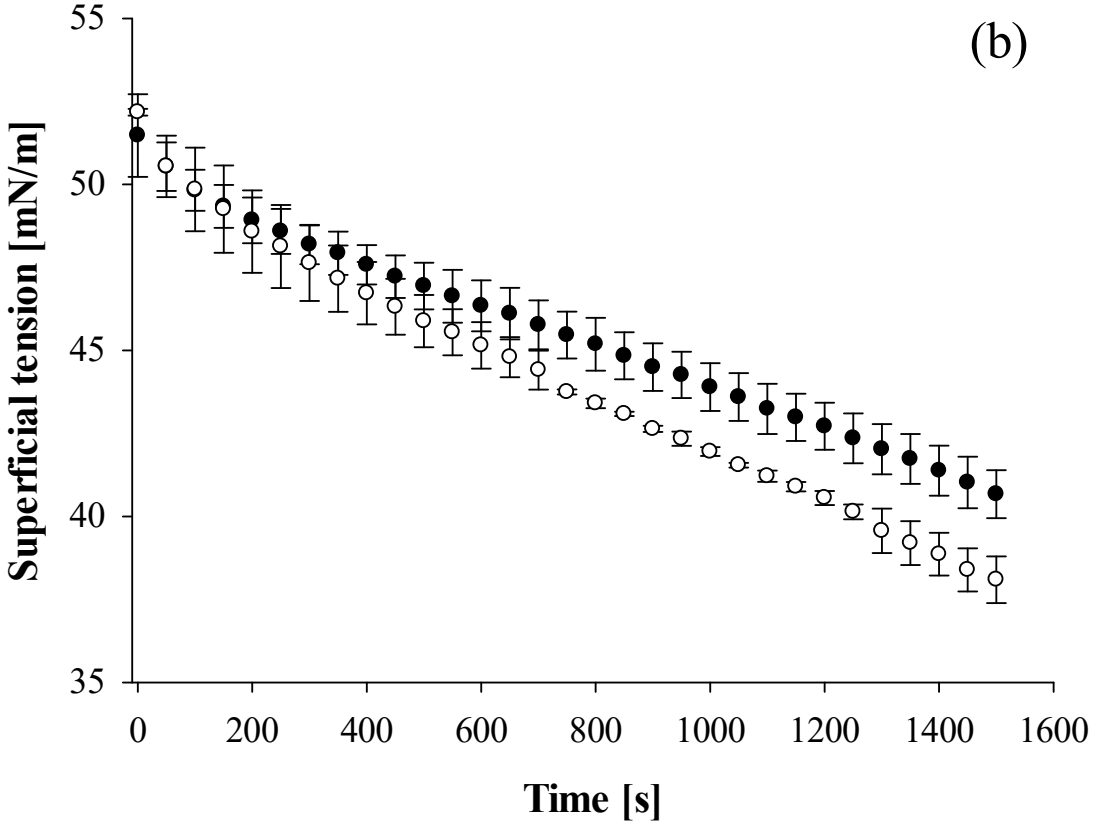
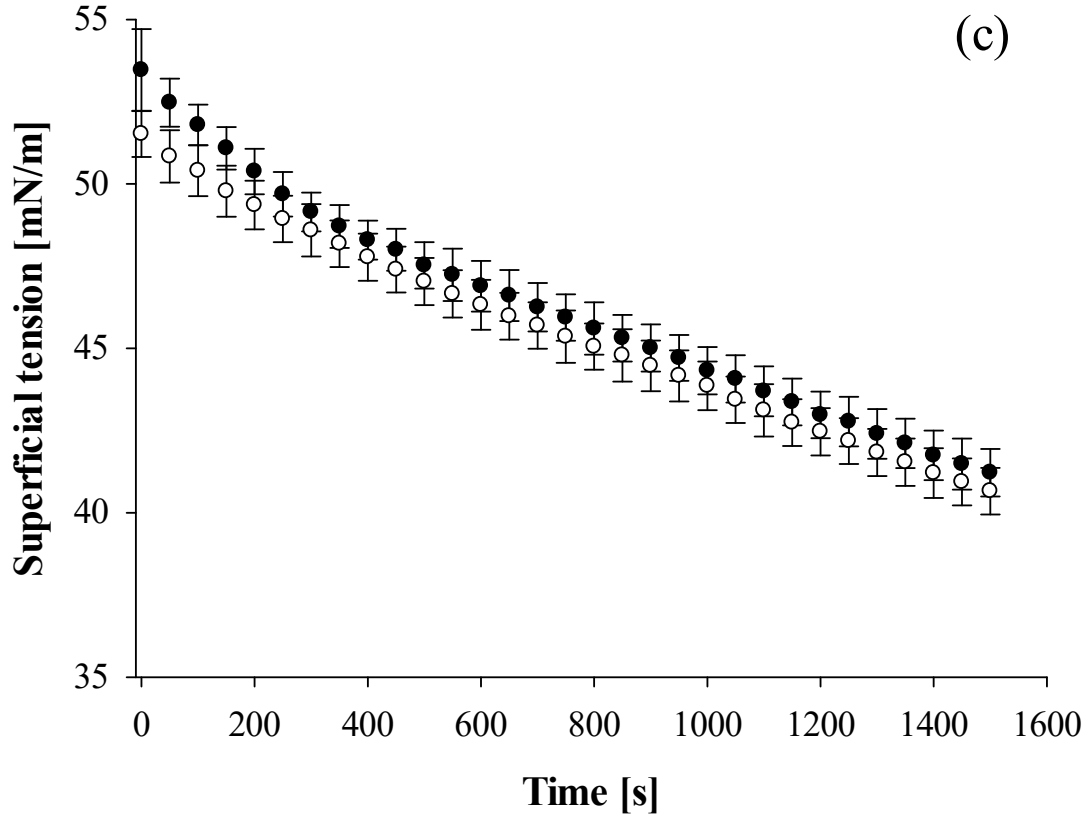

(b)
FS [%]
60
50
40
30
20
10
0
0
2
4
6
8
10
Storage time [day]

Figure 6


(a)
Superficial tension [mN/m]
55
50
45
40
35
0
200
400
600
800
1000
1200
1400
1600
Time [s]


(b)
Superficial tension [mN/m]
55
50
45
40
35
0
200
400
600
800
1000
1200
1400
1600
Time [s]


(c)
Superficial tension [mN/m]
55
50
45
40
35
0
200
400
600
800
1000
1200
1400
1600
Time [s]

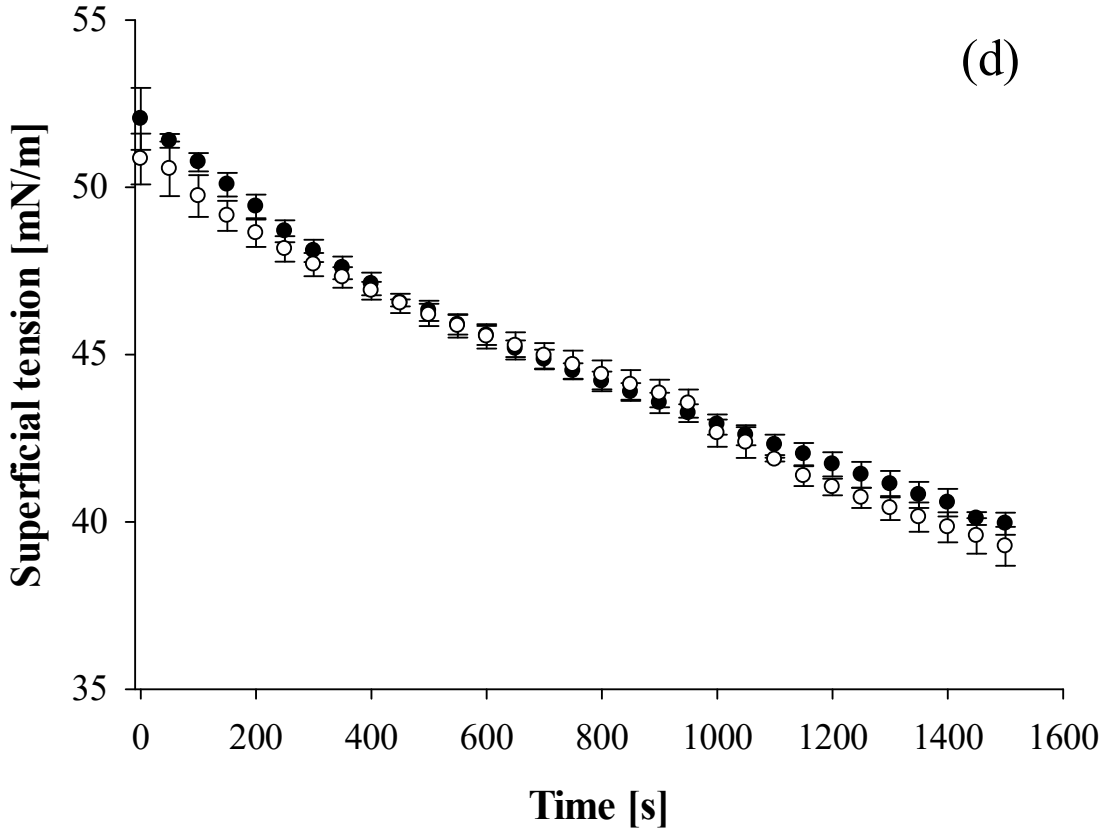

(d)
Superficial tension [mN/m]
55
50
45
40
35
0
200
400
600
800
1000
1200
1400
1600
Time [s]

Figure 7

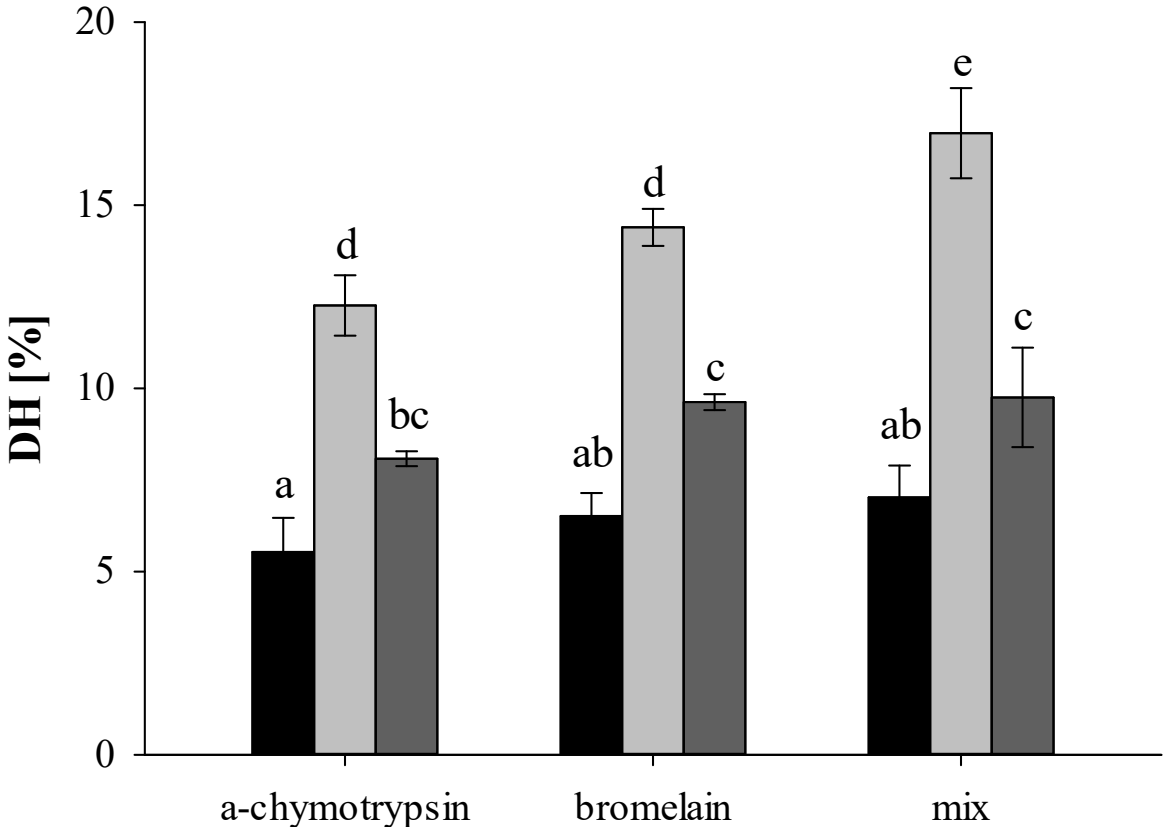


Figure 8

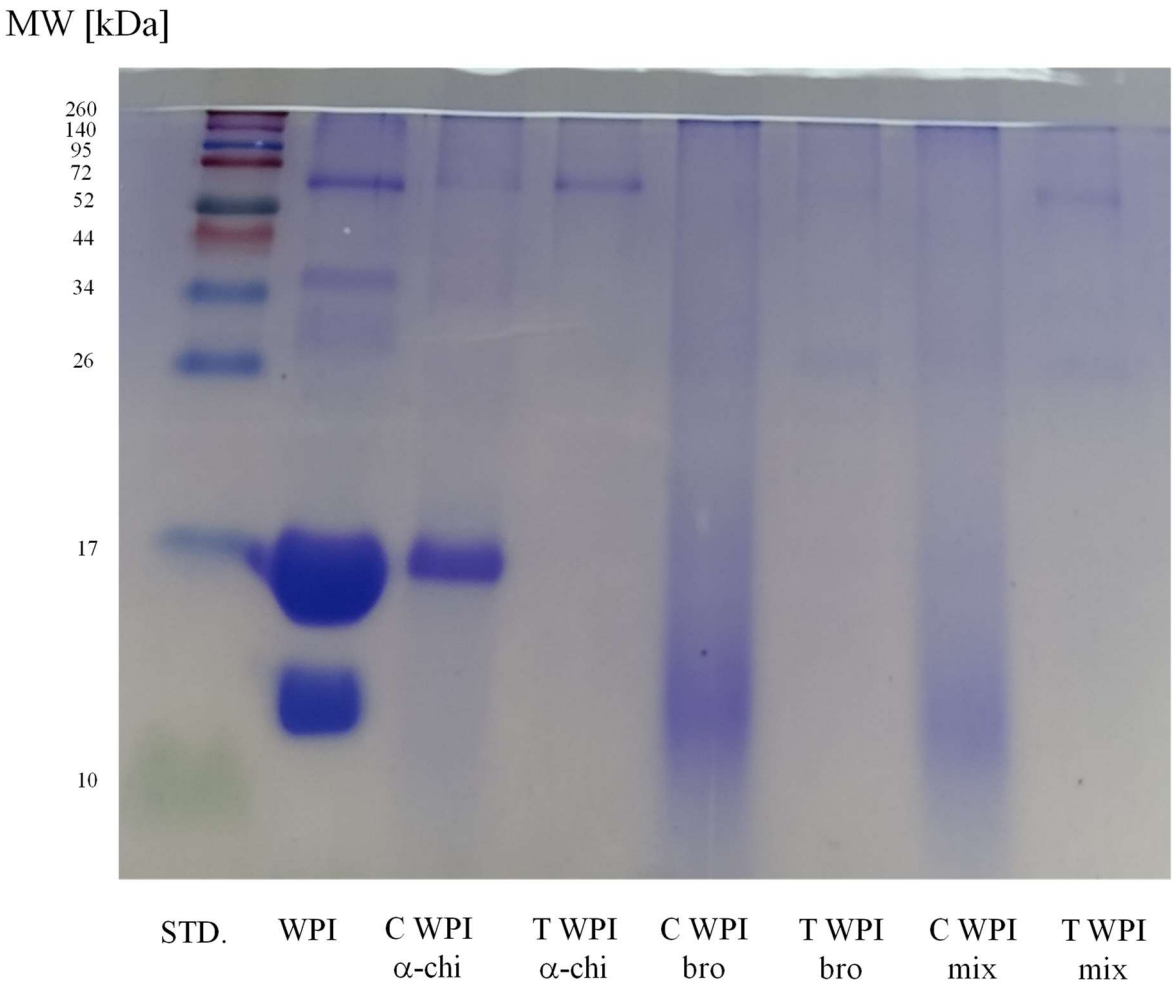

MW [kDa]
260
140
95
72
52
44
34
26
17
10
STD.
WPI
C WPI α-chi
T WPI α-chi
C WPI bro
T WPI bro
C WPI mix
T WPI mix

Table 1 Correlation coefficient among structural and techno-functional properties, namely free -SH groups concentration (μmol/$g_{prot.}$), foamability (%), foam stability (%) and air-water superficial tension (mN/m), of untreated (control) and HHP treated (P = 400 MPa, t = 15 min) WPI dispersions, observed along the whole refrigerated storage phase (0 – 10 days).

Properties	Free -SH groups	Foamability	Foam stability	Air-water superficial tension
Free -SH groups	-	0.959**	0.952**	-0.983**
Foamability	0.959**	-	0.956**	-0.946**
Foam stability	0.952**	0.956**	-	-0.951**
Air-water superficial tension	-0.983**	-0.946**	-0.951**	-

ns = not significant for $p > 0.05$; * = significant for $p \leq 0.05$; ** = significant for $p \leq 0.01$; *** = significant for $p \leq 0.001$.