Effect of ultrasound pretreatment and Maillard reaction on structure and antioxidant properties of ultrafiltrated smooth hound viscera proteins-sucrose conjugates

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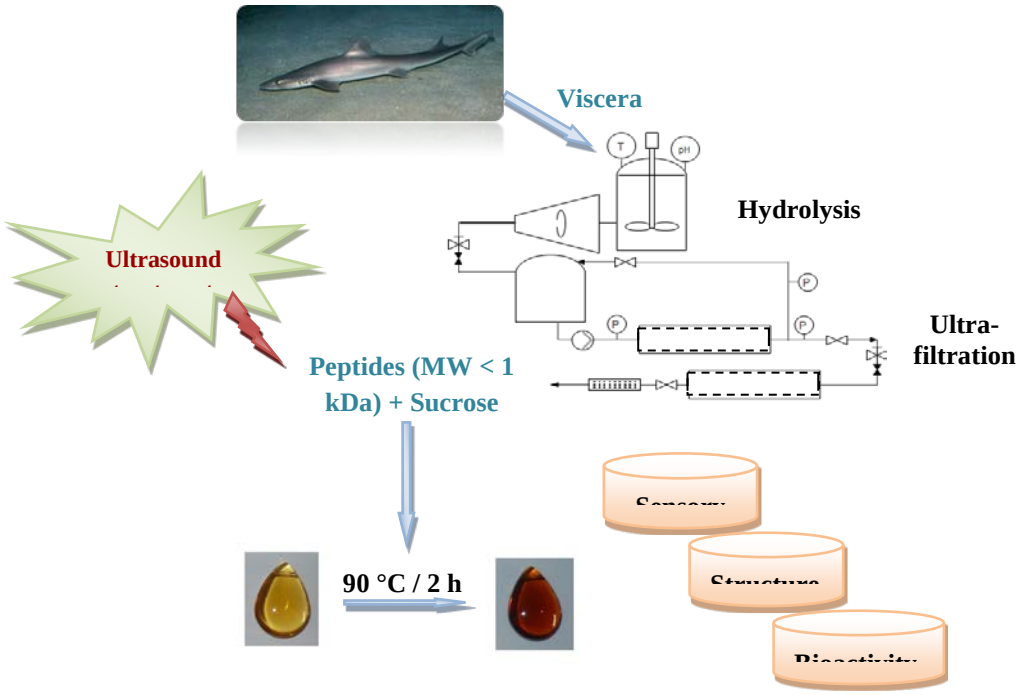
Running Title:

Ultrasound effect on ultrafiltrated peptides-sucrose properties

Graphical abstract



Abstract

The effect of ultrasound (US) pre-treatment on the evolution of Maillard reaction (MR) induced between low molecular weight (LMW) peptides and sucrose was studied. LMW peptides (<1 kDa) were obtained by the ultrafiltration of smooth hound viscera protein hydrolysates, derived produced by Neutrase, Esperase and Purafect. MR was induced by heating the LMW peptides in the presence of sucrose for 2 h at 90 °C, without or with US pre-treatment. During reaction, a marked decrease in pH values, coupled to the increase in color of the Maillard reaction products (MRPs), were recorded. In addition, after sonication, the glycation degree was significantly enhanced in Esperase-derived peptides/sucrose conjugates ($p<0.05$). Moreover, results showed that US treatment reduced the bitter taste and enhanced the antioxidant capacities of the resulting conjugates. Hence, it could be concluded that US leads to efficient mixing of sugar-protein solution and efficient heat/mass transfer, contributing to increase the MR rate.

Keywords: Ultrasound; Maillard reaction; Low molecular weight peptides; Glycation degree; Antioxidant activities.

1. Introduction

Among the numerous existing protein modification techniques, the glycation of proteins induced by sugar conjugation, via naturally-occurring Maillard reaction (MR), is commonly known as the best process on improving functional properties of food proteins (Seo, Karboune, L'Hocine & Yaylayan, 2013). Furthermore, the MR is one of the most complex reactions in food chemistry, due to the variety of compounds generated throughout the reaction. In fact, the amine-carbonyl condensation occurring during the early stages of the MR leads to form primordial intermediary products (Amadori products), which tended to be transformed during the advanced stages. These products can undergo numerous reactions giving rise to protein-sugar conjugates and then the formation of brown and polymeric products (Horvat & Jakas, 2004).

Different factors are involved in the MR evolution, the final glycation degree and MRPs composition, including the protein/carbohydrate ratio, temperature, incubation time, pH, as well as the interaction rate between the free amino groups of proteins and the carbonyl groups of sugars (Lan et al., 2010). Besides conventional thermal treatment, the ultrasound (US) is a new processing technology that has been recently reported to promote the interaction between proteins and reducing sugars (Corzo-Martínez, Moreno, Megías-Pérez, Olano, Moreno & Villamiel, 2014). In addition, it was reported that high-intensity sonication improve the propensity of proteins to aggregate (Chandrapala, Zisu, Palmer, Kentish & Ashokkumar, 2011) and can modify the secondary structure of proteins leading to increase their surface hydrophobicity (Stanic-Vucinic et al., 2012). On the other side, US treatment was found to modify the functional properties of carbohydrates (Sun, Hayakawa & Izumori, 2004) and enhance the isomerization of glucose to fructose (Wang, Pan, Zhang, Sun, Fang & Yu, 2012).

Depending on the operating conditions, different MRPs with various biological and functional properties and flavor sensory will be generated. Bioactive properties of MRPs include

93 the anti-cancer (Marko et al., 2003), anti-inflammatory (Chen & Kitts, 2015), antihypertensive
94 (Rufia´n-Henares & Morales, 2007) and anti-oxidative (Kitts, Chen, & Jing, 2012) activities.
95 Matmaroh, Benjakul and Tanaka (2006) have shown that the capacity of MRPs to reduce reactive
96 oxygen species is influenced by various factors such as the concentration of reactants, duration of
97 reaction, temperature, starting pH, water activity and the presence of metal ions or salts. In addition,
98 it has been previously reported that peptides below 1000 Da showed higher reactivity than high
99 molecular weight (HMW) peptides (Zhao, Zeng, Cui & Wang, 2007) and they are considered as a
100 key taste enhancer of flavor sensory, including umami, continuity and mouthfulness (Ogasawara,
101 Katsumata & Egi, 2006).

102 Proteins and peptides from animal origin are of great importance in food formulations such
103 as sausages from poultry, bovine or porcine meats (Hathwar, Rai, Modi & Narayan, 2012; Nasri et
104 al., 2013). Particularly, proteins from fish meat or its by-products are considered as premium
105 quality proteins for their high content in essential amino acids and their numerous nutritional
106 characteristics. In order to obtain new products from fish by-products, the major conventional
107 researches are still restricted to fishmeal production for animal feed (Péron, Mittaine & Le Gallic,
108 2010) and more recently for protein hydrolysates production (Abdelhedi et al., 2016; Zhou et al.,
109 2012). Recently MR was described to modify odor in salmon byproducts hydrolysates (Christelle,
110 Jean-Pascal, Regis, Laurent, Carole & Mireille, 2014) and to enhance the physicochemical,
111 antioxidant and hepato-protective properties of fish byproducts protein hydrolysate-ribose
112 conjugate (Yang, Lee, Pyo, Jeon, Kim & Lee, 2016).

113 Among byproducts, fish viscera represent a huge part of the total discards rejected in the
114 local fish markets and fish processing industries. Smooth hound (*Mustelus mustelus*) is the most
115 abundant hound shark in Tunisian coasts, with an interesting catch count of 192 tons (FAOSTAT,
116 2015). Regarding the huge quantities of visceral mass generated from freshwater fish marketing

and the dearth of their scientific exploitation, the present investigation aims to obtain protein hydrolysates from smooth hound viscera and their fractionation using ultrafiltration (UF) process in order to produce low molecular weight peptides.

Because sucrose is the cheapest sugar and the most commonly used in food industries for the fabrication of sweet products, the fractionated peptides were thereafter conjugated to sucrose via the MR. On the other hand, the impact of US pre-treatment on the final products was also investigated by the study of the physicochemical, sensory, structural and antioxidant properties of the resulting MRPs.

2. Materials and methods

2.1. Preparation of LMW peptides

Viscera were obtained following the processing of fresh filleted smooth hound (*M. mustelus*) fish available in the local fish market of Sfax City, Tunisia. The biological material was brought to the research laboratory in polyethylene bags, in iced conditions, within 30 minutes. Upon arrival, they were immediately rinsed with tap water to remove contaminants, and then weighed and stored in plastic bags at -20 °C until being used for protein hydrolysates elaboration.

Hydrolysis was carried out as previously described in our previou study (Abdelhedi et al., 2016). Smooth hound viscera were first cooked in distilled water at 95 °C for 15 min, with a solid/solvent ratio of 1:1 (w/v), to inactivate endogenous enzymes. After being well homogenized, the pH of the mixture was adjusted to the optimum value of the enzymatic activity by adding 4 N NaOH solution. Thereafter, viscera treated mass was subjected to enzymatic hydrolysis, using three different microbial enzymes, Neutrase® (pH 7.0), Esperase® (pH 9.0) and Purafect® (pH 10.0), added at an enzyme/protein ratio of 6/1 (U/mg of protein). During the reaction (50 °C), the pH of the mixture was maintained constant at the desired value by continuous addition of NaOH solution.

The degree of hydrolysis (DH) was calculated based on the volume of NaOH added during the reaction, as described by Adler-Nissen (1986) using the following formula:

$$DH\,(\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

where B is the amount of NaOH consumed (ml), *Nb* is the normality of the base, *MP* is the mass (g) of the protein (N = 6.25), and α represents the average degree of dissociation of the α-NH_2 groups in protein substrate expressed as:

$$\alpha = \frac{10^{pH\text{-}pK}}{1+10^{pH\text{-}pK}}$$

where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (*h*tot) in protein substrate was assumed to be 8.6 meq/g (Adler-Nissen, 1986).

After the achievement of the digestion process, the reaction was stopped by heating the different solutions for 20 min at 95 °C to inactivate enzymes. Protein hydrolysates were then centrifuged at 9500 g for 20 min to separate soluble fractions (peptides) and insoluble fractions (non-hydrolyzed proteins). Hydrolysates obtained through the digestion of smooth hound viscera proteins with Neutrase, Esperase and Purafect were noted NH, EH and PH, respectively.

Hydrolysates were fractionated by ultrafiltration (UF), as illustrated in Fig. 1. Two successive UF steps were applied on the liquid hydrolysates to obtain the LMW peptide fractions. First, a tangential flow filtration (Millipore, Labscale™ TFF System, USA) was performed using a 50 kDa molecular weight cut-off (MWCO) membrane to remove HMW proteins and contaminants. Then, permeates were separated by UF through 1 kDa MWCO membrane using a stirred cell (Amicon Mini membrane system, 1-800-Millipore, USA). The inlet and the outlet pressure were 10 psi and 5 psi, respectively. UF experiments were carried out in batch mode, where the initial volume was reduced to one-quart, *i.e.* to a volume concentration factor (VCF) = 4. After the UF processes,

permeates (MW < 1 kDa) were freeze-dried (Bioblock scientific Christ ALPHA 1-2, IllKrich-Cedex, France) and then stored at -20 °C until use.

2.2. Preparation of Maillard reaction products

The conjugation between LMW peptides from smooth hound protein hydrolysates and sucrose (S) was performed by suspending the freeze-dried peptide fractions and sucrose powder (1:1; w/w) in distilled water (1:5; w/v). Then, the mixtures were homogenized at room temperature (25 ± 1 °C) using a magnetic stirrer to form a uniform dispersion. After suspension, ultrasonic treatment (10 W/cm^2) was performed at 40 °C for 30 min with a frequency of 25 kHz and a maximum nominal power of 1,600 W, using SCIENTZ Electronics ultrasound device (JY98-3, Ningbo, China). Thereafter, the mixtures were incubated at 90 °C during 2 h to induce MR. All samples were then cooled immediately in iced water and then kept at 4 °C. The heated peptides-sucrose mixtures were termed as MRPs. A reference of lysine-sucrose conjugate (1:1, w/w) was prepared with the same manner and heated under the same conditions.

MRPs prepared from LMW-NH, LMW-EH and LMW-PH conjugated to sucrose without US pre-treatment were named LMW-NH/S, LMW-EH/S and LMW-PH/S, respectively, while those treated by US were referred to LMW-NH/S-Us, LMW-EH/S-Us and LMW-PH/S-Us, respectively. The control groups consisted of the protein hydrolysate fractions (< 1 kDa) heated alone during 2 h at 90 °C without sugar addition or US pretreatment.

During heating, products were sampled every 30 min in order to evaluate physiochemical properties, color, free amino acid contents, taste score and structural changes produced owing to caramelization process. Antioxidant activities of the resulting MRPs were also investigated.

184 **2.3. pH and browning intensity measurements of the MRPs**

185 The pH values of MRPs, obtained at 0, 30, 60, 90 and 120 min of heating, were measured
186 using a 827-pH meter (Metrohm, Swiss made) calibrated with buffer solutions at pH 4.0 and 10.0.

187 The UV-absorbance and the browning intensity of MRPs were measured at 294 nm and 420
188 nm, respectively (Guan et al., 2011), using an UV-visible spectro-photometer (T70, UV/VIS
189 spectrometer, PG Instruments Ltd., China). Appropriate dilutions of the MRPs were made when
190 required. The analyses were carried out in duplicate.

191 **2.4. Color measurement of the MRPs**

192 The color of the protein-sucrose conjugated samples was determined using a Color Flex
193 spectrocolorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA), readably calibrated
194 using a white standard plate (L* = 92.84, a* = -1.25 and b* = 0.49). The results of the different
195 samples were displayed as L*, a* and b* values which indicated lightness, redness and yellowness,
196 respectively. L*, a* and b* values were determined in triplicate from three dependent samples.
197 Whiteness (W*), differences in color (ΔE*) and chroma (C*) were calculated using the following
198 equations:

199 $$W^* = 100 - \left[\left(100 - L^*\right)^2 + a^{*2} + b^{*2} \right]^{1/2}$$

200 $$C^* = (a^{*2} + b^{*2})^{1/2}$$

201 $$\Delta E^* = \left[\left(L^* - L_0\right)^2 + \left(a^* - a_0\right)^2 + \left(b^* - b_0\right)^2 \right]^{1/2}$$

202 where L_0, a_0 and b_0 are the color of the sample at t = 0.

203

204 **2.5. Free amino acids content and degree of glycation**

Free amino acid contents of the freeze-dried LMW peptides and their MRPs (100 mg/ml) were determined after being derivatizated with phenyl isothiocyanate (PITC) according to the method described by Aristoy and Toldrá (1991). The PITC derivates were quantified by reverse phase HPLC with a 1200 Agilent liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector, using a PicoTag® column (300 mm × 3.9 mm, Waters, USA). The temperature was set at 52 °C and the detection was carried out at 254 nm. The eluents used were (A) 0.07 M sodium acetate adjusted to pH 6.55 and containing 2.5% acetonitrile and (B) 45:40:15 acetonitrile: water: methanol, with a flow rate of 1 ml/min. The following eluent gradients: initially 0% B; several consecutive linear gradients of (B) as follows (3% at 13.5 min; 3.5% at 19 min; 4.5% at 21 min; 33% at 40 min; then a linear change to 40% at 50 min). Results were expressed as mg of each amino acid per g of sample. For the glycation degree of the conjugated protein hydrolysate fractions (*i.e.* after prolonged glycation reaction for 120 min), results were expressed as the percentage of free amino acids content of the conjugates compared with those of untreated protein hydrolysate fractions.

2.6. Taste score of MRPs

The taste characteristics of the MRPs were assigned depending on the amino acid contents. Three categories of taste were selected (umami, bitter and sweet) using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) and bibliographic literature. Umami, bitter and sweet sensory attributes were evaluated based on Asp and Glu contents (Kabelová, Dvořáková, Čížková, Dostálek & Melzoc, 2009), Arg, Pro, Val, Ile, Leu, Lys, Phe and Trp contents (Yoshida & Saito, 1969) and Gly, Ala, Ser, Thr, Pro, Val and Lys contents (Yoshida & Saito, 1969), respectively.

2.7. Fourier Transform Infrared (FTIR) analysis

The infrared analysis was performed using the FTIR technique as previously reported by Liu, Kong, Han, Sun and Li (2014a) for MRPs. The spectroscopic measurements were performed using approximately 1 mg of each sample mixed with 100 mg of dried KBr The FTIR spectra at the mid IR region (500 and 4000 cm^{-1}) were recorded using a Thermo-Nicolet 5700 FTIR spectrometer equipped with an attenuated total reflection (ATR) accessory, with a resolution 4 cm^{-1} and scan. The background noise was corrected using the data for pure KBr.

2.8. Evaluation of the antioxidant activity

2.8.1. β-carotene bleaching assay

The prevention of β-carotene from bleaching was determined according to the method of Koleva, van Beek, Linssen, de Groot and Evstatieva (2002). First, the emulsion of β-carotene/linoleic acid was freshly prepared by dissolving 0.5 mg of β-carotene, 25 µl of linoleic acid and 200 µl of Tween 80 in 1 ml of chloroform. The chloroform was then completely evaporated under vacuum in a rotatory evaporator at 50 °C; then, 100 ml of distilled water were added and the resulting mixture was vigorously stirred. Thereafter, 2.5 ml of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 ml of each sample. Control tube was prepared in the same conditions by adding 0.5 ml of water, instead of sample, to the emulsion. The absorbance of every test tube was measured at 470 nm, before and after incubation for 1 to 2 h at 50 °C. The antioxidant activity was evaluated in terms of β-carotene bleaching and expressed as µM Trolox equivalent per mg of sample. The equation obtained for Trolox standard curve was $y = 0.0121\ x + 0.4003$ and the R^2 value was 0.99. The test was carried out in triplicate.

2.8.2. Reducing power assay

The capacity of protein hydrolysate fractions and their MRPs to convert Fe^{3+} into Fe^{2+} was determined as described by Yildirim, Mavi and Kara (2001). Samples (0.5 ml) were mixed with 1.25 ml of 0.2 M potassium phosphate buffer (pH 6.6) and 1.25 ml of 1% (w/v) potassium ferricyanide solution. After incubation for 30 min at 50 °C, 0.5 ml of 10% (w/v) trichloroacetic acid was added and the reaction mixtures were then centrifuged at 2700 g for 10 min. Finally, 1.25 ml of the supernatant solution, from each sample mixture, was mixed with 1.25 ml of phosphate buffer (0.2 M; pH 6.6) and then 0.25 ml of 0.1% (w/v) ferric chloride was added. After a-10 min reaction time, the absorbance of the resulting solutions was measured at 700 nm. The reducing power was expressed as µM Trolox equivalent per mg of sample. The equation obtained for Trolox standard curve was $y = 0.0283\ x + 0.0571$ and the R^2 value was 0.99. The control was conducted in the same manner, except that distilled water was used instead of sample. Values presented are the mean of triplicate analysis.

2.9. Statistical analysis

Data were expressed as mean ± SD (Standard Deviation) and statistically analyzed using SPSS ver. 17.0, professional edition. A one-way analysis of variance (ANOVA) was then performed and means comparison was carried out by Duncan's multiple range test to estimate the significance among the main effects at the 5% probability level.

3. Results and discussion

3.1. Smooth hound viscera protein hydrolysates and MRPs preparation

Three protein hydrolysates from smooth hound viscera were elaborated using three different microbial enzymes, Neutrase, Esperase, and Purafect, with different final degrees of hydrolysis (DH) of 4%, 10% and 14%, respectively. The differences in the HD could be due to the difference in the specificity of enzymes used during hydrolysis. Hydrolysates were separated by UF using 1

kDa MWCO membrane and LMW peptides were recovered from NH, EH and PH with respective yields of 13.25, 13.5 and 22.9 g per kg of fresh viscera. Thereafter, peptides (< 1 kDa) and sucrose mixtures, with or without US-pretreatment, were heated at 90 °C for 2 h to induce MR.

3.2. pH change of the MRPs

The pH change of MRPs subjected or not to US pretreatment (25 kHz for 30 min at 40 °C) was monitored to evaluate the MR evolution. As shown in Table 1, US pretreatment did not affect the pH value of peptides-sucrose mixtures at t = 0, whereas the heat treatment induced its increase during the first steps of the MR to be then gradually decreased ($p<0.05$) with advanced heating time. MR is known to make changes in the final products (Nooshkam & Madadlou, 2016). Meanwhile, for US pre-treated samples, the pH increased during the first 60 min of dry-heating and then values were significantly decreased ($p<0.05$), which indicated that US induced the acceleration of MR rate by enhancing the isomerization of glucose and fructose during the thermal hydrolysis of sucrose. In the same context, Wang, Pan, Zhang, Sun, Fang and Yu (2012) studied the combination of US application and ionic liquid to enhance the enzymatic isomerization of glucose to fructose. Also, Corzo-Martínez, Moreno, Megías-Pérez, Olano, Moreno & Villamiel (2014) investigated the effect of US on the isomerization of lactose, as a tool to accelerate the formation of lactulose to enhance MR.

The initial heating stage resulted in the hydrolysis of intact peptides by thermal treatment coupled to the progressive exposure of hidden amino groups, leading to increasing pH values. However, in a later stage, the peptides-sugar conjugation took place and the pH decreased gradually, as it was expected during the MR evolution. This decrease might be the result of either, the release of organic acids or the consumption of free N-terminal amino groups of peptides during protein-sucrose conjugation (Jiang, Rai, O'Connor & Brodkorb, 2013). These findings are in

agreement with those obtained by Nooshkam and Madadlou (2016), who assumed that the heating process induced the pH increase, at the early stages of the reaction, by the denaturation of whey protein hydrolysates.

3.3. Color measurement of the MRPs

The color changes of the MRPs prepared without or with US pre-treatment and heated at 90 °C for 2 h are shown in Table 1. No significant differences were detected among all the color parameters of the protein fractions heated alone ($p>0.05$) (Data not shown). Unlike control samples, the protein-sucrose combination, preceded by ultra-sonication, induced marked changes of the conjugated fractions color. In fact, whiteness values (W*) of the different samples decreased significantly with the thermal processing time, and this decrease was more pronounced in the US pre-treated samples rather than conventionally heated ones. Similarly, the color difference (ΔE*) values markedly increased over time ($p<0.05$) to reach their maximal levels in the US treated systems after 120 min of thermal treatment. Furthermore, increasing chroma (C*) values were observed with the increase of time, indicating the formation of brown compounds. Similar findings were obtained by Małgorzata, Konrad and Zieliński (2016) after buckwheat groats roasting for 50 min at 160 °C. The observed chromacity and whiteness changes of the MRPs demonstrated the occurring of the MR stages, characterized by the formation of brown colored pigments, which was relatively accelerated by US pretreatment.

According to the obtained results, the US treatment at relatively high temperature (40 °C) for 30 min was suitable to improve the MR rate in LMW peptides-sucrose model system. Corzo-Martínez, Moreno, Megías-Pérez, Olano, Moreno & Villamiel (2014) also observed an increase in the intermediate and advances stages of MR with US treatment at 40 °C with 50% of amplitude. In addition, Yu, Seow, Ong and Zhou (2017) showed that the ultrasonic MR process conducted at low

temperatures (below 60 °C) leads to generate higher levels of intermediate MRPs and melanoidins, than those produced at 65-75 °C.

3.4. UV-absorbance and browning intensity measurement of the MRPs

The MR rate was monitored based on the evaluation of the color intensity of the intermediate and final products, measured at 294 and 420 nm, respectively. In fact, carbonyl group-amino acid conjugation is associated to the development of intermediate colorless compounds (Amadori), which didn't absorb in the visible spectrum, prior to generation of brown pigments (melanoidins), the final products of the MR. Fig. 2 (A, B) shows A_{294} and A_{420} evolution as a function of the reaction time. As observed, the intermediate products and browning pigments formation began to increase in parallel after 60 min of dry-heating; particularly in LMW-NH/S and LMW-NH/S-Us, which were the most suitable systems for the production of melanoidins ($A_{420\ nm}$). In addition, products resulted from the US-assisted MR contained higher amount of final brown products within the first hour of dry-heating compared to conventional treated ones. However, the content of these advanced products still lower than that obtained in LMW-NH/S and LMW-NH/S-Us. Unlike A_{420nm} values, the US pre-treatment did not affect the formation of colorless intermediate compounds.

It could be concluded that, in the present systems, the browning development occurs after an induction period, characterized by the production of fluorescent uncolored intermediates, and then the brown color appeared. Interestingly, US pre-treatment gave final products with darker color. Similar evolution results were reported by Kim and Lee (2009) and Nooshkam and Madadlou (2016).

3.5. Free amino acid contents

Free amino acid (FAA) contents of the initial peptide fractions and their MRPs, evaluated each 60 min of thermal treatment, are summarized in Table 2. Data showed that there is a significant difference in the amounts of FAA between LMW peptides and the resulting MRPs. In addition, FAA contents of all MRPs decreased with the incubation time progress, confirming the occurrence of the MR during heating. The increase in the FAA amount in the heated peptides, without sucrose addition, is mainly due to the thermal protein hydrolysis occurred during dry-heating for 2 h. However, a significant decrease in total FAA, following the addition of sucrose, was observed, which resulted from the strong cross-linking between sucrose and free amino residues via the MR. Similar results were reported by Eric et al. (2013) and Lan et al. (2010) who studied the MRPs derived from xylose/cysteine-sunflower protein hydrolysate and xylose–soybean peptide model systems, respectively. In addition, Zhao, Zeng, Cui and Wang (2007) (2007) reported that in MR, peptides below 1 kDa showed higher reactivity than peptides with higher MW and that the direct peptide–sugar cross-linking via MR decreased the free amino acids content.

Moreover, results showed that sonication markedly promoted the MR between LMW peptides and sucrose compared to the non-sonicated samples. In fact, the final total FAA level of LMW-NH/S-Us, LMW-EH/S-Us and LMW-PH/S-Us, after 120 min of heating, were estimated at about 0.95, 1.55 and 0.97 mg/g, respectively, vs.1.08, 2.36 and 0.93 mg/g for LMW-NH/S, LMW-EH/S and LMW-PH/S, respectively. Similar data were found by Stanic-Vucinic, Prodic, Apostolovic, Nikolic and Velickovic (2013) showing that high-intensity ultrasound in aqueous solution promoted the glucose and fructose isomerization after the thermal hydrolysis of sucrose, leading to the MR rate acceleration. Particularly, a fast rate of Lys free residue loss was observed, proving the successful protein-sugar linking during MR. The high reactivity of Lys was also observed in the Lys/S reference system, where the total content of available Lys has been decreased by 57.15, 69.26, and 84.69% after 30, 60, and 90 min of heat processing, respectively. In the same

365 context, Golon, Kropf, Vockenroth and Kuhnert (2014) have shown that, in the presence of sucrose
366 and after heating, lysine and tyrosine yielded in simple spectra of MRPs, followed by arginine and
367 aspartic acid, whereas cysteine and serine produced the highest number of compounds.

368 It has been reported that US induced significant loss of protein amino groups due to free
369 radical reactions generated by water sonolysis, and lysine residue is prone to be oxidized to lysine
370 aldehyde, resulting in loss of its amino group (Meltretter & Pischetsrieder, 2008). These aldehydes
371 are highly reactive and they can undergo spontaneous MR in the presence of sugars.

372 **3.6. Degree of glycation**

373 The degrees of glycation in the final MRPs were determined and the results are shown in Fig.
374 2c. The ultrasound pre-treatment was found to enhance significantly only the LMW-EH/S system.
375 However, LMW-NH/S and LMW-PH/S systems, sonicated or not, had similar glycation degrees
376 (about 80%) ($p>0.05$), which were higher than those recorded in the LMW-EH/S-Us sample.
377 Greater glycation degrees reflected the high reactivity of LMW peptides and their derived free
378 amino groups with sucrose hydrolysates. These data are in accordance with those reported by
379 Nooshkam and Madadlou (2016) showing that the final MRPs, obtained from milk ultrafiltration
380 permeate and whey protein isolate heated at 90 °C for 45 min, exhibited a glycation degree of 46.73
381 ± 6.29%.

382 **3.7. Taste score of MRPs**

383 Flavor and aroma development throughout the MR depends, particularly, on the type of
384 sugars and amino acids involved in the reaction. In the present study, sweet, bitter and umami
385 scores of the different MRPs were determined as a function of the reaction time. As shown in Fig.
386 3, the original peptides contained high amounts of sweet amino acids, which were gradually
387 decreased from the first 30 min of heating, particularly in the US-pretreated samples. According to

388 Table 2, the observed sweetness decrease is mainly due to the consumption of free Gly and Lys
389 residues during MR, known as the most reactive amino acids. In fact, Guan et al. (2011) and
390 Vhangani and Wyk (2013) have described the high reactivity of glycine and lysine in glucose-
391 glycine, fructose–lysine and ribose–lysine model systems.

392 Similarly, hydrophobic FAA residues, responsible to the bitter taste, were significantly
393 reduced by 41.18%, 6.74% and 19.31%, for LMW-NH/S, LMW-EH/S and LMW-PH/S,
394 respectively, compared to the initial hydrolysates, after 60 min of heating. However, this reduction
395 was more significant for US-derived MRPs and reached 54.55%, 19.69% and 51.45% in LMW-
396 NH/S-Us, LMW-EH/S-Us and LMW-PH/S-Us, respectively, after the same period of heating. The
397 reduction in bitter attribute reflects the decrease of bitter taste in the final products, which is an
398 important criterion in food formulations.

399 For the umami amino acids, a similar behavior was observed, and a minimum content of
400 umami residues was kept constant until the end of the reaction, which promises the sensorial quality
401 of the final MRPs. In the same context, it has been reported that the Maillard peptides (MW = 1–5
402 kDa) are good enhancers of flavor, including umami, continuity and mouthfulness, and then they
403 could be considered as key taste enhancers in food formulations (Ogasawara, Katsumata & Egi,
404 2006). Hence, the obtained LMW peptides-sucrose derived MRPs could serve as potential flavor
405 promoting agents in thermally processed foods.

406

407

408 **3.8. Fourier transform infra-red analysis**

409 Fourier transform infra-red (FT-IR) spectroscopy is a useful technique to study protein–
410 carbohydrate systems. In order to investigate the interaction between sugars and peptides before
411 and after dry-heating, FT-IR analysis was performed (Fig. 3d).

412 As shown in FT-IR spectra, there are several typical regions in both, protein fractions and
413 their sucrose conjugates corresponding, respectively, to the N-H stretching at ~ 3301-3237 cm^{-1} for
414 the amide A and C–H stretching at ~ 2914-2939 cm^{-1} for the amide B (Chang & Tanaka, 2002).
415 No significant changes were observed in the FT-IR spectra of the different protein hydrolysate
416 fractions, before and after heat treatment, particularly in the amide A and B regions. Nevertheless,
417 the thermal treatment for 2 h affected the FT-IR spectra of the resulting conjugates, as compared
418 to the initial spectra of LMW peptide fractions. Particularly, US-pretreatment was found to affect
419 significantly the amide A band wavenumbers, which decreased from 3274 to 2998 cm^{-1} for LMW-
420 NH/S and LMW-NH/S-Us, respectively. Furthermore, US-treated MRPs showed the lowest
421 intensities at these regions among all the other samples, suggesting that the free -OH groups of
422 sucrose and amino acids (3380–3340 cm^{-1}) were consumed during the heating process.

423 Moreover, after MR induction, important intensity reductions were noted in the C=O
424 stretching (1600-1700 cm^{-1}) and N-H deformation (1500-1550 cm^{-1}) of the amide I and II,
425 respectively, especially in the NH and PH-derived MRPs. In addition, after 2 h of heating, marked
426 deformations of the bands attributable to C–N stretching and N–H bending of amide III vibrations
427 (1220-1400 cm^{-1}) were observed, particularly in the US-pretreated samples. The present changes
428 in the amide III band may be attributed to the complicated cross-linking occurred between peptides
429 and sucrose (Rhim, Mohanty, Singh & Ng, 2006). In this context, Stanic-Vucinic et al. (2013)
430 reported that strong sheer forces generated during sonication led to efficient mixing of sugar-
431 protein solution and efficient heat/mass transfer, contributing to increase the rate of intermediate
432 and advances stages of the MR. In addition, Corzo-Martinez, Moreno, Megías-Pérez, Olano,

Moreno and Villamiel (2014) found greater protein-sugar interactions in the ultrasonicated systems as compared to conventional treated samples under the same conditions.

Results of the FT-IR analysis reflected the interactions bonds between proteins and sugar formed during dry-heating, which were typically caused by the MR and proved the effect of the US-pretreatment on accelerating the reaction rate.

3.9. Evaluation of the antioxidant activity of MRPs

The antioxidant activities of peptides/sucrose model systems, before and after thermal treatment, were assayed in terms of their reducing power and β-carotene bleaching protection (Fig. 4). The three protein hydrolysates showed varying degrees of antioxidant activities, which is mainly due to their initial amino acids composition and their sequences. Data showed that LMW-EH exhibited the highest reducing power, while LMW-PH displayed the highest β-carotene protection effect. Also, after 120 min of heating, the antioxidant activities of all peptides remained constant, except for LMW-EH/S where the reducing power decreased by 20% after 120 min. On the other hand, the addition of sucrose and US pretreatment, without heating process, did not affect significantly the antioxidant capacity of unheated peptides ($p>0.05$). In contrast, the protein-sucrose conjugation (2 h at 90 °C) enhanced the antioxidant potential of the resulting MRPs that showed stronger reducing power and β-carotene bleaching protection effect, compared to the initial peptides ($p<0.05$). Interestingly, US treatment was found to enhance considerably the antioxidant potential of MRPs. Among the three US-treated MRPs, the highest reducing power enhancement was obtained with LMW-NH/S-Us, which increased by 200% compared to LMW-NH/S, while the greater β-carotene bleaching protection improvement was found with LMW-EH/S-Us (+99% in comparison with LMW-EH/S). Interestingly, US pre-treated MRPs exhibited higher antioxidant potential than those derived from conventional heating (without US).

This study revealed that the physicochemical and structural changes greatly correlated with the increase in the antioxidant activity. In addition, our results are in agreement with those obtained by Huang et al. (2012) and Liu, Kong, Han, Sun and Li (2014a) who found that the glycation of LMW peptides enhanced their reducing power and antiradical activity, compared to the peptides alone. The increase of antioxidant properties might be attributed to generation of new products, resulting from peptides/sucrose cross-linking. Furthermore, Liu, Li, Kong, Jia and Li (2014b) proposed that the MR could be a useful method to improve the free radical scavenging, iron chelating activity and reducing power of protein hydrolysates, and they explained this finding by the caramelization reaction induced between an NH_2-protein and a sugar-reducing compound.

Moreover, Daglia, Papetti, Aceti, Sordelli, Gregotti and Gazzani (2008) suggested that advanced MRPs are considered as a particular complex mix that contained numerous compounds, which contributed mostly to the antioxidant activity of the product mixture. Hence, the present results suggested that the conjugates obtained from LMW peptides-sucrose system could be a potential food antioxidant ingredient in functional food products.

4. Conclusion

In the present study, LMW peptides-sucrose conjugates were elaborated through the MR process, with or without ultrasonic pre-treatment. Neutrase-derived peptides were the most reactive agents with sucrose, resulting in higher COOH-NH_2 interaction. Physical (pH, color and browning intensity) and structural changes showed that, in the different studied systems, US pre-treatment accelerated the rate of the MR evolution and give the higher glycation degrees as compared to conventional heating process. In addition, MRPs showed better taste attributes (low amount of bitter amino acids) and increased antioxidant capacities, evaluated based on their reducing power and β-carotene protection activities, compared to the un-conjugated proteins. As a result, the

proposed food model systems, formed based on LMW peptides (< 1 kDa) from smooth hound by-products and sucrose, could serve as aromatic and bioactive ingredients for preparing foods.

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Table 1. pH and color evolution of protein hydrolysates fractions and their MRPs during heating for various times

	Without ultrasonic treatment					With ultrasonic treatment				
Time (min)	**0**	**30**	**60**	**90**	**120**	**0**	**30**	**60**	**90**	**120**
pH	5.42±0.11^{c}	5.32±0.01^{c}	5.53±0.02^{c}	6.93±0.01^{a}	6.53±0.02^{b}	5.38±0.02^{b}	6.17±0.05^{a}	6.20±0.01^{a}	5.56±0.03^{b}	5.49±0.09^{b}
W*	92.55±0.01^{a}	90.90±0.25^{b}	90.41±1.2^{b}	89.94±1.30^{bc}	87.42±1.36^{c}	91.26±0.00^{a}	80.15±0.43^{b}	74.98±0.31^{c}	71.01±0.71^{d}	71.61±0.80^{d}
C*	1.22±0.02^{c}	1.48±0.13^{b}	1.61±0.14^{a}	1.48±0.21^{ab}	1.77±0.1^{a}	1.20±0.01^{c}	2.13±0.2^{a}	1.77±0.1^{b}	1.78±0.01^{b}	2.22±0.1^{a}
ΔE*	-	1.68±0.06^{d}	2.40±0.15^{c}	2.80±0.17^{b}	5.17±0.38^{a}	-	11.77±0.29^{a}	16.48±0.21^{b}	20.33±0.50^{a}	20.43±0.43^{a}
pH	6.30±0.1^{bc}	6.20±0.01^{c}	6.37±0.01^{b}	6.40±0.01^{a}	5.62±0.01^{d}	6.30±0.01^{b}	6.73±0.03^{a}	6.81±0.04^{a}	6.16±0.05^{b}	5.20±0.01^{c}
W*	89.22±1.03^{a}	84.02±2.31^{c}	87.15±0.63^{a}	87.35±1.09^{a}	87.87±0.68^{b}	90.22±1.00^{a}	79.83±1.32^{b}	75.42±0.39^{c}	74.45±0.19^{c}	71.74±0.1^{d}
C*	1.00±0.12^{b}	1.26±0.10^{ab}	1.15±0.11^{ab}	1.28±0.13^{ab}	1.35±0.04^{a}	1.02±0.21^{d}	1.79±0.03^{b}	1.64±0.02^{c}	1.63±0.01^{c}	2.19±0.07^{a}
ΔE*	-	1.93±0.72^{b}	2.30±0.29^{b}	3.03±1.57^{b}	5.47±0.22^{a}	-	11.02±0.05^{d}	15.22±0.04^{c}	16.20±0.01^{b}	19.96±0.06^{a}
pH	6.03±0.01^{e}	6.33±0.02^{d}	6.38±0.01^{c}	7.08±0.03^{a}	6.53±0.02^{b}	6.00±0.00^{cd}	6.71±0.14^{b}	7.02±0.10^{a}	6.62±0.1^{b}	6.25±0.3^{b}
W*	85.95±2.69^{a}	83.81±0.99^{a}	84.17±1.31^{a}	83.20±0.01^{b}	79.94±0.76^{c}	86.00±2.68^{a}	82.56±1.67^{a}	84.22±2.61^{a}	76.58±1.30^{b}	77.26±0.7^{b}
C*	1.48±0.04^{c}	1.53±0.02^{b}	1.53±0.05^{b}	1.62±0.13^{ab}	1.65±0.01^{a}	1.36±0.05^{b}	2.05±0.1^{a}	1.53±0.1^{b}	1.64±0.25^{ab}	1.77±0.4^{ab}
ΔE*	-	1.91±0.43^{d}	3.84±0.92^{c}	5.57±0.03^{b}	6.32±0.32^{a}	-	3.80±0.05^{c}	3.87±0.03^{c}	9.44±0.02^{b}	10.88±0.01^{a}

LMW-NH/S, LMW-EH/S and LMW-PH/S indicate the MRPs prepared from LMW peptides derived from NH, EH and PH and conjugated to sucrose (S), respectively.

Different letters in the same sample within different incubation time indicate significant differences at $p < 0.05$.

$$W^* = 100 - \left[\left(100 - L^*\right)^2 + a^{*2} + b^{*2} \right]^{1/2}$$

$$C^* = (a^{*2} + b^{*2})^{1/2}$$

$$\Delta E^* = \left[\left(L^* - L_0\right)^2 + \left(a^* - a_0\right)^2 + \left(b^* - b_0\right)^2 \right]^{1/2}$$

where L_0, a_0 and b_0 are the color of the sample at t = 0.

663 **Table 2.** Changes in free amino acid contents of LMW peptides and their MRPs

664

Free amino acids (mg/g)			Asp	Glu	Hyp	Ser	Gly	Tau	His	Thr	Ala	Arg	Pro	Tyr	Val	Met	Ile	Leu	Phe	Trp	Lys	**Total**
		0	0.2	0.24	0.02	0.16	0.83	0.1	0.07	0.22	0.87	0.05	0.32	0.07	0.42	0.11	0.19	0.34	0.16	0.03	0.36	4.76
		120	0.2	0.25	0.02	0.16	0.85	0.11	0.06	0.22	1.07	0.06	0.4	0.11	0.61	0.12	0.22	0.32	0.15	0.02	0.31	5.26
		60	0.07	0.13	0.01	0.06	0.35	0.03	0.03	0.1	0.35	0.02	0.17	0.06	0.36	0.06	0.13	0.22	0.11	0.02	0.07	2.35
		120	0.03	0.07	0.01	0.03	0.17	0	0.01	0.06	0.17	0.01	0.09	0.03	0.18	0.02	0.05	0.09	0.04	0	0	1.08
		60	0.03	0.09	0.01	0.05	0.29	0.02	0.02	0.08	0.31	0.01	0.16	0.05	0.34	0.06	0.12	0.22	0.11	0.02	0.04	2.04
		120	0.02	0.04	0.01	0.03	0.12	0	0.01	0.03	0.13	0	0.08	0.02	0.3	0.02	0.04	0.07	0.04	0	0	0.95
		0	0.1	0.27	0.02	0.06	0.41	0.07	0.03	0.14	0.49	0.04	0.35	0.08	0.28	0.1	0.25	0.45	0.12	0.04	0.4	3.69
		120	0.1	0.29	0.02	0.1	0.45	0.09	0.03	0.13	0.54	0.03	0.3	0.09	0.41	0.11	0.2	0.52	0.12	0.05	0.4	3.98
		60	0.16	0.2	0.03	0.12	0.06	0.11	0.08	0.1	0.49	0.09	0.2	0.16	0.23	0.16	0.22	0.4	0.18	0.04	0.44	3.47
		120	0.08	0.15	0.02	0.06	0.04	0.05	0.03	0.1	0.49	0.03	0.1	0.1	0.12	0.09	0.15	0.42	0.13	0.04	0.15	2.36
		60	0.16	0.12	0.02	0.1	0.03	0.1	0.05	0.19	0.49	0.06	0.25	0.11	0.15	0.12	0.15	0.44	0.11	0.03	0.36	3.05
		120	0.04	0.1	0.01	0.03	0.01	0.02	0.02	0.07	0.32	0.03	0.07	0.07	0.02	0.07	0.12	0.34	0.11	0.03	0.07	1.55
		0	0.11	0.17	0.02	0.12	0.48	0.06	0.03	0.1	0.61	0.1	0.19	0.09	0.34	0.08	0.14	0.35	0.13	0.03	0.17	3.32
		120	0.11	0.18	0.02	0.13	0.51	0.09	0.03	0.11	0.68	0.1	0.22	0.11	0.31	0.11	0.15	0.4	0.16	0.04	0.19	3.65
		60	0.2	0.17	0.03	0.1	0.3	0.12	0.08	0.26	0.5	0.08	0.11	0.09	0.25	0.12	0.21	0.21	0.1	0.09	0.12	3.15
		120	0.01	0.02	0.01	0.03	0.12	0	0.01	0.05	0.14	0.01	0.08	0.04	0.06	0.03	0.06	0.16	0.07	0.01	0.02	0.93
		60	0.05	0.07	0.01	0.05	0.23	0	0.01	0.07	0.21	0.02	0.11	0.04	0.1	0.04	0.08	0.2	0.08	0.01	0.07	1.46
		120	0.01	0.03	0.01	0.03	0.12	0	0.01	0.07	0.14	0.01	0.07	0.04	0.07	0.03	0.07	0.16	0.07	0.01	0.02	0.97

665

Figure captions:

Fig. 1. Experimental set-up of ultrafiltration process before Maillard reaction induction, where P: pressure and T: temperature points of measurement.

Fig. 2. Evolution of the absorbance at 294 nm (A) and 420 nm (B) as indicators of intermediate and advanced stages of MR, respectively, after different intervals of incubation time.
Glycation degree value of the final MRPs (C); different letters in the same system with different treatments (without or with US) indicate significant differences ($p < 0.05$).

Fig. 3. Evolution of the taste scores of MRPs prepared without or with ultrasonic treatment, after different intervals of incubation time: sweet (a), bitter (b) and umami (c) amino acid contents. Effect of ultrasonic treatment on the infra-red spectra of the LMW peptides heated alone or in conjugation to sucrose (d).

Fig. 4. Comparison of the antioxidant activities of LMW peptides and their sucrose conjugates, without or with US pre-treatment, before and after heat treatment at 90 °C for 2 h; Reducing power assay (a, b, c) and inhibition of β-carotene bleaching activity (d, e, f). Different letters in the same sample within different incubation times indicate significant differences ($p < 0.05$).

Fig. 1

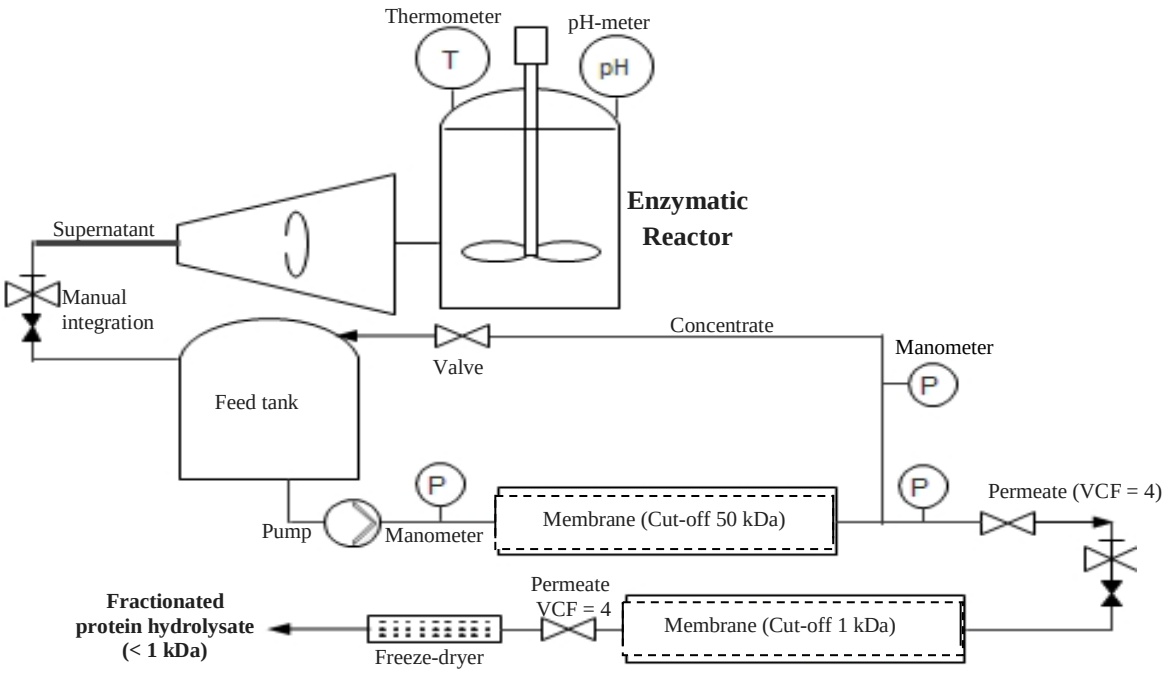
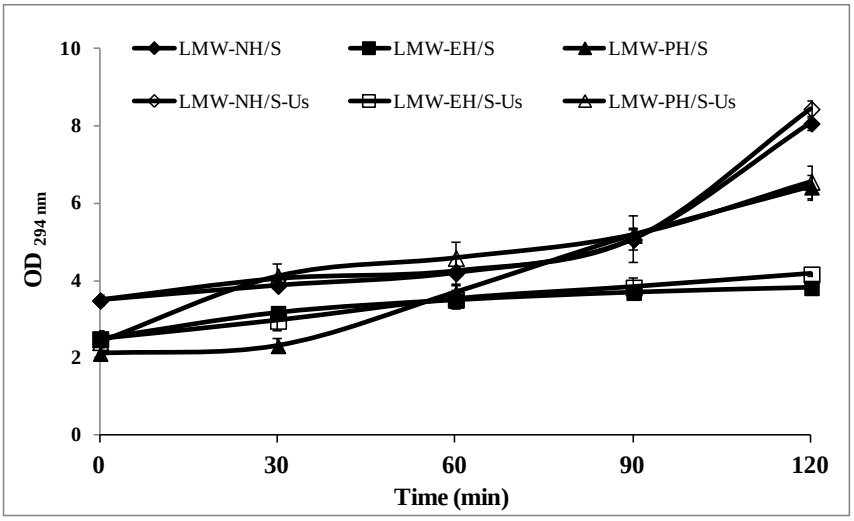
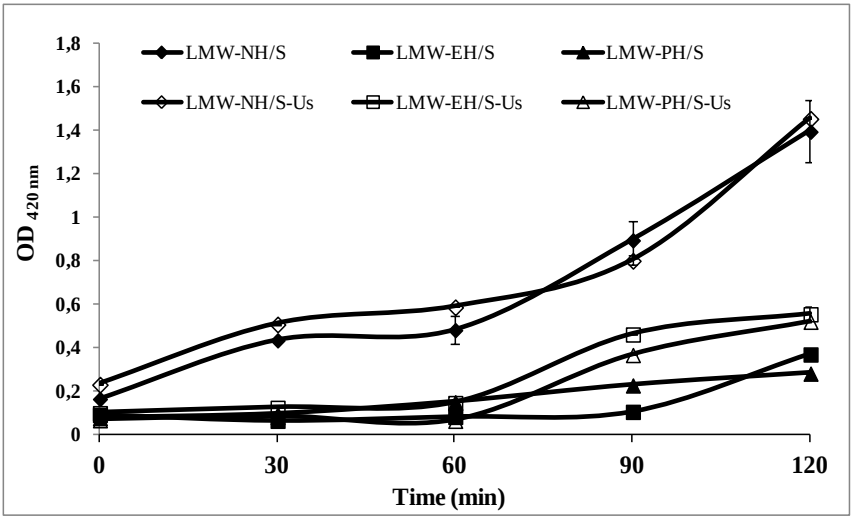

Thermometer
pH-meter
T
pH
Enzymatic Reactor
Supernatant
Manual integration
Concentrate
Manometer
Valve
Feed tank
P
P
P
Pump
Manometer
Membrane (Cut-off 50 kDa)
Permeate (VCF = 4)
Permeate VCF = 4
Membrane (Cut-off 1 kDa)
Fractionated protein hydrolysate (< 1 kDa)
Freeze-dryer

Fig. 2

A


LMW-NH/S
LMW-EH/S
LMW-PH/S
LMW-NH/S-Us
LMW-EH/S-Us
LMW-PH/S-Us
OD 294 nm
Time (min)
0
2
4
6
8
10
0
30
60
90
120

B


LMW-NH/S
LMW-EH/S
LMW-PH/S
LMW-NH/S-Us
LMW-EH/S-Us
LMW-PH/S-Us
OD 420 nm
Time (min)
0
0,2
0,4
0,6
0,8
1
1,2
1,4
1,6
1,8
0
30
60
90
120

C

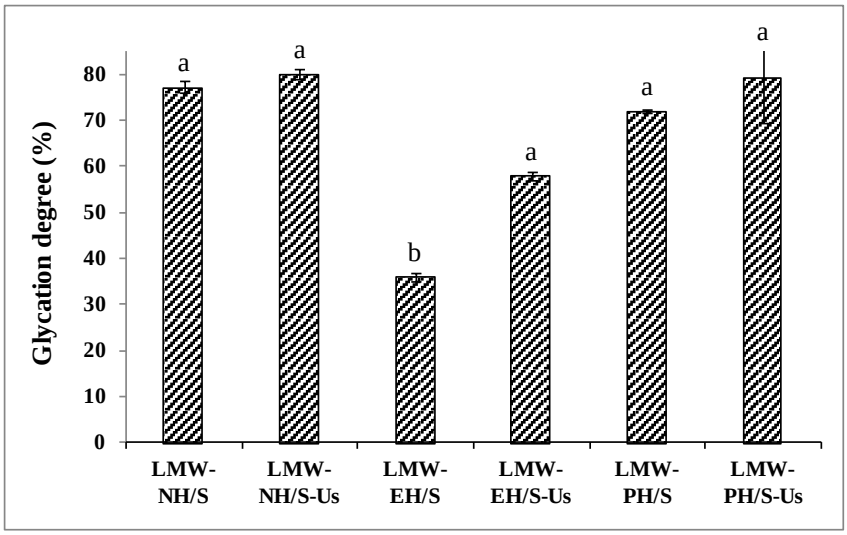

Glycation degree (%)
0
10
20
30
40
50
60
70
80
a
a
b
a
a
a
LMW-NH/S
LMW-NH/S-Us
LMW-EH/S
LMW-EH/S-Us
LMW-PH/S
LMW-PH/S-Us

Fig. 3

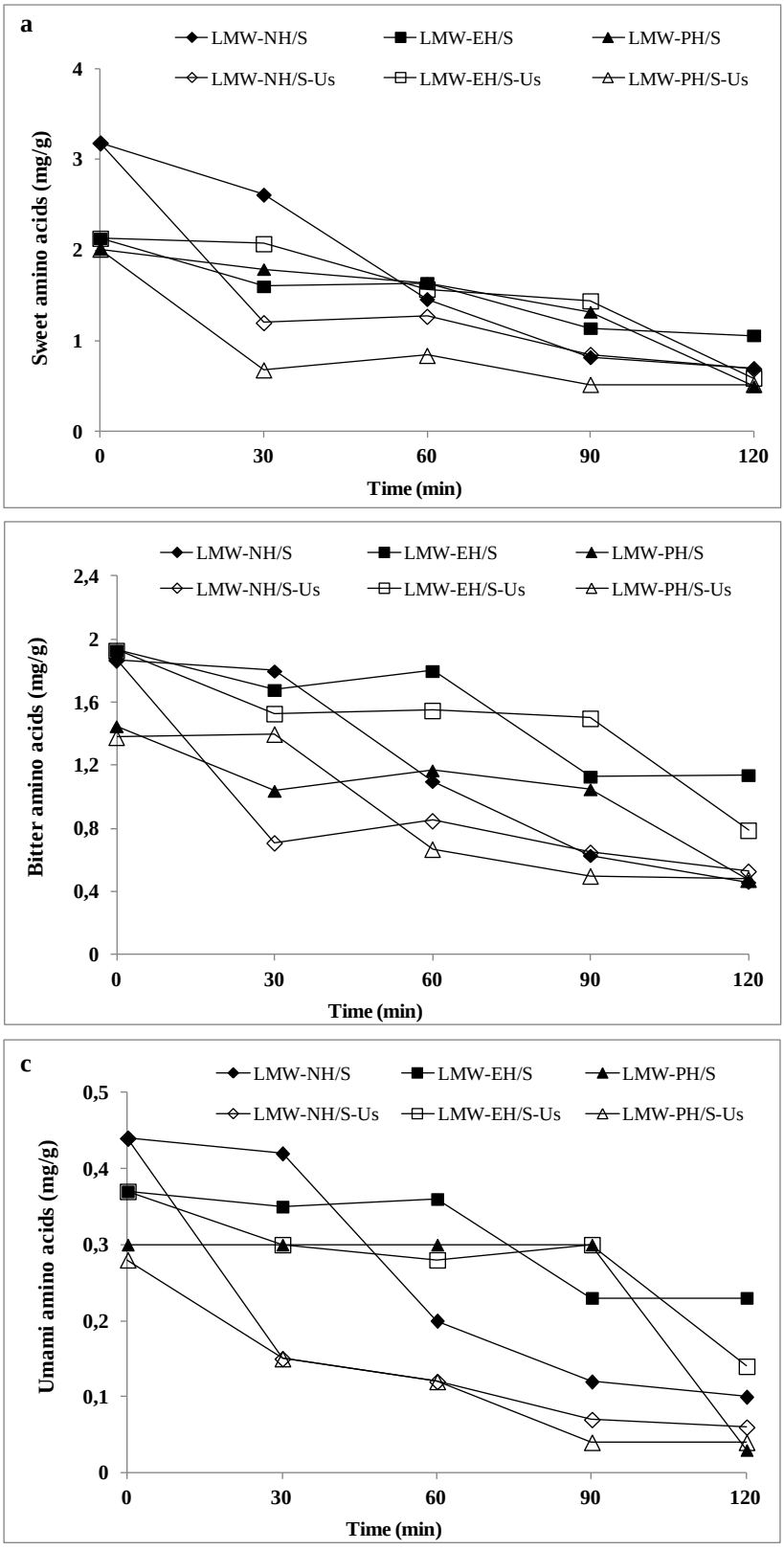

a
LMW-NH/S
LMW-EH/S
LMW-PH/S
LMW-NH/S-Us
LMW-EH/S-Us
LMW-PH/S-Us
Sweet amino acids (mg/g)
Time (min)
Bitter amino acids (mg/g)
Time (min)
c
Umami amino acids (mg/g)
Time (min)

Fig. 3d

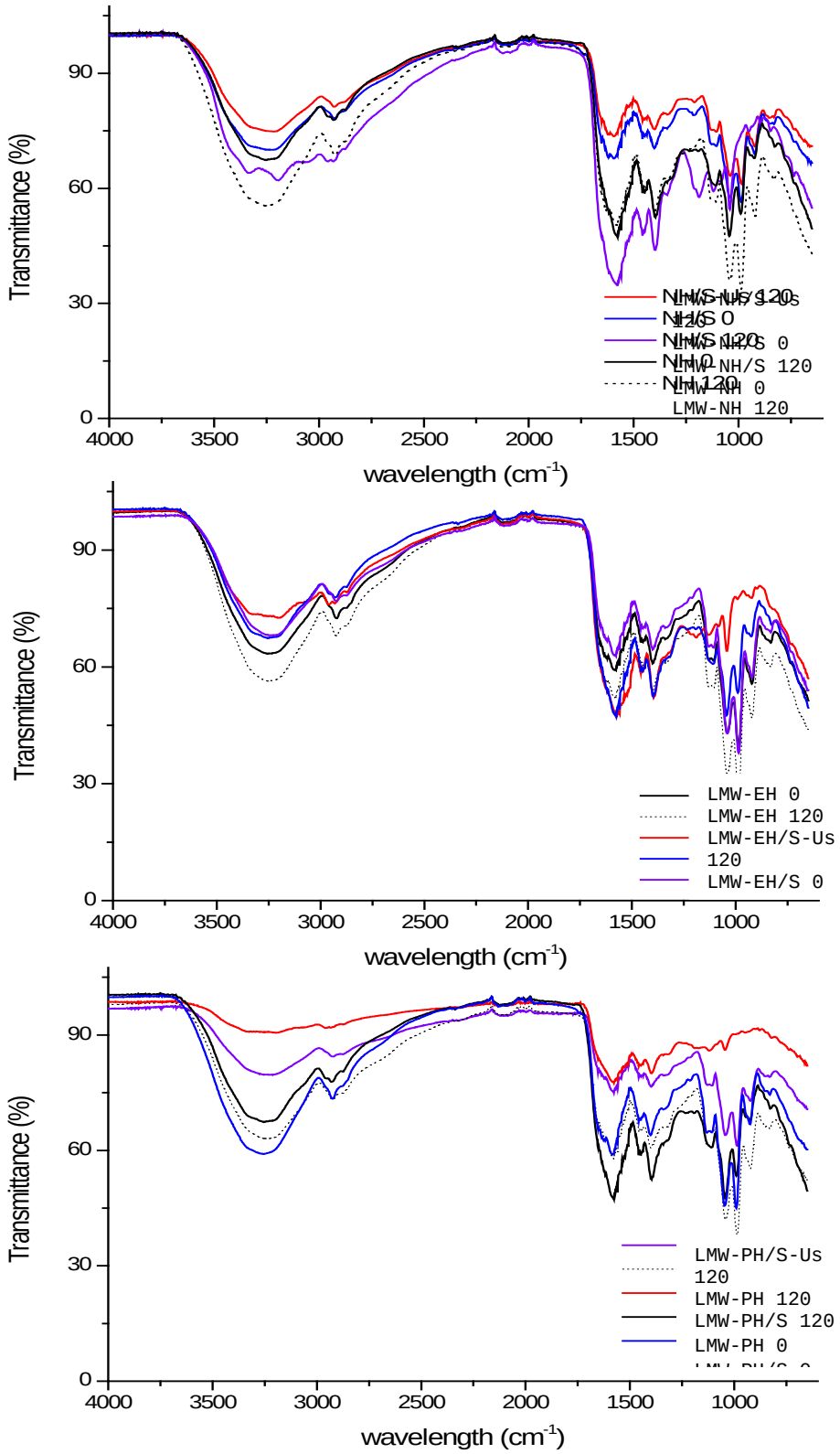
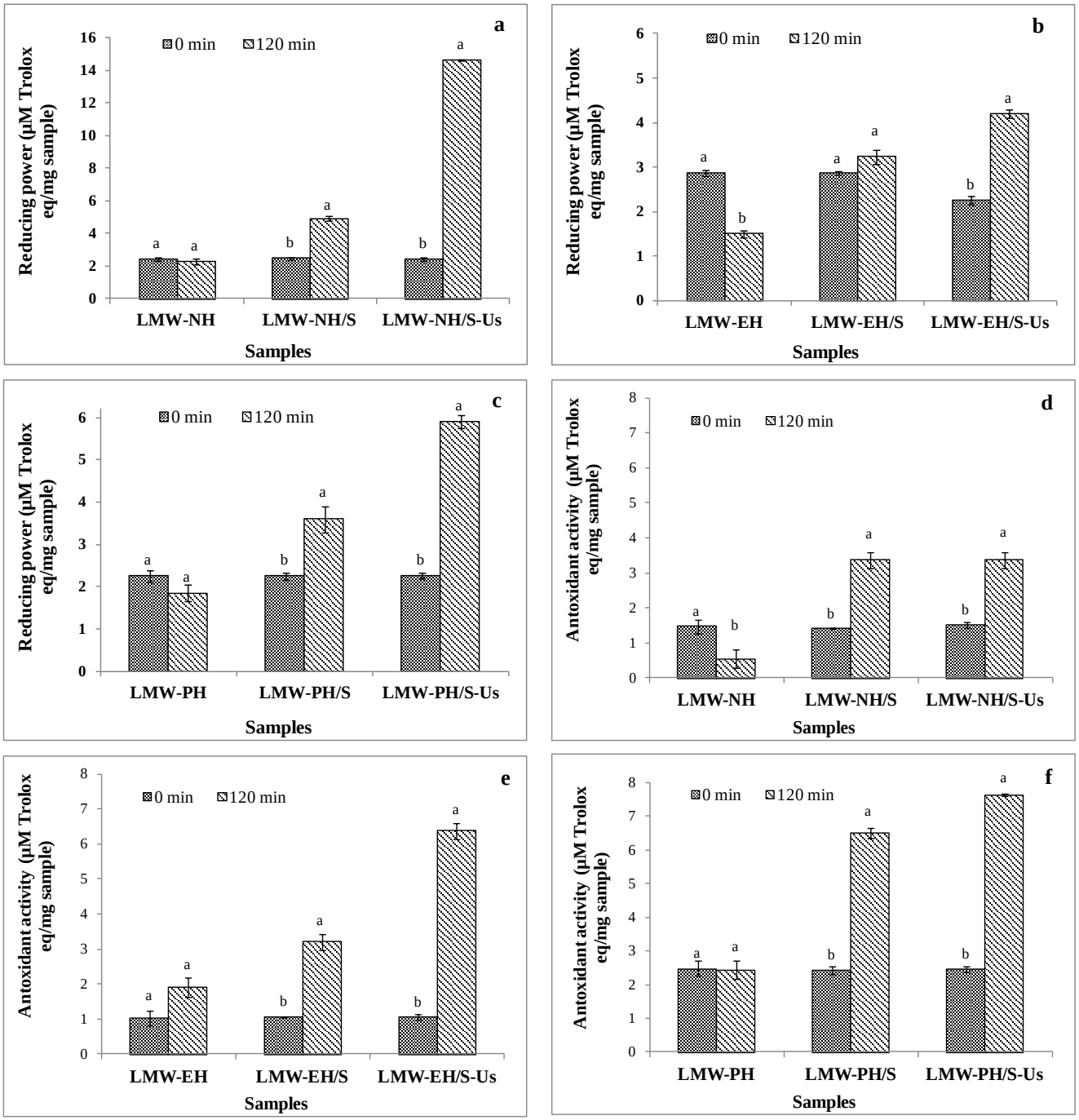

Transmittance (%)
wavelength (cm-1)
LMW-NH/S-Us
LMW-NH/S 120
LMW-NH/S 0
LMW-NH 120
LMW-NH 0
LMW-EH 0
LMW-EH 120
LMW-EH/S-Us
120
LMW-EH/S 0
LMW-PH/S-Us
120
LMW-PH 120
LMW-PH/S 120
LMW-PH 0

Fig. 4


a
Reducing power (µM Trolox eq/mg sample)
0 min
120 min
LMW-NH
LMW-NH/S
LMW-NH/S-Us
Samples
b
Reducing power (µM Trolox eq/mg sample)
LMW-EH
LMW-EH/S
LMW-EH/S-Us
Samples
c
Reducing power (µM Trolox eq/mg sample)
LMW-PH
LMW-PH/S
LMW-PH/S-Us
Samples
d
Antoxidant activity (µM Trolox eq/mg sample)
LMW-NH
LMW-NH/S
LMW-NH/S-Us
Samples
e
Antoxidant activity (µM Trolox eq/mg sample)
LMW-EH
LMW-EH/S
LMW-EH/S-Us
Samples
f
Antoxidant activity (µM Trolox eq/mg sample)
LMW-PH
LMW-PH/S
LMW-PH/S-Us
Samples