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## SPR Screening of Metal chelating Peptides in a Hydrolysate for their Antioxidant Properties

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# 1                    **SPR Screening of Metal chelating Peptides in a** 2                    **Hydrolysate for their Antioxidant Properties**

3                    Abbreviated running title: Screening of antioxidant metal chelating peptides using SPR

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# SPR Screening of Metal chelating Peptides in a Hydrolysate for their Antioxidant Properties

## Highlights.

- Metal chelating peptides were screened in protein hydrolysates using SPR.
- An apparent affinity constant and a maximal resonance can be determined with SPR.
- $K_A$  should represent the proportion of metal binding peptides in the hydrolysate.
- A correlation was established between SPR-determined  $K_A$  and metal chelation tests.

## ABSTRACT

There is a growing need in the industrial sector (health, nutrition and cosmetic) to discover new biomolecules with various physico-chemical and bioactive properties. Various beneficial effects of peptides - notably those produced from protein hydrolysis - are reported in the literature. The antioxidant activity involves various mechanisms, among them metal chelation, studied by UV-visible spectrophotometry. In this paper, we set up an original method of screening metal chelating peptides in a hydrolysate using Surface Plasmon Resonance (SPR) for their antioxidant properties. To date, the empirical approach used several cycles of hydrolysate fractionation and bioactivity evaluation until the isolation of the pure bioactive molecule and its identification. Besides, the detection of metal-chelating peptide is not sensitive enough by spectrophotometry. For the first time, metal chelating peptides were screened in hydrolysates using SPR and a correlation was established between affinity constant determined in SPR and metal chelation capacity determined from UV-visible spectrophotometry.

## 1. INTRODUCTION

Food protein-derived bioactive peptides (BPs) have been reported to trigger certain physiological responses in the body, thereby influencing health positively. Those peptides have attracted high

50 research and consumer interest due to their huge potential in functional foods and other dietary  
51 intake to promote health. However, successful product development is limited by the fact that  
52 current manufacturing processes are either difficult to scale up, expensive, or may affect the  
53 structure-activity relations and thus the properties of those peptides.

54 The industrial sector (especially in health, nutrition and cosmetics) needs to discover new  
55 biomolecules presenting various physico-chemical or bioactive properties. Various beneficial  
56 effects of peptides are reported in the literature such as anti-hypertensive (Pihlanto-Leppälä,  
57 2001; Marzak et al, 2003), anti-thrombosis (Ariyoshi, 1993), anti-carcinogeneous (Messina &  
58 Messina, 1991), opioid (Zioudrou, Streaty & Klee, 1979), anti-microbial (Broeckaert, Cammue,  
59 De Bolle, Thevissen, De Samblanx, & Osborn, 1997), or growth factor for cell culture (Franek,  
60 Hohenwarter & Katinger, 2000; Deparis et al, 2003; Farges-Haddani et al, 2006; Chabanon et al,  
61 2008).

62 Peptides can be produced from enzymatic hydrolysis of proteins extracted from vegetable or  
63 animal co-products. Antioxidant peptides were already isolated from protein hydrolysate of  
64 various origins, notably from vegetable resources such as rapeseed proteins (Pan, Jiang & Pan,  
65 2011; Zhang, Wang, Xu & Gao, 2009), chickpea (Zhang, Li, Miao & Jiang, 2011), sweet potatoes  
66 (Zhang, Mu & Sun, 2012), or lucerne (Xie, Huang, Xu, & Jin, 2008).

67 Antioxidant capacity is a generic term, which recovers various chemical mechanisms. On total,  
68 about twenty antioxidant tests are reported in the literature (Huang, Ou & Prior., 2005) and  
69 various complementary tests must be carried out to obtain a representative result of the whole  
70 antioxidant capacity of a given molecule.

71 Nevertheless, three main activities describe antioxidant power. The first one is the scavenging of  
72 various free radicals and involves either an electron transfer (ET) mechanism or a hydrogen atom  
73 transfer (HAT) mechanism. Radical scavenging activity is mainly followed by ABTS or DPPH  
74 tests, with  $ABTS^{\circ+}$  or  $DPPH^{\circ+}$  radicals, respectively. The second mechanism relies on the  
75 inhibition of lipid peroxidation (Farvin, Baron, Nielsen & Jacobsen, 2010), which can be followed

76 by oxygen consumption or MalonDiAldehyde test. Finally, metal chelation such as iron or copper  
77 ions complexation (Decker & Welch, 1990; Xie et al, 2008) is often studied as an indirect  
78 antioxidant mechanism since upon metal complexation, radical reactions in chain are inhibited  
79 and oxidation phenomena are delayed. Indeed, in living systems, the presence of transient metal  
80 ion, such as  $Fe^{2+}$  and  $Cu^{2+}$  involve the formation of Reactive Oxygen Species (ROS) as for  
81 instance, hydroxyl radicals ( $OH^\bullet$ ) formed by the Fenton reaction. In addition, ferrous ions ( $Fe^{2+}$ )  
82 can catalyse the formation of hydroxyl radicals *via* the Haber-Weiss reaction, which occurs in the  
83 presence of superoxide anions. These free radicals, produced locally, can react with adjacent  
84 biomolecules and are responsible of serious damages in the biological tissue. Due to some  
85 environmental conditions (*i.e.* pollution, UV, non-equilibrated food), defence mechanisms  
86 involving antioxidants are sometimes overcome; an oxidant stress is generated, which can lead to  
87 the development of several pathologies in humans such as diabetes, rheumatoid arthritis, and  
88 cancer (Abuja & Albertini, 2001; Collins, 2005; Halliwell, 2000; Halliwell & Whiteman, 2004;  
89 Hoelzl, Bichler, Ferik, Simic, Nersesyan, & Elbling, 2005).

90 Yet, the low concentration of target bioactive peptides in an hydrolysate (a complex mixture of  
91 peptides sequences) and the difficulty to associate their bioactivity to one or several specific  
92 physicochemical properties constitute one of the main factors limiting their separation and their  
93 use. To valorise these bioactive peptides, one of the principal stakes is then the separation of  
94 peptides in a complex mixture, according to the desired bioactivity. The empirical approach  
95 consists in separating each hydrolysate in various fractions and in evaluating their bioactivity.  
96 These sequential steps are reiterated until reaching pure bioactive molecules identified by mass  
97 spectrometry. To date, the separation methods settled up (*i.e.* chromatography, ultrafiltration) are  
98 time-consuming. Besides, depending on the size of the peptide of interest, and its low  
99 concentration in the hydrolysate - whether present -, a detection using UV-visible  
100 spectrophotometry is often not sensitive enough.

101 Surface Plasmon Resonance (SPR) enables the monitoring of molecular interactions in real time.  
102 Its main advantage is the label free detection based on the phenomenon of surface plasmon  
103 resonance. While the interacting molecule (ligand) is immobilized on the surface of a sensor chip,  
104 the analyte is injected in solution and flow over the surface. The binding of the analyte to the  
105 immobilized ligand involves alteration of the refractive index at the sensor surface, which is  
106 proportional to the change in mass concentration. From sensorgram (SPR response in resonance  
107 unit plotted against time) studied at various analyte concentrations, the affinity constant of the  
108 analyte for the ligand can be determined.

109 Several studies reported the use of SPR for the affinity comparison of different tagged proteins to  
110 metal ions (Nieba et al, 1997; Bernaudat & Bülow, 2005; Kurzatkowska et al, 2014). Yet, Knecht  
111 et al, (2009) were the only ones to study the affinity of His-tag peptides to Ni<sup>2+</sup> ions.

112 Hence, a direct screening of metal chelating peptides using SPR would enable to detect more  
113 rapidly their presence in a hydrolysate before launching a separation phase, that should be  
114 engaged solely in the case of positive bioactivity screening. To the author's knowledge, SPR has  
115 never been used as a screening method of metal chelating peptides related to their antioxidant  
116 capacities.

117 The aim of this study is to propose a new proof-of-methodology on a screening procedure of  
118 metal chelating peptides in hydrolysates for their antioxidant properties. Such approach uses SPR  
119 with a M<sup>2+</sup>-NTA sensor chip. The main advantage of such technique is based on the higher  
120 sensitivity and label free detection of SPR detector as compared to classical UV-Vis spectrometry.

121 Thanks to this methodology, the most interesting sources of hydrolysates in terms of metal  
122 chelating peptides could be screened rapidly. Moreover, another aim of this study is to correlate  
123 affinity constant determined by SPR methodology and antioxidant chemical tests based on metal  
124 chelation using UV-visible spectrophotometry.

125 To overcome potential interferences of non-peptide contaminants with SPR, the SPR  
126 methodology was first set up on one hydrolysate, taken as a model, with or without purification.

127 Once the methodology validated, screening was carried out on raw samples for further industrial  
128 applications. Then, correlation was established between affinity constants and metal chelation  
129 capacities determined on raw hydrolysates.

130

## 131 **2. MATERIALS AND METHODS**

### 132 **2.1. Hydrolysates**

#### 133 **2.1.1. Industrial specification**

134 Peptide hydrolysates were provided from Kerry group (Ingredients and Flavours Department,  
135 USA). They were obtained from various vegetable sources such as soy (*i.e.* H1510, H1512, and  
136 UP Soy), wheat (H4601N) and rice proteins (H5603). The total and free amino acids composition  
137 is given in the industrial specification (**Supplementary data, Table SD 1**). Besides, for each  
138 hydrolysate, the global amino acid composition was presented by class of amino acids, with its  
139 main characteristics in terms of total nitrogen (TN, %), protein and degree of hydrolysis  
140 (**Supplementary data, Table SD 2**).

#### 141 **2.1.2. OPA quantification**

142 As complex mixture of peptides, hydrolysates cannot be determined in molar concentration. To  
143 compare them, peptide concentration was quantified using *ortho*-phthaldialdehyde test (OPA) and  
144 expressed in mM glycine equivalent. This spectrophotometric dosage involves a reaction between  
145 the N-terminal extremity of each peptide present in the mixture and OPA reagent (OPA;  
146 ThermoFisher Scientific, Loughborough, UK) in the presence of *N,N*-dimethyl-2-mercaptoethyl-  
147 ammonium (ThermoFisher Scientific). The chemical group obtained absorbs at a wavelength of  
148 340 nm.

149 The OPA quantification was performed according to Frister, Meisel, & Schlimme (1988). The  
150 OPA solution was prepared by dissolving 40 mg of OPA in 1 mL of pure methanol and 100 mg  
151 of *N,N*-dimethyl-2-mercaptoethyl ammonium in a few milliliters of Borax buffer (sodium  
152 tetraborate 100 mM, 1% w/v sodium dodecyl sulfate, pH 9.3, Sigma-Aldrich). These two  
153 solutions were mixed in a 50 mL-volumetric flask, qsf Borax buffer. The molar concentration of  
154 1 mg.mL<sup>-1</sup> peptide hydrolysate solutions (20 µL) qsf Borax buffer was measured in 96-well plate  
155 by spectrophotometry at 340 nm after 3 min incubation with stirring at room temperature  
156 (ThermoFisher Scientific) with the OPA solution (200 µL). For each hydrolysate, molar  
157 concentration (expressed in equivalent α-NH<sub>2</sub>) was determined using a calibration curve  
158 performed with glycine.

159

### 160 **2.1.3. Determination of total phenolics**

161 The total content of phenolic compounds was quantified by the method of Folin-Ciocalteu  
162 according to the colorimetric method described by Singleton & Rossi (1965) and adapted to a 96-  
163 well microplate by Mussato, Ballesteros, Martins & Teixeira (2011). Briefly, 5 µL of an  
164 hydrolysate solution (25 g.L<sup>-1</sup>) were mixed with 60 µL of sodium carbonate solution at 7.5%  
165 (w/v) and 15 µL of Folin-Ciocalteu reagent (1N; Sigma Aldrich). Then, 200 µL of ultrapure  
166 water (18 mΩ.cm<sup>-1</sup>) were added and the solutions were mixed. Samples were incubated at 60°C  
167 for 5 min and cooled down at room temperature. Absorbance was measured at 700 nm using a  
168 microplate reader. Calibration of this test was carried out with gallic acid standard solutions (0,  
169 200, 400, 600, 1000 et 1500 mg.L<sup>-1</sup>) and the blank was performed with ultrapure water. The total  
170 content of phenolic compounds was expressed in mass percentage of hydrolysate powder (w/w).

171

172



#### 173 2.1.4. Purification of hydrolysates

174 Samples were purified using Solid Phase Extraction column (CC6 polyamide column,  
175 Chromabonb PA 6 mL/1000g, Macherey-Nagel, Duren, Germany) to remove polyphenols.  
176 Purification step was adapted from Collins, Ng, Fong, Wan, & Yeung, (1998). Briefly, the column  
177 was conditioned with  $2 \times 5$  mL of ultrapure water, removed upon centrifugation (1000 g, 1 min).  
178 Then, 5 mL of hydrolysate solution ( $25 \text{ g.L}^{-1}$ ) was deposited onto the column and percolated at  
179 atmospheric pressure. The peptide fraction was eluted with 5 mL water and the column was then  
180 successively eluted with 5 mL MeOH 50%, 5 mL of pure MeOH and several washes of 0.1 M  
181 NaOH. The peptide fraction was then lyophilized and frozen until analysis.

182

#### 183 2.2. Metal chelation tests

184 The antioxidant capacity determined by  $\text{Cu}^{2+}$  chelation was measured by spectrophotometry  
185 using murexide as colored indicator. A mini-review on metal chelation tests used in the literature  
186 and their experimental conditions is presented in supplementary data (**Table SD3**). This test was  
187 adapted from literature (Wu, Shiau, Chen, & Chiou, 2003; Wong, Leong & Koh, 2006 and Li,  
188 Wang, Chen & Chen, 2011). Absorbance was measured at two wavelengths, 485 nm and 520 nm,  
189 for the copper-murexide complex and the murexide alone, respectively. The ratio of absorbance  
190 ( $A_{485}/A_{520}$ ) was considered proportional to the free copper ion ( $\text{Cu}^{2+}$ ) concentration (Barges,  
191 Cravotto, Gianolio & Fedeli, 2006).

192 This test was adapted for the microplate study to reduce the time of experiments and the volume  
193 of studied solution. EDTA (EDTA disodium salt dihydrate, Fluka Chemie, Buchs, Switzerland)  
194 and carnosine (99%, Sigma Aldrich), both studied as positive controls, were prepared in  
195 hexamine buffer (10 mM Hexamine, 10 mM KCl, pH 5) first in a range of concentration 0-40  
196 mM to determine saturation, then in the range 0-4 mM for linearization. Peptide hydrolysates  
197 were prepared similarly in a concentration range varying between 0-40  $\text{g.L}^{-1}$ , concentration then

198 expressed in mM equivalent glycine. Experiment was repeated 5 times. Each sample (EDTA,  
 199 carnosine and hydrolysate solutions) was directly diluted in microplate with hexamine buffer for a  
 200 total volume of 143  $\mu\text{L}$ . Then, 143  $\mu\text{L}$  of a 3 mM  $\text{CuSO}_4$  solution prepared in hexamine buffer  
 201 and 14  $\mu\text{L}$  of 1 mM murexide solution were added in each well (total volume: 300  $\mu\text{L}$ ). The 96-  
 202 well plate was incubated for 3 min at ambient temperature and absorbance measured at 485 nm  
 203 and 520 nm. For each sample, the  $\text{Cu}^{2+}$  chelation capacity was determined according to equation  
 204 (3):

$$205 \text{ Cu}^{2+} \text{ complexation (\%)} = \frac{[(A_{485}/A_{520})_0 - (A_{485}/A_{520})_s]}{(A_{485}/A_{520})_0} \times 100 \quad (3)$$

206  
 207 With  $(A_{485}/A_{520})_0$  = ratio of absorbances measured in the absence of sample (negative control:  
 208 hexamine buffer),

209 And  $(A_{485}/A_{520})_s$  = ratio of absorbances measured in the presence of sample (EDTA, carnosine or  
 210 hydrolysate).

211 For each sample, a linearization was performed in the linear part of the curve in order to  
 212 determine, after calculation, the indices characterizing its capacity for  $\text{Cu}^{2+}$  chelation: the EECC  
 213 (EDTA Equivalent copper Chelation Capacity) or the CECC (Carnosine Equivalent copper  
 214 Chelation Capacity). Such indices are calculated according to equations (4) and (5):

$$215 \quad EECC = \frac{a_s}{a_{EDTA}} \quad (4)$$

$$216 \quad CECC = \frac{a_s}{a_{Car}} \quad (5)$$

217 With  $a_s$  = the slope of the linearization line, for hydrolysate (% of copper chelated as a function of  
 218 peptide concentration).

219  $a_{EDTA}$  = the slope of the linearization line for EDTA (% of copper chelated as a function of  
 220 EDTA concentration).

221  $a_{car}$  = the slope of the linearization line for carnosine (% of copper chelated as a function of  
222 carnosine concentration).

223

### 224 **2.3. Affinity constant determination**

225 Binding of peptides present in hydrolysate to  $Ni^{2+}$ -NTA was analysed by Surface Plasmon  
226 Resonance (SPR). The SPR measurement was performed on a Biacore X100 instrument (Biacore  
227 AB, Uppsala, Sweden) equipped with NTA (nitrilotriacetic acid) sensor chips at 25°C. The  
228 method was adapted according to Knecht et al (2009) with slight modification on tri-histidine  
229 peptide (HHH) before the study of peptide hydrolysate.

230 The eluent buffer (PBS 1X, pH 7.4) and the dispenser buffer (PBS 1X, pH 7.4, 0.005% Tween  
231 20) were filtered (0.22  $\mu$ m, support membrane, low protein binding, non pyrogenic, PALL) and  
232 degassed by the Biacore instrument. The PBS 1X running buffer was prepared from a 10X  
233 concentrate made of 67 mM  $Na_2HPO_4 \cdot 2H_2O$ , 12.5 mM  $KH_2PO_4$ , 150 mM NaCl with a pH value  
234 of 6.65. When the 10X stock solution was diluted to 1X, the final pH of PBS 1X was of 7.4.

235 Peptide hydrolysates were dissolved at various concentrations in freshly prepared PBS 1X buffer  
236 before each experiment. Loading of  $Ni^{2+}$  onto the NTA chip was performed with a  $NiCl_2$   
237 solution (500  $\mu$ M, Biacore kit, Uppsala, Sweden) and the chip surface was regenerated with  
238 imidazole (500 mM) dissolved in ultrapure water (18.2  $m\Omega \cdot cm^{-1}$ ), followed by a regeneration  
239 solution (10 mL buffer added with 87  $\mu$ L EDTA at 50  $\mu$ M).

240 All binding experiments were performed at a flow rate of 20  $\mu$ L $\cdot$ min $^{-1}$ , starting with a 1-min  
241 injection of aqueous  $NiCl_2$  solution to load the NTA chip, with a 1-min stabilisation period. A  
242 NTA flow cell without  $Ni^{2+}$  was used as a reference cell. Then each studied peptide hydrolysate  
243 was injected on both flow path for 270 s followed by 270 s of undisturbed dissociation time. The  
244 regeneration procedure consisted of 1-min injections of imidazole (500 mM) at 20  $\mu$ L $\cdot$ min $^{-1}$

245 followed by an extra-wash with buffer-EDTA (50  $\mu\text{M}$ ). Upon the second regeneration  
246 procedure, the surface was washed with SDS 0.5% v/v for 1 min at a flow rate of 40  $\mu\text{L}\cdot\text{min}^{-1}$   
247 followed by an extra-wash after injection with running buffer. Each regeneration and washing  
248 cycle was repeated twice. One buffer blank before and between each sample series were used for  
249 double referencing during data processing (Myszka, 1999; mentioned in: Knecht et al, 2009).

250 The sensorgrams obtained from the SPR experiments were processed with BIAevaluate software.  
251 The isotherms obtained were expressed in Resonance Unit (RU, corrected by the offset value) as  
252 a function of the concentration of peptide hydrolysate (expression in mM equivalent glycine  
253 according to the OPA quantification). The dissociation constants ( $K_D$ , M equivalent glycine) were  
254 determined at equilibrium by fitting the experimental data with the 1:1 binding model. The  
255 affinity constant ( $K_A$ ,  $\text{M}^{-1}$  equivalent glycine) was calculated as the inverse of the dissociation  
256 constant.

257

## 258 **3. RESULTS AND DISCUSSION**

### 259 **3.1. Hydrolysate characterization**

#### 260 **3.1.1. Raw hydrolysates**

261 Hydrolysates are composed of a mixture of peptides, whose sequences are unknown. For their  
262 comparison, peptide concentration of hydrolysates solutions (1  $\text{mg}\cdot\text{mL}^{-1}$ ) were quantified using  
263 OPA test in order to express their molar concentration in mM equivalent glycine. For raw  
264 hydrolysates, peptide concentration varied between 1.23 and 1.82 mM equivalent glycine (**Table**  
265 **1**).

266 According to their specification, industrial hydrolysates have been submitted to ultrafiltration, the  
267 method commonly used to remove polyphenols (Bornman, Gokemen and Nyhuis, 2001;

268 Gokmen, Acar & Karahman, 2003). Thus, raw hydrolysates were supposed to be free of  
269 polyphenols. Surprisingly, total polyphenols were quantified between 3.6% (w/w) for H1510 and  
270 H1512 hydrolysates up to 5.9% (w/w) for H5603 (**Table 1**). The fact that similar value was  
271 observed for a trypsin casein hydrolysate used as a negative control ( $5.3 \pm 0.2$  % w/w) suggests  
272 the existence of interferences in quantification method due for example to tyrosine residues in  
273 relation with their phenol group, but the presence of residual polyphenols in hydrolysates can not  
274 be rule out.

### 275 **3.1.2. Purified hydrolysates**

276 The purification method adapted from Collins et al (1998) was carried out on polyamide resins to  
277 eliminate non-peptides contaminants such as polyphenols. After a purification step, peptide  
278 concentration of hydrolysate samples was not significantly modified on the whole as proved by  
279 OPA quantifications (**Table 1**). In the meantime, there was practically no loss of total  
280 polyphenols. Nevertheless, as observed in Collins et al (1998), a significant amount of binding  
281 was seen as a brown coloration of resin, which eluted with sodium hydroxide (result not shown).  
282 Besides, the comparison of the OPA quantification test carried out on raw hydrolysate powder  
283 ( $1\text{g.L}^{-1}$ ) and on a powder of hydrolysate purified onto CC6 polyamide column, lyophilised and  
284 then reconstituted ( $1\text{g.L}^{-1}$ ) showed a slight increase in peptide concentration (**see**  
285 **Supplementary data. Figure SD1**).

286

## 287 **3.2. Determination of metal chelating capacity/affinity by SPR**

### 288 **3.2.1. Set up of SPR method on purified H5603 hydrolysate**

289 Validation of the SPR method has been realised on the H5603 hydrolysate as a model, and in  
290 order to overcome potential interferences, in a first approach, hydrolysate purified on CC6  
291 polyamide column was used. The initial methodology set up on HHH peptide by Knecht *et al.*

292 (2009) was adapted for the study of hydrolysate. Binding to Ni<sup>2+</sup>-NTA was analyzed both  
293 qualitatively and quantitatively with surface plasmon resonance (SPR) using commercially  
294 available NTA sensor chips from Biacore. Purified H5603 hydrolysate was injected at a  
295 concentration varying from 0 to 40 g.L<sup>-1</sup> for 4.5 min at a flow rate of 20 μL.min<sup>-1</sup> to detect the  
296 association phase, followed by a dissociation time of 4.5 min at a flow rate of 20 μL.min<sup>-1</sup>. This  
297 led to a clear detection of binding signals for all tested concentrations of hydrolysate with very  
298 fast association, reaching equilibrium immediately after injection, and dissociation rates, and with  
299 an excellent reproducibility of the triplicate injections (not shown) (**Figure 1**). Association and  
300 dissociation phases were shown to occur in a dose-dependent manner, suggesting the existence  
301 of an apparent affinity of the hydrolysate (peptides) for Ni<sup>2+</sup>. The hyperbolic profile of the level  
302 of binding at equilibrium as a function of the sample concentration showed a saturating  
303 behaviour, suggesting the formation of peptide–Ni<sup>2+</sup> complexes.

304 The experimental isotherm, *i.e.* the level of binding expressed in Resonance Unit (RU) in function  
305 of hydrolysate concentration expressed in mM equivalent glycine according to the NH<sub>2</sub> terminal  
306 concentration quantified using OPA test, was fit to the 1:1 binding model using BIAevaluation  
307 (version 2) software (**Figure 1**). It enabled to determine the value of the apparent dissociation  
308 constant ( $K_D$ ), the maximal signal ( $R_{max}$ ) at saturation of the Ni<sup>2+</sup>-NTA chip and the offset. The  
309 offset value (expressed in RU), which represents the difference in response between the reference  
310 (no Ni<sup>2+</sup>) and the sample canal (with Ni<sup>2+</sup>) of the chip, was used to calculate the true maximal  
311 response ( $R_{maxc}$ ). From the value of the  $K_D$ , the value of the apparent affinity constant ( $K_A$ ) was  
312 calculated (**Table 2**). The lowest the  $K_D$  value, the highest the affinity of metal chelating peptides  
313 for Ni<sup>2+</sup> ions. Due to heterogeneity of the hydrolysate, in terms of peptide sequences and  
314 concentration of metal chelating peptides, it may be assumed that the value of the  $K_A$  should  
315 represent the proportion of metal binding peptides within the hydrolysate, whereas the value of  
316 the  $R_{maxc}$  would be more representative of the size of the formed complexes. Thus, the results

317 show that metal binding capacity of a hydrolysate can be quantified on purified peptide  
318 hydrolysate using Surface Plasmon Resonance.

319 In an industrial perspective, for screening various hydrolysates for their metal chelating peptides,  
320 metal-chelating properties should directly be explored on raw hydrolysates. Indeed, SPR  
321 experiments were performed similarly than previously on raw H5603 hydrolysate. The fact that  
322 similar values of  $K_A$  and  $R_{max}$  were obtained for the purified and non-purified H5603 hydrolysate  
323 (**Figure 2, Table 2**) shows that metal binding capacity can be determined directly on raw  
324 hydrolysate using Surface Plasmon Resonance.

325

### 326 3.2.2. Affinity constant determination on raw hydrolysates

327 In order to set up a direct screening method of metal chelating peptide in complex mixture, raw  
328 hydrolysates constitute the most appropriate materials. Hence, SPR experiments were performed  
329 similarly than previously on H1510, H1512, H4601N and UP Soy raw hydrolysates.

330 For all hydrolysates, association and dissociation phases were shown to occur in a concentration  
331 dependent manner (Results not shown). Plots of response at equilibrium in function of  
332 hydrolysate concentration present a hyperbolic profile, whatever the hydrolysate (**Figure 2**).  
333 From the fitting of these experimental binding isotherms, values of the different parameters were  
334 obtained (**Table 2**). Considering the  $K_A$  values, the apparent affinity of raw peptide hydrolysate  
335 for  $Ni^{2+}$  ions varied according to the samples studied and evolved in the following rank: H1512 <  
336 H5603 < H4601N < UP Soy < H1510. The highest affinities were measured for the H1510  
337 hydrolysate and then for UP Soy in comparison to the other three hydrolysates.

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### 342 3.3. Metal chelation

343 Metal chelation tests are usually performed on hydrolysate using spectrophotometric tests. A  
344 mini-review on metal chelation tests used in the literature and their experimental conditions is  
345 presented in supplementary data (**Table SD3**). Nevertheless, according to the size of the peptides  
346 of interest, and their low concentration in the hydrolysate - whether present -, a detection using  
347 UV-visible spectrophotometry may be sometimes not sensitive enough for such application.

348 The test of metal chelation capacity ( $\text{Cu}^{2+}$ ) was validated on EDTA and carnosine using UV-  
349 visible spectrophotometry (**Figure 3, panels A and B**) and then, tested on raw hydrolysates  
350 (**Figure 3, panels C and D**). The  $\text{Cu}^{2+}$  chelation capacity (%) was plotted as a function of molar  
351 ratio (mM carnosine or EDTA /mM  $\text{CuSO}_4$ ) (**Figure 3, panels A and B**). Saturation was  
352 observed at 87% of  $\text{Cu}^{2+}$  chelated for EDTA and 82% for carnosine. Since EDTA is known as a  
353 really good complexing agent, the totality of  $\text{Cu}^{2+}$  is considered fully chelated for a calculation  
354 corresponding to 87% chelation capacity. For a 1:1 stoichiometry between EDTA and  $\text{Cu}^{2+}$ , the  
355 copper chelated was of 62%. For a 1:1 stoichiometry between carnosine and  $\text{Cu}^{2+}$ , the copper  
356 chelated was about 30%. In comparison of the study of Wu and co-workers (2003), these authors  
357 determined the chelating ability of a 4 mM-EDTA solution at 68% and the chelating effect of a 5  
358 mM-carnosine solution at 32%.

359 The  $\text{M}^{2+}$  chelation capacities (%) were plotted as a function of the molar ratio of the hydrolysate  
360 over the metal concentration (mM equivalent glycine / mM  $\text{CuSO}_4$ ; **Figure 3, panels C and D**).  
361 In **Figure 3** (panels C), a Langmuir-shaped isotherm was observed for all the hydrolysates  
362 studied with saturation at about 80% copper chelation, which fit to the totality of the copper  
363 complexed as for positive controls (EDTA and carnosine). Nevertheless, as a complex mixture of  
364 peptides, all the peptides present in the hydrolysate are not able to complex the  $\text{Cu}^{2+}$  ions. In  
365 such conditions and despite the Langmuir-shaped isotherm, we cannot be sure that the binding  
366 of metal chelating peptides with metal ions can be approximated with a 1:1 model. Then, to



367 determine, the most interesting source of hydrolysate in terms of metal chelating peptides, an  
368 indice expressed in equivalent chelation capacity (ECC) was calculated, considering either EDTA  
369 (EECC) or carnosine (CECC) as reference (part 2.2.), using the slope of the linear part of the  
370 curve (**Table 2**). Whatever the reference was, the rank remained the same: H1510 > UP Soy >  
371 H5603 > H1512 > H4601N. The variations between hydrolysates observed by UV  
372 spectrophotometry is less pronounced than those observed by SPR, when we compare  
373 respectively the results of metal chelation indices and the affinity constants, which seems to  
374 confirm once more the interest of SPR for the screening of metal chelating peptides in  
375 hydrolysates.

376 According to the literature, amino acids preferentially involved in metal chelation are known. A  
377 mini-review on amino acids and their functional groups involved in metal chelation is presented  
378 in supplementary data (**Table SD4**). Several amino acids are concerned especially (1) aspartic and  
379 glutamic acid, through their carboxylate function, (2) histidine *via* the imidazole ring, (3) arginine  
380 and lysine through the amino group, (4) cysteine with its thiol group (5) serine and threonine with  
381 hydroxyl. Nevertheless, over the five studied hydrolysates, relationship between the copper  
382 chelation capacity (%) and the given industrial amino acid composition cannot be stated (See  
383 **SD1**. Amino acid composition given in the industrial specification (**SD2**. Global amino acid  
384 composition and main characteristics of the hydrolysate).

385

### 386 **3.4. Correlations between affinity constants and metal chelation tests**

387 From the former data, the relation between the affinity constants ( $K_A$ ,  $M^{-1}$ ) and the copper  
388 chelation indices was plotted (**Figure 4, panels A and B**, for EECC and CECC, respectively). A  
389 satisfactory correlation was observed with a  $R^2$  value of 0.88. Hence, in addition to the  
390 determination of  $K_A$  for peptide hydrolysate, SPR methodology can be used for the screening  
391 method of metal chelating peptides in a protein hydrolysate with more accuracy than the classical

392 spectrophotometric method used to date. Nevertheless, no correlation was established between  
393 affinity constant and the amino acid content in each hydrolysate given in industrial specifications  
394 (see **supplementary data SD1 and SD2**). This could be due to the fact that the amino acid  
395 residue content is not the only factor involved upon the mechanism of complexation occurring  
396 between peptide and metal ion. Indeed, the position of one amino acid residue to another one is  
397 also important in the complexation mechanism. Hence as perspective of this work, SPR  
398 experiments will be coupled to Mass Spectrometry (SPR-MS), which could help us to establish  
399 correlations between affinity and amino acid composition in regards of the peptides sequence.  
400 Such study could be extended to model peptides of known sequence to better understand the  
401 link between affinity constant and peptide concentration.

402

#### 403 **4. CONCLUSION**

404 Surface Plasmon Resonance is a powerful tool to screen antioxidant metal chelating peptides in  
405 hydrolysate before launching time-consuming separation. Besides, SPR can directly be used on  
406 industrial hydrolysates formerly treated by ultrafiltration without an additional step of  
407 purification. A good correlation was obtained between  $K_A$  and metal chelation capacity, when  
408 both analyses were carried out on raw industrial hydrolysates. According to the results of this  
409 study, SPR is an innovative method that can be used for screening various sources of  
410 hydrolysates in terms of metal chelating peptides and could be considered for various industrial  
411 applications such as nutraceuticals or cosmetics.

412

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418

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515



516 **List of Tables and Figures**

517

518 **Table 1.** Characterization of hydrolysates: peptide concentration expressed in equivalent glycine,  
 519 total polyphenol quantification (in mass percentage, w GAE/w hydrolysate powder; GAE: Gallic  
 520 Acid Equivalent), and respective yields.

521

Hydrolysate	Peptide concentration (mM equivalent glycine)		Total polyphenol quantification in mass % (w/w)		Yields in fraction collected after purification	
	Raw hydrolysate	Purified hydrolysate	Raw hydrolysate	Purified hydrolysate	Peptide - OPA quantification	Polyphenol (Folin-ciocalteu quantification)
H1510	1.23	1.29	3.6 ±0.2	3.7 ±0.2	105	103
H1512	1.82	1.92	3.6 ±0.2	3.6 ±0.4	99	99
H4601N	1.38	1.74	4.6 ±0.3	4.1 ±0.2	107	87
H5603	1.50	1.74	5.9 ±0.2	5.5 ±0.4	101	93
UP Soy	1.46	1.52	4.9 ±0.3	4.2 ±0.0	101	86
Hcasein			5.3 ±0.2	4.1 ±0.3	104	82

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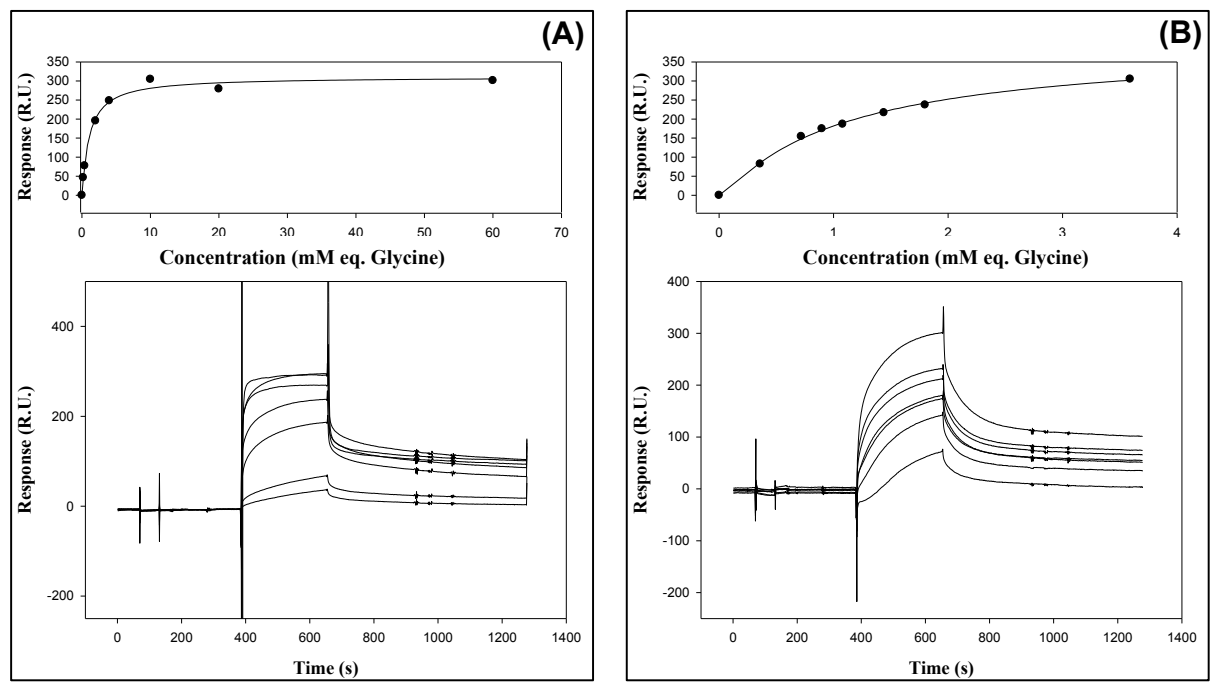
526 **Table 2.** Dissociation ( $K_D$ , mM), Affinity constants ( $K_A$ , mM<sup>-1</sup>),  $R_{maxc}$  determined by SPR and  
 527 metal chelation capacity determined by spectrophotometry. EDTA (EECC) and Carnosine  
 528 Equivalent Chelation Capacity (CECC) determined for Copper (II) on raw hydrolysate.

Nature of the hydrolysate	Sample	$K_D$ (mM eq. Glycine)	$K_A$ (mM <sup>-1</sup> eq. glycine)	$R_{maxc}$ (RU)	Slope of the tangent at the origin (Chelated Cu <sup>2+</sup> % / molar ratio)	EECC for copper (II)	CECC for copper (II)
<b>Purified</b>	H5603	0.90	1.11	319			
	H1510	0.10	9.92	60	91.9	2.23	3.21
	H1512	2.58	0.39	125	56.5	1.37	1.97
<b>Raw</b>	H4601N	0.78	1.29	64	51.5	1.25	1.80
	H5603	1.04	0.96	418	59.4	1.44	2.07
	UP Soy	0.14	6.95	129	69.8	1.70	2.44
EDTA					41.2	1	/
Carnosine					28.6	/	1

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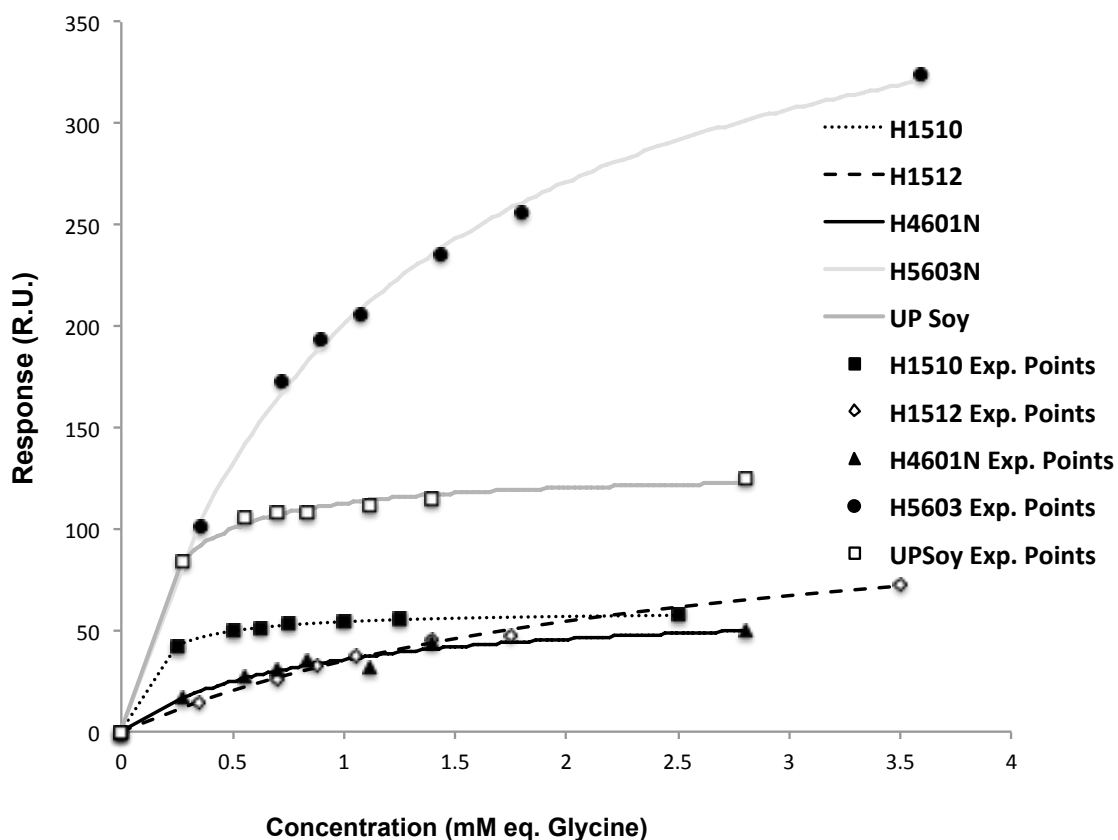
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531 **Figure 1.** Sensorgram (down graph) and corresponding sorption isotherm (top graph) of purified  
532 (A) and raw (B) H5603 hydrolysate.



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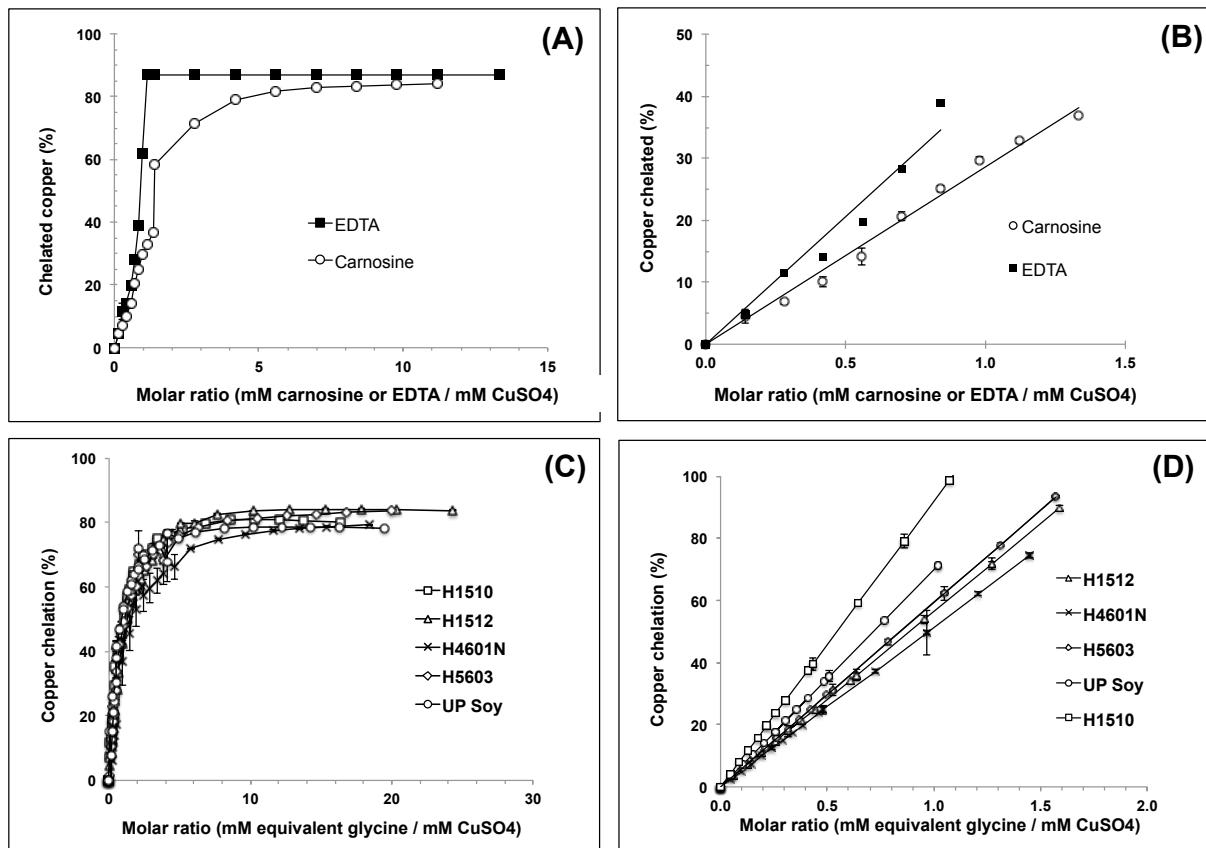
535 **Figure 2.** Sorption isotherms enabling the determination of the dissociation constant for various  
536 raw hydrolysates. Data corrected by the offset and hydrolysate concentration expressed in mM  
537 equivalent glycine. Experimental points and their respective fits.



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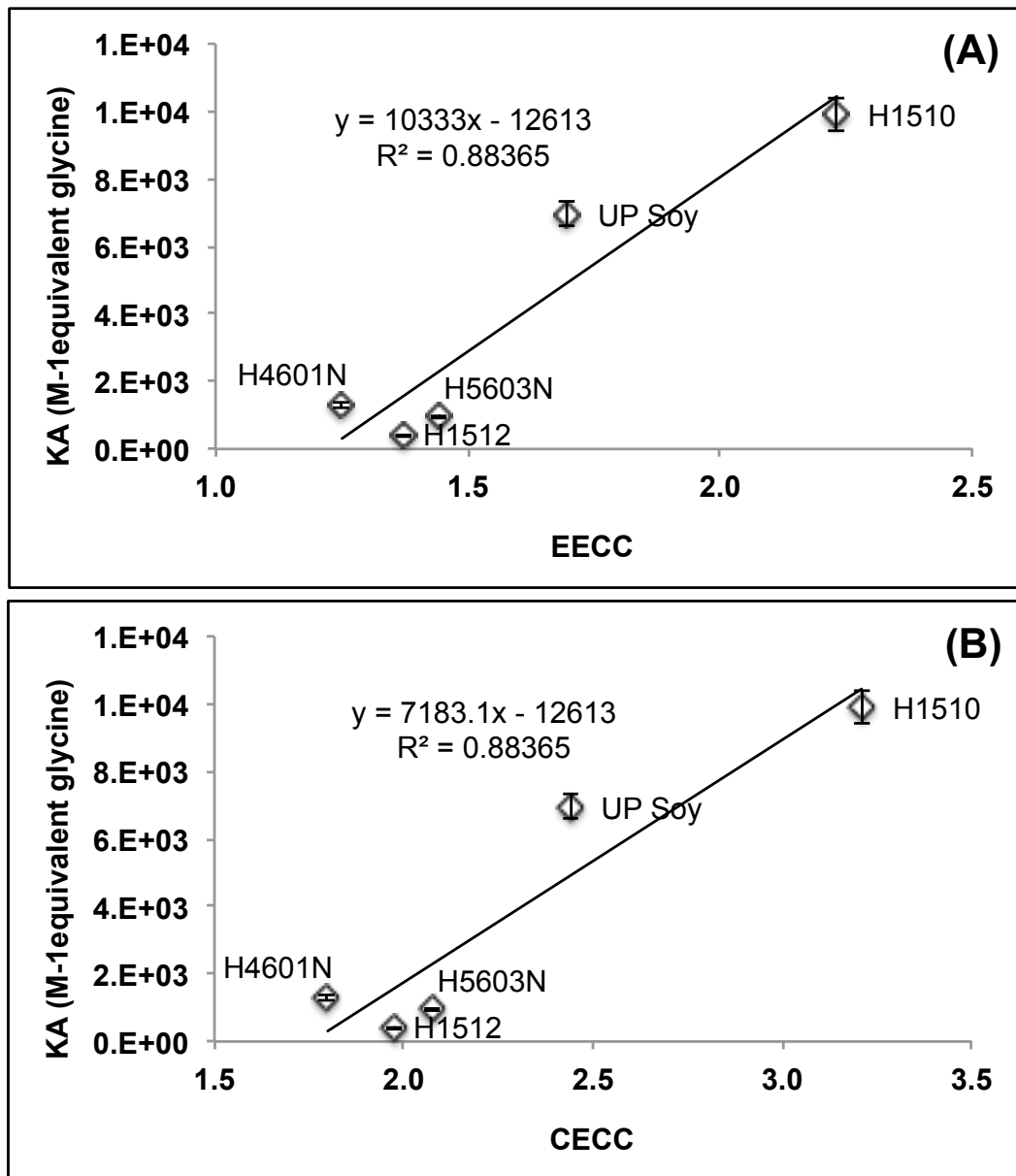
539

540 **Figure 3.** Results of metal chelation obtained by spectrophotometry. Panels A and B for EDTA  
 541 and carnosine. Panels C and D for hydrolysates.



542  
 543

544 **Figure 4.** Correlation between affinity constant ( $K_A$ ,  $M^{-1}$  equivalent glycine) and copper (II)  
545 chelating activity in raw hydrolysate. (A) Metal chelation expressed as EECC. (B). Metal chelation  
546 expressed as CECC.



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552 **Supplementary Data**

553 **Table SD1.** Amino acid composition given in the industrial specification.

Characteristics of samples	1510	1512	4610N	5603	UP Soy
Ala	26.0	25.0	21.0	48.0	19.0
Arg	41.0	37.0	27.0	72.0	30.0
Asn					0.0
Asp	73.0	66.0	24.0		51.0
Cys	4.0	3.0	11.0	8.0	0.0
Gln					
Glu	122.0	112.0	319.0		79.0
Gly	25.0	24.0	27.0	37.0	13.0
His	14.0	13.0	15.0	20.0	9.0
Ile	23.0	22.0	32.0	34.0	19.0
Leu	40.0	38.0	57.0	66.0	30.0
Lys	38.0	32.0	11.0	35.0	33.0
Met	7.0	7.0	12.0	15.0	2.0
Phe	26.0	24.0	46.0	43.0	20.0
Pro	28.0	27.0	115.0	38.0	39.0
Ser	31.0	29.0	38.0	44.0	21.0
Thr	24.0	22.0	21.0	31.0	17.0
Trp					
Tyr	21.0	20.0	29.0	43.0	17.0
Val	25.0	24.0	32.0	49.0	19.0
<b>Free AA (mg/g)</b>					
Ala	2.6	3.8	1.9	0.6	5.6
Arg	9.2	9.8	8.6	1.0	9.9
Asn		2.4	1.8	0.9	4.0
Asp	2.1	3.1	0.7	0.4	5.1
Cys				2.9	0.1
Gln	3.5	0.2	1.2	0.4	
Glu		5.2	0.4	2.9	6.9
Gly	2.5	3.6	1.0	0.1	1.1
His	1.4	1.3	3.2	0.1	2.6
Ile	0.5	1.6	4.8	0.2	4.8
Leu	6.3	11.0	10.7	1.9	13.9
Lys	4.6	6.4	2.3	0.6	10.8
Met	0.9	1.7	2.0	0.2	1.3
Phe	2.7	4.4	4.9	1.4	7.7
Pro		0.3	0.5	0.3	0.6
Ser	4.0	4.9	3.9	1.1	5.4
Thr	1.7	2.2	5.7	0.3	4.8
Trp	1.5	2.5	3.0		4.5
Tyr	1.1	1.6	1.3	0.1	1.7
Val	1.0	2.0	5.0	0.1	5.0

554

555

556 **Table SD2.** Global composition in amino acids (AA; mg/g) by class and main characteristics of  
 557 the studied hydrolysates. TN: Total Nitrogen (%). DH: ratio AN/TN x 100.

		1510	1512	4610N	5603	UP Soy
<b>Global amino-acid (AA) composition (mg/g)</b>						
Positively charged AA (RHK)	Total	93	82	53	127	72
	Free	15	18	14	2	23
Negatively charged AA (DE)	Total	195	178	343	0	130
	Free	2	8	1	3	12
Polar uncharged AA (STNQ)	Total	55	51	59	75	38
	Free	9	10	13	3	14
Sulfured AA (CM)	Total	11	10	23	23	2
	Free	1	2	2	3	1
Hydrophobic AA (AVILMFYW)	Total	168	160	229	298	126
	Free	17	29	34	5	45
Aromatic AA (FYW)	Total	47	44	75	86	37
	Free	5	9	9	2	14
<b>Other parameters</b>						
	Total Nitrogen (TN, %)	9.2	8.8	14.2	12.8	8.0
	DH (ratio AN/TN x100)	16-29	27.5	15.4	14-22	25-35
558	DH (ratio AN/TN x100), mean value	22.5	27.5	15.4	18.0	30.0

559



560 **Table SD3.** Mini-review on copper chelation tests carried out on peptides and on hydrolysate in  
 561 the literature and their experimental conditions.

	Wu et al, 2003	Saiga et al, 2003	Carasco-Castilla et al, 2012	Carasco-Castilla et al, 2012b	Guo et al, 2015	Torres-Fuentes et al, 2011	Zhang et al, 2011
<b>Sample nature</b>	Peptides	Hydrolysate	Hydrolysate	Hydrolysate and peptide fraction	Peptide hydrolysate	Chickpea protein hydrolysate	Hydrolysate
<b>Range of concentration, volume sample</b>	0.5-40 mM, 2 mL		100 µg	100 µg for hydrolysate or 50 µg for peptide	Sample, 100 µg/mL, 200 µL	n.d.	1 mL
<b>Method adapted from</b>	Shimada et al, 1992		Saiga et al, 2003	Saiga et al, 2003	Saiga et al, 2003	Saiga et al, 2003	Kong and Xiong, 2006
<b>Positive control</b>	n.d.	EDTA at 0.045%	n.d.	n.d.	CPPs***	n.d.	n.d.
<b>Negative control (blank)</b>	Deionized water, treated with reverse osmosis		n.d.	n.d.	Deionized ater	n.d.	n.d.
<b>Buffer</b>	Hexamine buffer (10 mM), KCl (10 mM)		Na acetate buffer, 50 mM, 290 µL	Na acetate buffer, 50 mM, 290 µL	Na acetate buffer, 50 mM,	Na acetate buffer, 50 mM,	Pyridine, 10%, 1 mL
<b>pH adjusted</b>	No		pH 6.0		pH 5.0	pH 6.0	
<b>Copper source and concentration</b>	CuSO <sub>4</sub> , 3 mM, 2 mL	CuSO <sub>4</sub> , 2 mM, 1 mL; mixed with pyridine (pH 7.0)	CuSO <sub>4</sub> ·5H <sub>2</sub> O, 1 µg	CuSO <sub>4</sub> ·5H <sub>2</sub> O, 10 µg	CuSO <sub>4</sub> , 1 mg/mL, 20 µL	CuSO <sub>4</sub> , 0.1 µg/µL, 100 µL	CuSO <sub>4</sub> , 2 mM, 1 mL
<b>Metal chelating indicator, concentration, volume</b>	TMM*, 1 mM, 0.2 mL	PV**, 20 µL	PV**, 4 mM, 6 µL	PV**, 4 mM, 6 µL	PV**, 2 mM, 10 µL	PV**, 4 mM, 25 µL	PV** 0.1%, 20 µL
<b>Incubation: time, temperature</b>	3 min, RT				5 min, 37°C		
<b>Wavelength (s)</b>	485 nm	632 nm	632 nm	632 nm	632 nm	632 nm	632 nm
<b>Cu<sup>2+</sup> chelating ability (%)</b>	$\frac{(A_0 - A_s)}{A_0} \times 100$		$\frac{(A_0 - A_s)}{A_0} \times 100$	$\frac{(A_0 - A_s)}{A_0} \times 100$		$\frac{(1 - (A_s/A_0)) \times 100}{(A_s/A_0)}$	$\frac{(1 - (A_s/A_0)) \times 100}{(A_s/A_0)}$
<b>Standard curve</b>	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.
<b>Scale</b>	Cuvette		Microplate reader	Microplate reader	Microplate reader	n.d.	Cuvette

\* TetraMethylMurexide

\*\*Pyrocatechol Violet

CPPs:

CaseinoPhosphoPeptides

n.d.: not determined

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564 **Table SD4.** Amino acids and their functional group involved in metal chelation.

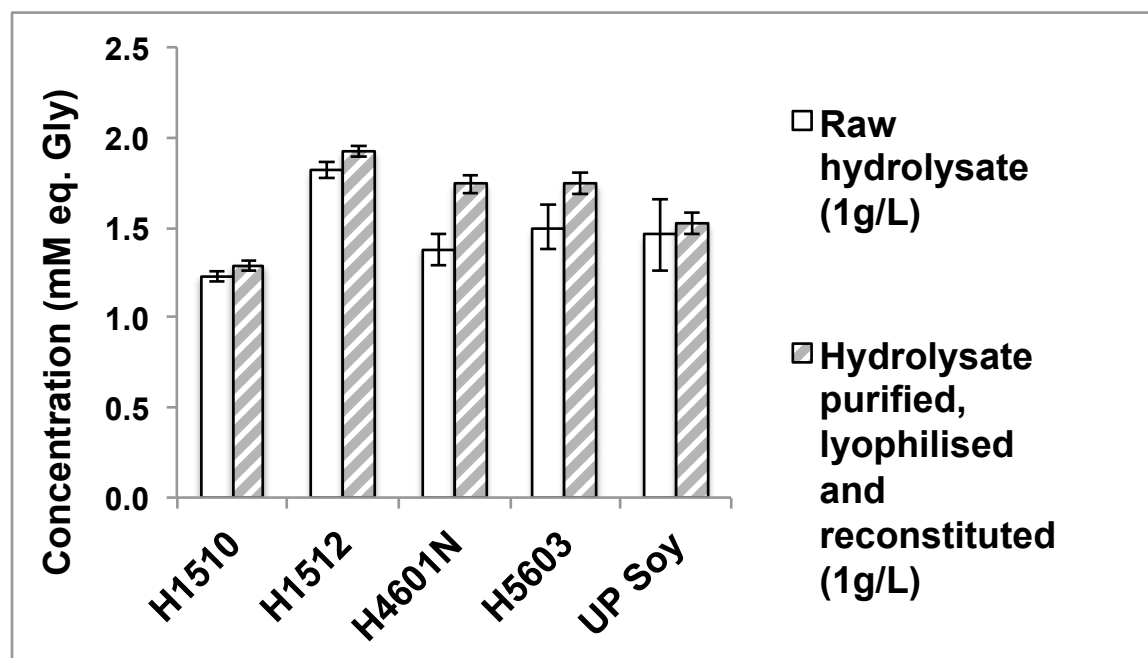
565

Aminoacid	Group involved	Atom involved	pK <sub>a</sub>	pK <sub>b</sub>	pK <sub>R</sub>	Reference
Aspartic acid	Carboxylate	Oxygen rich group	2.09	9.82	3.86	Zacchariou and Hearn, 1996
Glutamic acid			2.19	9.67	4.25	Lv et al, 2009
Histidine	Imidazole	Nitrogen rich group	1.82	9.17	6.00	Zacchariou and Hearn, 1996 Zoroddu et al, 2009 Lv et al, 2009 Wu et al, 2003
Arginine	Amino group	Nitrogen	2.17	9.04	12.48	Farvin et al, 2010
Lysine			2.18	8.95	10.53	Zhang et al, 2009
Cystein	Thiol	Sulfur	1.71	8.33	10.78	Lv et al, 2009
Serine	Hydroxyl	Oxygen	2.21	9.15		Lv et al, 2009
Threonine			2.63	10.43		Bamdad and Chen, 2013 Storckdieck et al, 2007 Swain et al, 2002 Taylor and Layrisse, 1986
Glycine	n.d.	n.d.	2.34	9.60		
Alanine			2.35	9.69		Wu et al, 2003

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568 **Figure SD1.** OPA quantification test carried out on raw hydrolysate powder (1 g/L) and on  
569 powder of hydrolysate purified onto CC6 polyamide column, lyophilised and reconstituted  
570 (1g/L).



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