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Influence of pH on colour and iron content of peptide fractions obtained from bovine Hb concentrate hydrolysates

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Summary The effect of pH on colour and iron content (Fe) of peptide fractions obtained from bovine haemoglobin concentrate (BHC) hydrolysates was studied. Four hydrolysates were obtained using three enzymes: Protex-6-L (P), Fungal–Protease–Concetrate (FC) and Flavourzyme (F). BHC and its hydrolysates (P, FC, P + F, FC + F) were fractioned at pH 4.5, 7.0 and 9.5. Solubility and Fe from different fractions were measured. Correlations between CIELAB colour parameters and Fe from different fractions were analysed. The colour from different fractions varied from red to yellow (a^* and b^* positives). Lightness values (L^*) ranged from twenty-four to seventy. FC4.5 and FC + F4.5 fractions were the clearest and yellow (higher L^*, b^*, h), while BHC9.5 and P + F9.5 fractions had the lowest values of L^*, b^* and h. There was an inverse linear relationship between b^* and L^* parameters and Fe from fractions. This relationship could be associated with the pH of extraction. As pH increases Fe significantly increases and lower b^* and L^* values were obtained.

Keywords Bovine haemoglobin concentrate, CIELAB colour parameters, hydrolysates, iron content.

Introduction

The use of protein-rich agricultural and industrial byproducts has been the subject of numerous investigations intended to obtain protein concentrates and isolates and to improve functional properties of these protein sources. Hydrolysis is one of the alternatives that allow protein modification of these products. It can be carried out either by chemical (acid or alkaline), physical, or enzymatic methods, the latter having remarkable advantages over the chemical ones (Cian *et al.*, 2010). Physicochemical properties of the hydrolysates depend on the protein substrate and the extent to which the protein is hydrolysed (Vioque *et al.*, 2001; Spellman *et al.*, 2002). Animal blood is a by-product of meat industry generated in large volumes. Red blood cell (RBC) fraction of bovine blood reaches 40% of the blood. Haemoglobin (Hb) constitutes 95% of RBC dry weight and has good nutritional and functional properties. The absorption rate of haeme iron is higher than that of non-heme form, which is mostly found in plant sources or in ferrous salts commonly used in food fortification (Toldrà *et al.*, 2008). Hb is mainly used in manufacture of pig, fish and pet foods, as well as sausages. Due to its good bioavailability it is also suggested as iron supplement (Duarte *et al.*, 1999). Several processes have been developed to produce heme iron from the hydrolysis of bovine Hb, in order to obtain a peptide product enriched with such prosthetic group. These heme enriched peptides have higher iron content than the initial heme protein, thus contributing to keep the heme group in a soluble state and favoring its bioavailability (Cian *et al.*, 2010).

Colouring agents are extensively used by the food and beverage industries to make their products more attractive to consumers. Although both artificial and natural colourants are approved for use in human food, consumers today are increasingly demanding the use of natural products because of reported adverse reactions to some synthetic food dyes and their impurities. Hb gives a red brownish colour when is used a food ingredient. Its red colour is unstable and very dependent on the oxygenation/oxidation state of the heme iron and pH (Salvador *et al.*, 2009).

The aim of this study was to assess the effect of pH on colour and iron content of peptide fractions

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obtained from bovine haemoglobin concentrate (BHC) hydrolysates.

Materials and methods

o-Phthaldialdehyde, dithiotreitol, sodium dodecyl sulfate, bovine serum albumin, L-serine and Flavourzyme (F) were obtained from Sigma Chemical Co. (St Louis, MO, USA). All reagents were analytical grade. The other enzymes Protex 6L (P) and Fungal–Protease–Concentrate (FC) were provided by Danisco S.A. (Arroyito, Córdoba, República Argentina). Commercial BHC was supplied by YERUVA S.A. (Esperanza, Argentina).

BHC chemical composition

BHC composition was determined using AOAC (1995) procedures. Total iron content was measured by atomic absorption spectroscopy after dry mineralisation. Ash was removed with 20% HCl (v/v). An atomic absorption spectrometer (IL 551 device; Instrumentation Laboratory, Norwood, MA, USA) was used.

Hydrolysate preparation

Hydrolysates were obtained using 800 mL batch thermostatised reactor. The reaction pH was continuously measured using an IQ Scientific Instruments pH-meter, and adjusted by adding base (NaOH) or acid (HCl) with a burette. The substrate concentration was 8% (w/w) in every case. Working conditions for the enzymes were: temperature 60 °C, pH 9.5, enzyme/substrate (E/S) ratio 0.1% (w/w) for P; temperature 55 °C, pH 4.3, E/S ratio 0.1% (w/w) for FC; and temperature 55 °C, pH 7.0, E/S ratio 1% (w/w) for F. Once the hydrolysis was finished, the enzyme was inactivated by thermal treatment following the manufacturer guidelines. BHC hydrolysates were prepared as follows:

- Simple hydrolysis systems:
  - Hydrolysis P, P enzyme (2 h).
  - Hydrolysis FC, FC enzyme (2 h).
- Sequential hydrolysis systems:
  - Hydrolysis P + F, P enzyme (2 h) + F enzyme (4 h); total reaction time, 6 h.
  - Hydrolysis FC + F, FC enzyme (2 h) + F enzyme (4 h); total reaction time, 6 h.

The hydrolysates were lyophilised.

Free amino groups were measured using o-phthaldialdehyde, according to Nielsen *et al.* (2001), and the degree of hydrolysis (DH) was calculated as:

$$DH(\%) = \frac{(h - h_0)}{h_{tot}} \times 100,$$

where h_{tot} is the total number of peptide bonds in the protein substrate (8.3 mEq g^{-1} protein); h is the number of peptide bonds cleaved during hydrolysis and h_0 is the content of free amino groups of substrate.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) samples profiles were generated following the method of Molina Ortiz & Añón (2001). Briefly, 2 mg (db) of sample were weighed out, placed in an aluminum pan for dry samples (Perkin-Elmer, No. 0219-0041) and hermetically sealed. Thermal transitions were then determined in a differential scanning calorimeter (Perkin-Elmer, DSC-6) between 30 and 130 °C at a rate of 10 °C min^{-1} and a nitrogen flow of 20 mL min^{-1}.

Hydrolysates and fractions characterisation

Fractions from BHC and its hydrolysates at different pHs (4.5, 7.0 and 9.5) were obtained according to Drago & González (2001). Briefly, a solution of the different hydrolysates at 5% (W/W, dry basis) was prepared. The pH was achieved by adding 0.8N HCl or 0.8N NaOH. The samples were stirred for 1 h at room temperature, and then centrifuged during 15 min at 8000 ***g*** at room temperature. The supernatant (the extract at each pH) was freeze-dried and protein content determined by semimicro-Kjeldahl method. Protein solubility at each pH was calculated as the percentage of proteins in the soluble fraction respect to the protein content of the sample. Soluble iron was determined after dry mineralisation as was mentioned before. The iron percentage extracted at each pH was calculated with respect to the hydrolysate total iron. Free amino group content was determined and average peptide chain length (PCL) at each pH was determinate according to Nielsen *et al.* (2001) and Adler-Nissen (1986), respectively.

The colour of fractions was measured with a colourimeter (MINOLTA CM-508D), with an angle of observer: 10°, illuminant: D65 and specular component excluded. Chroma (C^*) was calculated from a^* and b^* values and hue (h) by using the cylindrical coordinates system. The C^* value represents the length of vector and h its rotation angle. As C^* increases, higher the colour saturation is. An h equal 90º represents a yellow hue, while lower h values tend to orange or even red for a^* and b^* positives (Gnanasekharan *et al.*, 1992).

Statistical analysis

All analyses were performed in triplicate. Data were analysed by one-way analysis of variance using the software Statgraphics Plus 3.0 (Warrenton, VA, USA). The LSD test was used to determine statistical differences among samples ($P < 0.05$).

Table 1 Proximal composition of BHC

Composition of BHC (%)	BHC (g/100 g) (X ± SD)
Moisture	3.36 ± 0.02
Proteins	92.3 ± 0.3
Lipids (ether extract)	0.069 ± 0.02
Ash	2.77 ± 0.18
Iron (mg kg^{-1})	1700

Results and discussion

Proximal composition of bovine Hb concentrate (BHC)

Table 1 shows proximal composition of BHC. Similar results were reported by Duarte *et al.* (1999). BHC iron content is lower than that observed by Hurrell (1997) for Hb. This low value can be attributed to the fact that of BHC sample contains plasma proteins which diluted Hb.

Enzymatic hydrolysis of BHC

Figure 1a, b show the DH as a function of time for simple and sequential hydrolysis reactions, respectively. After 2 h of hydrolysis similar DH is reached with the two enzymes (P and FC). An important feature of FC is that this enzyme has endopeptidase and exopeptidase activity, that way explain the higher DH at earlier time of reaction in comparison with P, which only shows endopeptidase activity. DH reached by the use of both sequential hydrolysis reactions was higher than that of simple one. The higher DH can be attributed to the more exhaustive activity showed by Flavourzyme, because this enzyme has endopeptidase and exopeptidase activity (In *et al.*, 2002).

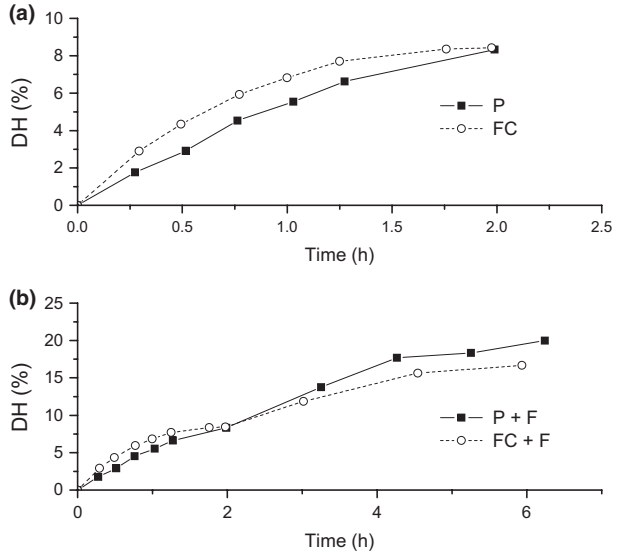

Figure 1 DH as a function of time for simple reaction (a) (P and FC) and sequential reaction (b) (P + F and FC + F).

DH corresponding to P + F and FC + F (19.98 and 16.67, respectively) were higher than those obtained by Chang *et al.* (2007), who worked with porcine Hb as substrate and Alcalase (E/S 2%, pH 8.5 and 4 h reaction time) + Flavourzyme (E/S: 1%, pH: 7.5 and 6 h reaction time). In this work, DH measured by formol titration after 10 h reaction time, was 13.14%, indicating that the enzyme systems P + F and FC + F employed in our work were more effective.

Characterisation of hydrolysates

Differential scanning calorimetry (DSC)

Hydrolysates do not showed endothermic peak. This difference respect to substrate thermogram may be due to enzymatic hydrolysis and/or post-processing heat inactivation enzyme (> 80 °C, 10 min).

Table 2 shows DSC results of protein denaturation transition corresponding to BHC at different pH used for enzyme hydrolysis. It is observed that pH significantly affects both enthalpies (ΔH in J g^{-1}) and pick temperatures (T_p °C) and that both characteristics are directly related. The lowest values correspond to pH 7. As indicating that at pH 7, protein structure is less stable and at pH 4.5 the structure is more compact and thus more thermal energy would be necessary for denaturation. A similar value of T_p at pH 7 was obtained by Michnik *et al.* (2005).

Solubility

Figure 2 shows the solubility (%) at pH 4.5, 7.0 and 9.5, of BHC and its hydrolysates. For BHC, lowest water solubility was observed at pH 7, as this pH is close to its isoelectric point. Beyond this point protein is charged and consequently water interaction is promoted. Also the repulsion among molecules increases and solubility is favored (Cheftel *et al.*, 1989).

Solubility of BHC hydrolysates is significantly higher than that of substrate at pH 7. Hydrolysis promotes exposition of more polar groups and also generates new polypeptides molecules smaller than the substrate and consequently solubility is increased (Puski, 1975; Kabirullah & Wills, 1981). At pH 4.5, hydrolysates showed a significant reduction of solubility in comparison with

Table 2 Denaturation transition enthalpy (ΔH in J g^{-1}) and pick temperature (T_p in °C) corresponding to BHC at different pHs

pH of sample	T_p (°C)	ΔH (J g^{-1})
4.5	73.2 ± 0.8^c	11.17 ± 0.75^c
7.0	63.0 ± 0.3^a	5.15 ± 0.67^a
9.5	65.7 ± 0.3^b	8.23 ± 0.68^b

X ± SD. Different letters means significant difference ($P < 0.05$).

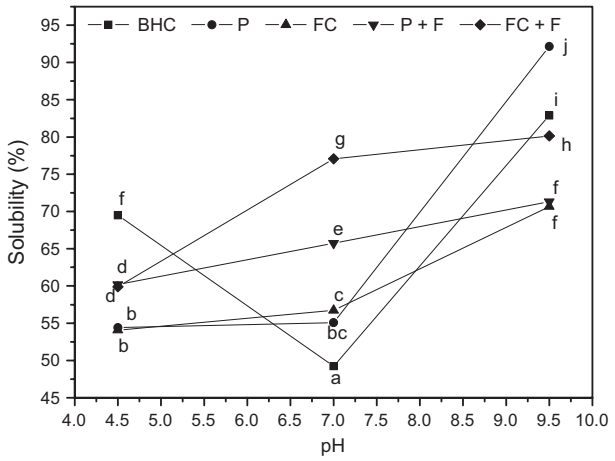

Figure 2 Solubility percentage at pH 4.5, 7.0 and 9.5 for bovine Hb concentrate (BHC) and its hydrolysates (P, FC, P + F and FC + F). Different letters mean significant difference ($P < 0.05$).

BHC. The precipitation of some polypeptides as presented in the hydrolysate would indicate that average isoelectric point of peptides fraction produced by hydrolysis is different from BHC and located in the acidic range. These fractions, when precipitates (mainly due to hydrophobic interactions) would produce a dragging effect on heme group, which solubility at acid pH depend directly on its interaction with peptides (Ericsson, 1983; Margalit & Rosenberg, 1984; Léonil *et al*., 1994; Liu *et al*., 1996, 2010).

Lebrun *et al*. (1998) found a reduction in solubility of bovine Hb hydrolysates at pH 5.5 (average isoelectric point of the peptides mixture) in comparison with the substrate. Hydrolysates were heat treated after proteolysis to inactivate the enzyme. The treatment was done at 85 or 90 °C, during 5 or 10 min (depending on the enzyme), which could also affect solubility (Toldrà *et al*., 2004).

At pH 9.5, P hydrolysate sample showed significantly higher solubility than the other ones, including BHC.

Characterisation of the extracts obtained at different pH from BHC and its hydrolysates

Average PCL

Table 3 shows PCL values corresponding to the polypeptides mixture as presented in each extract. PCL from BHC or its hydrolysate was not affected significantly by the pH of extract. As expected, extract form P and FC contain peptides with higher average size than those from the sequential enzyme systems (P + F and FC + F), since F enzyme produces free amino acids and di- and tri- peptides that contributes to reduce PCL (Chang *et al*., 2007). Sakanaka *et al*. (2004) also reported a PCL value of 2.6, working with extract obtained from egg yolk hydrolysed with alcalase + exopeptidase, during 6 h.

Table 3 Average PCL corresponding to extracts (at pH 4.5, 7.0 and 9.5) obtained from BHC and its hydrolysates (P, FC, P + F and FC + F)

Sample	$PCL_{4.5}$	$PCL_{7.0}$	$PCL_{9.5}$
BHC	7.50 ± 0.44^{d}	7.22 ± 0.94^{d}	7.90 ± 0.95^{d}
P	4.60 ± 0.77^{bc}	4.52 ± 0.55^{bc}	4.94 ± 0.15^{c}
FC	4.44 ± 0.44^{bc}	4.05 ± 0.94^{b}	4.64 ± 0.65^{bc}
P + F	2.08 ± 0.33^{a}	2.31 ± 0.20^{a}	2.38 ± 0.02^{a}
FC + F	2.48 ± 0.20^{a}	2.76 ± 0.14^{a}	2.66 ± 0.30^{a}

X ± SD. Different letters means significant difference ($P < 0.05$).

Determination of iron content in the extracts obtained at different pHs

Figure 3 show the percentage of iron extracted (%Fe) from each hydrolysate. In all cases, %Fe significatively increases with the pH. In general, the higher values of %Fe corresponded to BHC at any pH.

It has been suggested that during hydrolysis of Hb, polymerisation of heme group can occur and that these polymers are soluble depending on the pH (Lebrun *et al*., 1998). Also, Fe solubility is affected by the nature of peptides as presented in the solution (Vaghefi *et al*., 2002).

At pH 9.5 higher proportion of iron is extracted, independently of the sample. This is because at pH higher than 8.0, polymers containing heme group are soluble, independently of peptide interactions (Lebrun *et al*., 1998). On the contrary, below pH 8.0 solubility of prosthetic group (heme) is more dependent on interaction between heme group and peptides, which is clearly

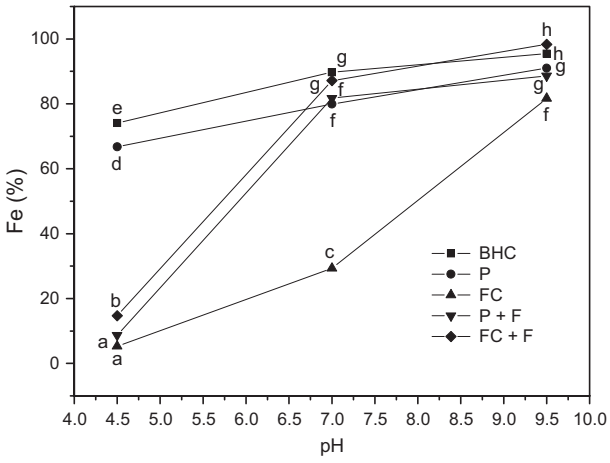

Figure 3 Fe (%) extracted at pH 4.5, 7.0 and 9.5 from BHC and its hydrolysates (P, FC, P + F and FC + F). Different letters mean significant difference ($P < 0.05$).

Table 4 Colour parameters (L^*, b^* and a^*) corresponding to extracts obtained from BHC and its hydrolysates at different pHs

Extracts	L^*	b^*	a^*	h	C^*
BHC 4.5	25.1 ± 0.6^{b}	4.6 ± 0.1^{c}	8.2 ± 0.2^{j}	29.1 ± 0.3^{e}	9.4 ± 0.2^{bc}
BHC 7.0	26.6 ± 0.1^{c}	5.0 ± 0.2^{c}	9.5 ± 0.1^{k}	27.4 ± 0.8^{g}	10.8 ± 0.1^{b}
BHC 9.5	24.6 ± 0.6^{b}	3.5 ± 0.7^{b}	6.1 ± 0.1^{f}	29.9 ± 0.5^{c}	7.0 ± 0.2^{c}
P 4.5	33.2 ± 0.1^{g}	9.0 ± 0.2^{g}	4.8 ± 0.1^{d}	61.9 ± 0.3^{f}	10.2 ± 0.2^{j}
P 7.0	27.4 ± 0.5^{d}	5.6 ± 0.1^{d}	5.5 ± 0.1^{e}	45.5 ± 1.4^{d}	7.8 ± 0.1^{f}
P 9.5	26.6 ± 0.1^{c}	4.6 ± 0.6^{c}	3.5 ± 0.1^{c}	52.7 ± 0.4^{b}	5.8 ± 0.5^{h}
FC 4.5	68.4 ± 0.1^{k}	22.1 ± 0.1^{j}	3.6 ± 0.1^{c}	80.7 ± 0.1^{j}	22.4 ± 0.1^{e}
FC 7.0	43.3 ± 0.1^{h}	18.1 ± 0.1^{h}	12 ± 0.1^{m}	57.5 ± 0.3^{i}	21.5 ± 0.1^{i}
FC 9.5	29.9 ± 0.1^{f}	7.9 ± 0.13^{f}	6.9 ± 0.1^{h}	49.0 ± 0.4^{fg}	10.5 ± 0.1^{g}
P + F 4.5	45.8 ± 0.1^{i}	21.4 ± 0.2^{i}	5.5 ± 0.1^{e}	75.5 ± 0.1^{j}	22.1 ± 0.2^{k}
P + F 7.0	28.4 ± 0.1^{e}	9.5 ± 0.1^{g}	10.1 ± 0.1^{l}	43.1 ± 0.3^{h}	13.9 ± 0.1^{e}
P + F 9.5	25.2 ± 0.2^{b}	1.0 ± 0.1^{a}	2.4 ± 0.1^{a}	23.3 ± 1.9^{a}	2.6 ± 0.1^{a}
FC + F 4.5	63.1 ± 0.4^{j}	24.8 ± 0.1^{k}	2.8 ± 0.1^{b}	83.6 ± 0.4^{k}	25.0 ± 0.1^{m}
FC + F 7.0	27.5 ± 0.1^{d}	6.3 ± 0.2^{e}	8.7 ± 0.1^{j}	36.0 ± 0.5^{g}	10.7 ± 0.1^{d}
FC + F 9.5	23.8 ± 0.1^{a}	4.7 ± 0.4^{c}	6.4 ± 0.1^{g}	36.1 ± 2.1^{d}	7.9 ± 0.3^{d}

X ± SD. Different letters in the same column mean significant difference ($P < 0.05$).

observed at pH 4.5 where the lowest %Fe is obtained. Also, as proteolysis increases, molecular size of the produced peptides is smaller and consequently much weaker is the interaction between heme and peptides, since small peptides cannot avoid heme group polymerisation (Vaghefi *et al.*, 2002). This is observed at pH 4.5 for FC + F and P + F extracts, which showed the lowest %Fe values. Because of these two samples having higher DH, the interactions heme-peptides are weaker and thus, heme groups polymerise and precipitate.

At pH 7.0, the lowest %Fe corresponded to FC extract. For this sample hydrolysis occurred at acid pH and probably heme group polymerised during the hydrolysis process, remaining insoluble during the extraction assays (Liu *et al.*, 2010), but becoming more soluble at alkaline pH.

Relation between colour parameters and iron content (%Fe) of extracts obtained from BHC hydrolysates at different pHs

Table 4 shows the results of colour parameters obtained for extracts of BHC and its hydrolysates at different pHs. Colour varied between yellow and red with their different hue values, since only positive values of a^* and b^* were obtained. On the other hand, lightness values (L^*) varied from twenty-four to seventy. The highest values of L^*, b^*, h and C^* corresponded to $FC_{4.5}$ and FC + $F_{4.5}$ extracts, while the lowest ones corresponded to P + $F_{9.5}$ and $BHC_{9.5}$. In fact, $FC_{4.5}$ and FC + $F_{4.5}$ extracts tend to be light yellow colour. On the contrary, the colour of P + $F_{9.5}$ and $BHC_{9.5}$ tend to be dark red brown, although rather diffuse, because C^* values are small.

Figure 4 shows linear correlation between L^* and b^* with Fe content (mg of iron kg^{-1} sample) in the extracts (r^2: 0.9813 and 0.9773, respectively). As iron content of extracts increases, L^* decreases, indicating that sample is much darker. On the contrary, as iron content decreases, higher values of b^* is observed and the colour tend to be more yellowish. This is in agreement with the results reported by Martínez-Graciá *et al.* (2000). They found that the addition of porcine heme iron in weaning foods produced a decrease of the parameters b^*, h and C^*, in comparison with the control.

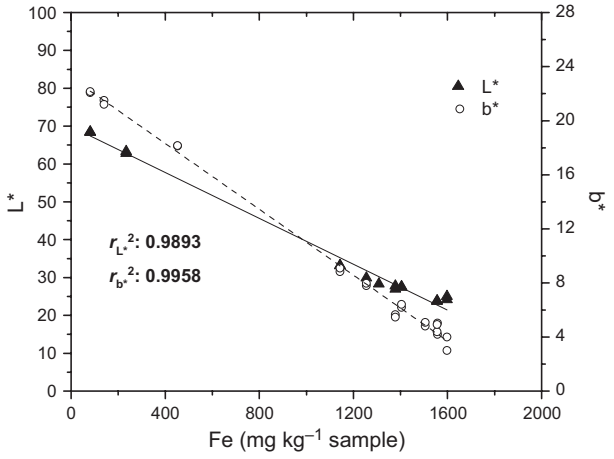

Figure 4 Relationships between colour parameters (L^* and b^*) with iron content (mg kg^{-1} sample) of extracts obtained from BHC and its hydrolysates at different pHs (4.5, 7.0 and 9.5).

Thus, different colours could be associated to the iron content (mg kg^{-1}) as presented in each extracts, which in fact, depended on the pH of extraction (4.5, 7.0 and 9.5) (Fig. 4). As pH increases, higher amount of iron is extracted, since at pH higher than 8.0 polymers of heme group become soluble (Lebrun *et al.*, 1998), which would give lower values of L^*, b^* and h and brownish colour is observed. On the contrary, at pH acid heme

solubility depends on its association with proteins, so that the content of Fe is lower and the values of L^*, b^* and h are high and more yellowish colour is observed in the extracts from hydrolysate.

Besides, it must be taken into account that the iron oxidation state depends also on pH (Gallais, 1950) and consequently, the pH changes affect iron oxidation state and the colour of extract.

Conclusion

The colour from different extracts depends on both, extraction pH and the capacity of peptide fractions generated during the hydrolysis to keep heme-group in solution.

Results allow the choice of the appropriate conditions in order to obtain a specific use for a definite product. In this way, if a protein-rich, iron-free, light colour product is wanted, FC and FC + F extracts at pH 4.5 would be the most convenient for a product whose functional properties, easy digestibility, great potential as flavoring, among others, would make it a proper ingredient. If, on the contrary, a red-brownish, soluble, iron and protein rich product is desired to be used as a food supplement, BHC, P and P + F extracted at pH 9.5 should be chosen.

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