

Research Article

Effects of Operation Conditions on Antioxidant and Iron Chelating Activity of Chemical Hydrolysates from Red Tilapia (*Oreochromis sp.*) Scales

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Abstract: The aim of this study was to optimize the alkaline hydrolysis to extract protein from RTS with antioxidant and iron chelating activity. Red Tilapia (*Oreochromis sp.*) scales (RTS) have essential protein contents; therefore, those represent an opportunity to obtain hydrolysates, which may present biological activities of interest. For the extraction of the protein, it is possible to use chemical methods such as alkaline hydrolysis. The factors of central composite design (DOE) were NaOH concentration (0.5 to 2M), temperature (40 to 60°C) and percentage of scales (2.5 to 7.5%). The response variables were: protein (g/L), antioxidant capacity (ABTS and FRAP) and percentage of iron chelating activity (ICH). The hydrolysis time was evaluated in 2, 4 and 8 h in a 500 mL reactor with constant stirring. It was possible to obtain significant models for the variables assessed and those showed that with high levels of temperature, NaOH concentration and substrate concentration get a higher concentration of soluble protein without affecting the biological activities evaluated. The quantity of the protein obtained for the optimal DOE conditions was 20 g/L, with an extraction yield of 98%. The best time to get proteins with bioactivity was 2 h. This study evidenced the obtainment of protein hydrolysates from RTS with antioxidant and iron chelating activity using alkaline hydrolysis.

Keywords: Alkaline, bioactive, extraction, hydrolysis, protein

INTRODUCTION

The animal production industry generates a considerable amount of waste (Martínez-Alvarez *et al.*, 2015), in fishery, for instance, only about 40% is destined for human consumption and the remaining 60% is usually discharged or used for low value added applications (Je *et al.*, 2007; Silva *et al.*, 2014). These residues mainly consist of filleting waste (15-20%), skin and fins (1-3%), bones (9-15%), heads (9-12%), viscera (12-18%) and scales (5%) (Martínez-Alvarez *et al.*, 2015), which are relevant sources of protein and lipids (He *et al.*, 2013), so, inappropriate disposal of these can cause adverse environmental effects (Chalamaiah *et al.*, 2012).

Fish scales have protein contents between 41%-84% (Sankar *et al.*, 2008), due to this, scales could be used to obtain functional proteins and peptides as in the case of antioxidant, antihypertensive, antimicrobial, anticoagulant peptides (Harnedy and FitzGerald, 2012), with calcium binding (Charoenphun *et al.*, 2012; Chen *et al.*, 2014) or antitumoral activities (Benjakul *et al.*, 2014).

The extraction of proteins is a significant step in the production of value-added products from aquaculture residues, such as hydrolysate and bioactive

peptides, which can be produced meanwhile protein extraction occurs. This removal can be made through basic compounds such as sodium hydroxide (Sanmartín *et al.*, 2009). However, chemical treatments could cause some damage on the amino acid present on the protein (Anal *et al.*, 2013); consequently, it is necessary to evaluate the quality protein after of extraction treatment, which can be made by analysis of the biological activity.

Alkaline hydrolysis has been used by several authors to obtain proteins with bioactive or functional effects from various aquaculture byproducts. Consequently, peptides with antihypertensive or antioxidant activity have been derived from fillets of Nile tilapia (*Oreochromis niloticus*) (Raghavan and Kristinsson, 2008, 2009) or calcium chelating peptides from whole nilotic tilapia (*Oreochromis niloticus*) (Charoenphun *et al.*, 2012). Despite the crucial findings regarding the extraction of the protein, there are no reports linking operation condition, like temperature, time or hydroxide concentration, with the activity of hydrolyzed (Piva *et al.*, 2001; Undeland *et al.*, 2002; Zhou and Regenstein, 2005; Wangtueai and Noomhorm, 2009; Rawdkuen *et al.*, 2009; Essuman *et al.*, 2014).

The objective of this research was to study the best operation conditions (temperature, sodium hydroxide concentration, substrate concentration and time) to obtain protein from RTS with antioxidant and iron chelating activity.

MATERIALS AND METHODS

Materials and reagents: Piscicola El Gaitero supplied fish scales, those were washed and disinfected with sodium hypochlorite 0.2 mg/L, were then placed in polyethylene bags and stored at -20°C until used. The reagents 2,2'-azino-bis (3-ethylbenzothiazolin) -6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromo-2-carboxylic acid (Trolox),3-(2-Pyridyl),5-6 diphenyl acid monosodium salt hydrate (Ferrozine) were obtained from (Sigma-Aldrich, USA), 2,4,6-Tri-2-pyridyl-s-triazine (TPTZ) was resourced from (Merck, USA). All other chemicals were of analytical grade.

Protein extraction: A glass reactor of 500 mL, with a water circulation jacket for temperature regulation was used. The reaction system kept under constant stirring at 500 rpm using a magnetic stirrer for 4 h. The hydrolysates were centrifugated at 3500 rpm for 20 min. Total protein was determined by the Kjeldahl method (Latimer, 2012) and the conversion factor used was 6.25. On the other hand, the extraction was evaluated at different time (2, 4 and 8 h) in the optimal conditions defined in experimental design.

Antioxidant activity: For the determination of bioactivity, the hydrolysates were adjusted to pH 7 and centrifugated at 3500 rpm by 20 min.

ABTS assay: It was determined as described Re *et al.* (1999), the ABTS•+ solution was prepared at 7mMABTS•+ and 2.45mMpotassium persulfate. Then 1 mL this solution was added to sample or Trolox and left in the dark for 1 h. Absorbance was measured at 730 nm. The results were calculated using Trolox standard curve and expressed as micromoles of Trolox equivalents (μmoLET /L).

FRAP assay: It was determined as described Pulido *et al.* (2000), the FRAP solution was prepared with TPTZ, FeCl₃ and acetate buffer, it was left at 37°C. A 900 μL solution was added to 30 μL sample or Trolox and 90 μL of water and it kept in the dark for 1,5 h. Absorbance was measured at595 nm. The results were calculated using Trolox standard curve and expressed as micromoles of Trolox equivalents (μmoLET /L).

Iron chelating activity: This assay was determined according to Choonpicharn *et al.* (2015), in which 1 mL of sample is mixed with 20 μL of ferrozine and 40 μL

of ferrous sulfate and left in the dark for 10 min. Absorbance was measured at 562 nm and the chelating activity percentage was calculated with the Eq. (1):

$$\% \text{chelating activity} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

Experimental design: A Central Composite Design (CCD) was carried out to evaluate the effects of factors NaOH concentration (N) (0.5 to 2M), Temperature (T) (40 to 60°C) and percentage of Scales (S) (2.5 to 7.5%) on response variables protein (g/L), antioxidant capacity (ABTS and FRAP) and percentage of iron chelating activity (ICH). The Design Expert software (version 7.0, Stat Easy Inc., Minneapolis, USA) was used. Table 1 shows the experimental matrix with 19 experimental runs in a random form, 14 design points and 5 repetitions on a central point.

Statistical analysis: The measurements were done at three independent assays. All statistical analyses were performed at a confidence level of 95% (value p<0.05).

RESULTS AND DISCUSSION

Effects of factors on the response variables: Table 1 shows randomly experimental data of CCD with the results for the response variables Protein, ABTS, FRAP and ICH in each run. Meanwhile, results of the ANOVA of the CCD are shown in Table 2, indicating the statistical significance of each factor in each response variable (Value-P).

The factors without significant effects (p>0.05) were eliminated from the ANOVA and the R² and the lack of fit of the resulting model were determined. Both data are presented in Table 2 for adjusted models of Protein, ABTS, FRAP and ICH, which appear in Eq. (2), (3), (4) and (5) respectively.

The ai coefficients in these equations correspond to the effects of the factors concerning their actual values. The R² values and lack of fit (p>0.05) in Table 2 suggest that these polynomials represent the relationship between responses and factors:

$$\text{Protein} = 15.72 + 1.21T + 4.38N + 4.29S + 1.96(N * S) - 2.63N^2 - 1.93S^2 \quad (2)$$

$$\text{ABTS} = 757.46 + 286.34T + 280.09N + 297.09S + 196.39(N * T) + 156.85(T * S) \quad (3)$$

$$\text{FRAP} = 174.36 + 82.70T + 63.33N + 96.59S \quad (4)$$

$$\text{ICH} = 47.81 + 5.08T - 0.79N + 7.97S - 11.65(N * T) \quad (5)$$

The ANOVA displays that the variables temperature and sodium hydroxide concentration are

Table 1: Central composite design in alkaline extraction of protein from scale

Run	T (°C)	N (M)	S (%)	Protein (g/L)	ABTS (µMET/L)	FRAP (µMET/L)	ICH (%)
1	60	0.5	2.5	0,6	25,3	0	55.3
2	50	1.3	9.2	22,7	1395,1	343,9	40.4
3	50	1.3	5	14,6	489,2	129	54.7
4	50	1.3	0.8	14,9	976,2	257,9	56.6
5	33,2	1.3	5	17,4	822,6	212,7	54.3
6	50	0	5	21,3	1195,1	263,1	56.6
7	50	1.3	5	0,7	63	0	21.2
8	50	1.3	5	9	526,4	79,7	33.4
9	40	2	2.5	1	140,5	8,2	42.8
10	66,8	1.3	5	4,1	117,6	0	30.8
11	60	0.5	7.5	15,7	1238,6	304,6	47
12	50	1.3	5	16,6	883,5	238,7	55.1
13	40	0.5	7.5	23,4	1590,7	412	47
14	60	2	2.5	8,6	698,8	51,9	41.4
15	60	2	7.5	17,4	1266,1	349,8	49.6
16	50	2.5	5	16	1084,9	277,2	61.4
17	50	1.3	5	9,5	154,6	38,2	28.7
18	40	2	7.5	15,1	1111	274,2	47.2
19	40	0.5	2.5	7,7	616,8	72,7	52.1

Table 2: P-Value of response variables of experimental design

Variable	Protein	ABTS	FRAP	ICH
Model	< 0.0001	< 0.0001	< 0.0001	0.0007
T	0.0475	0.0001	0.0005	0.016
N	< 0.0001	0.0002	0.0043	0.0008
S	< 0.0001	< 0.0001	0.0001	0.6741
T*N	-	0.0145	-	0.0004
T*S	-	0.0424	-	-
N*S	0.0109	-	-	-
N ²	0.0002	-	-	-
S ²	0.0016	-	-	-
Lack of fit	0.2245	0.7619	0.3329	0.0989
R-Squared	0.9635	0.8855	0.7921	0.7533
Adj R-Squared	0.9416	0.8411	0.7505	0.6774

significant in the four models evaluated; nevertheless, substrate concentration is significant for protein, ABTS and FRAP but no for ICH. Particularly, temperature has significant impact on all responses, mainly in its linear terms, but in the ICH case there is an interaction between N and T. The positive impact of T could be due the proteins are dissolved and hydrolysate between 40 to 100°C (Nolsøe and Undeland, 2008).

On the other hand, N has meaningful effect on all responses and a meaningful interaction with T in the ABTS and ICH cases, it could be due because there is a denaturation reaction in which rapid nucleophilic attack occurs in the carbonyl groups of the peptide, which can be accelerated by heat (Silva *et al.*, 2015). However, it is interesting the fact that N increase ABTS and FRAP but decreased protein and ICH, could be a consequence of the denaturation of protein during the chemical exposition (Essuman *et al.*, 2014). It is possible that protein is partly unfolding during alkali process, which means that it could be due to changes in its activity, nevertheless, if the pH is adjusted to 7, then protein recovers its folding and therefore its activity (Nolsøe and Undeland, 2008).

The polynomial Eq. (2)-(5) were graphed in response surface way (Fig. 1 to 4), with the aim to analyze the graphic behavior of every response. For

each graph, the independent variable not depicted in the graph was positioned its higher level.

Finally, substrate concentration has a significant effect on the four responses, which is logical because when the solution has a high content of protein, the possibility to obtain bioactive peptides increases.

The response surface graphs for extracted protein from RTS as a function of NaOH concentration, temperature and substrate concentration are shown in Fig. 1. The graph indicates that the amount of obtained protein increased with the increase of all factors being more pronounced the effect of NaOH concentration and substrate concentration, than temperature. The fact that the extracted protein was more pronounced at the high alkaline side is similar that to Batista (1999) result in protein extractions using NaOH from hake and monkfish, also (Nurdiyana *et al.*, 2008) in protein extraction from the waste of sardine (*Sardina pilchardus*).

On the other hand, the response surface graphs evidenced that all biological activities increase with increasing of the factors, which was already observed with the polynomial equations.

Optimization process: The optimization criteria were defined to obtain a hydrolysate with higher contents

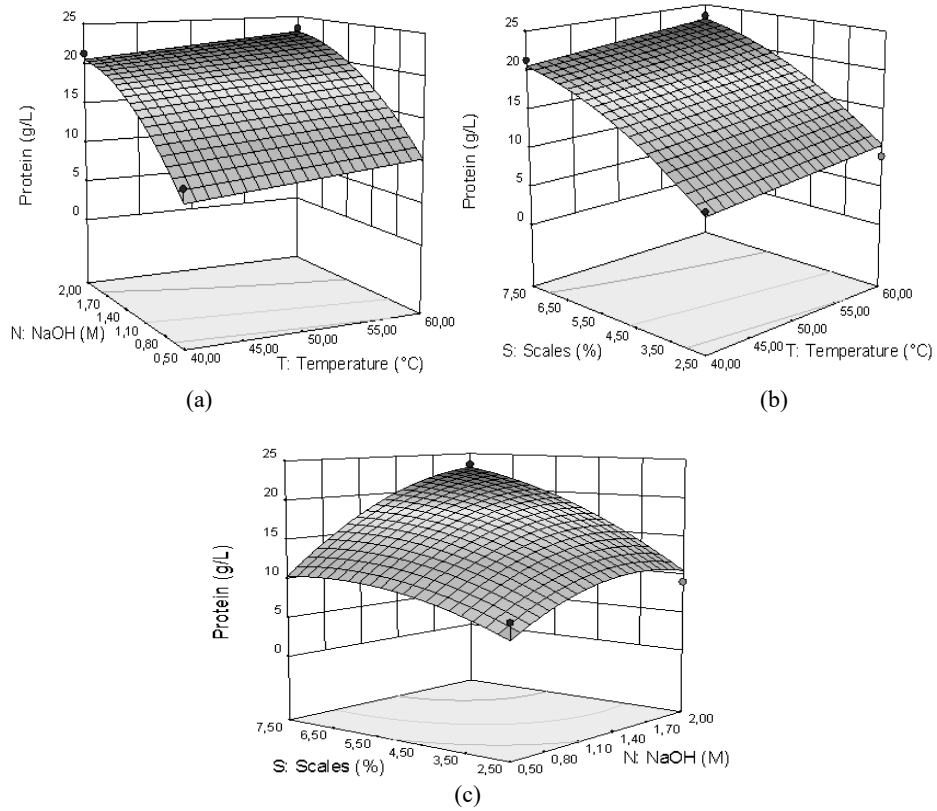


Fig. 1: Response surface for Protein; a) %Scales: 7,5; b) NaOH:2M; c) Temperature: 60°C

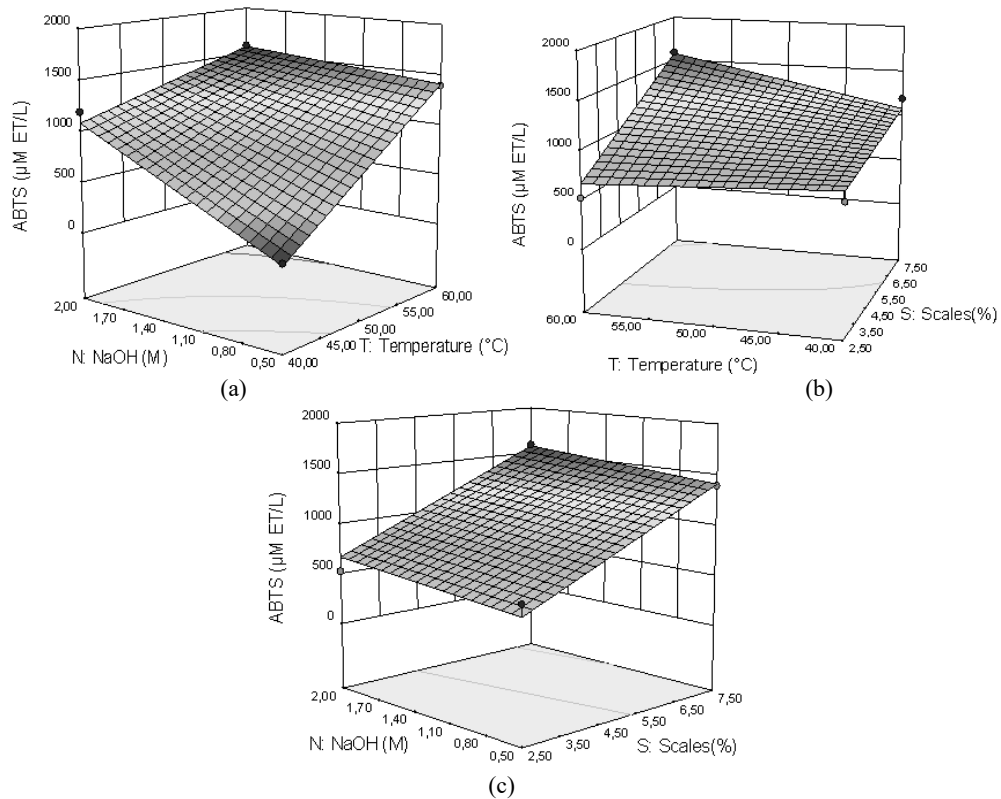


Fig. 2: Response surface for ABTS; a) %Scales: 7,5; b) NaOH:2M; c) Temperature: 60°C

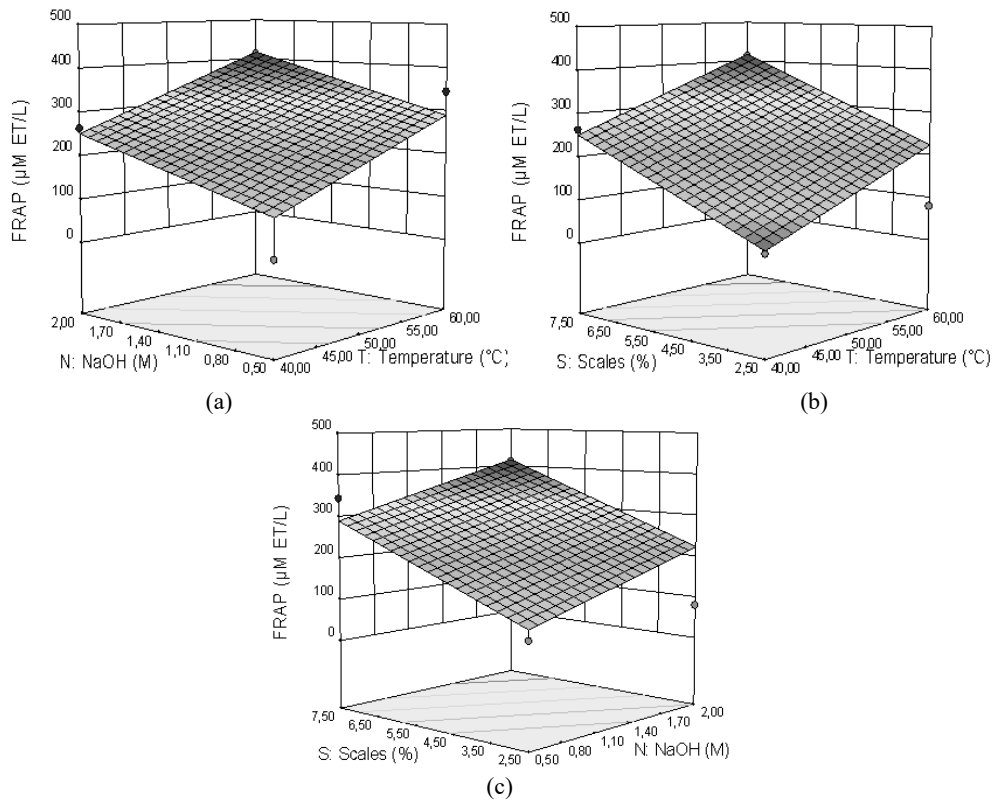


Fig. 3: Response surface for FRAP; a) %Scales: 7,5; b) NaOH:2M; c) Temperature: 60°C

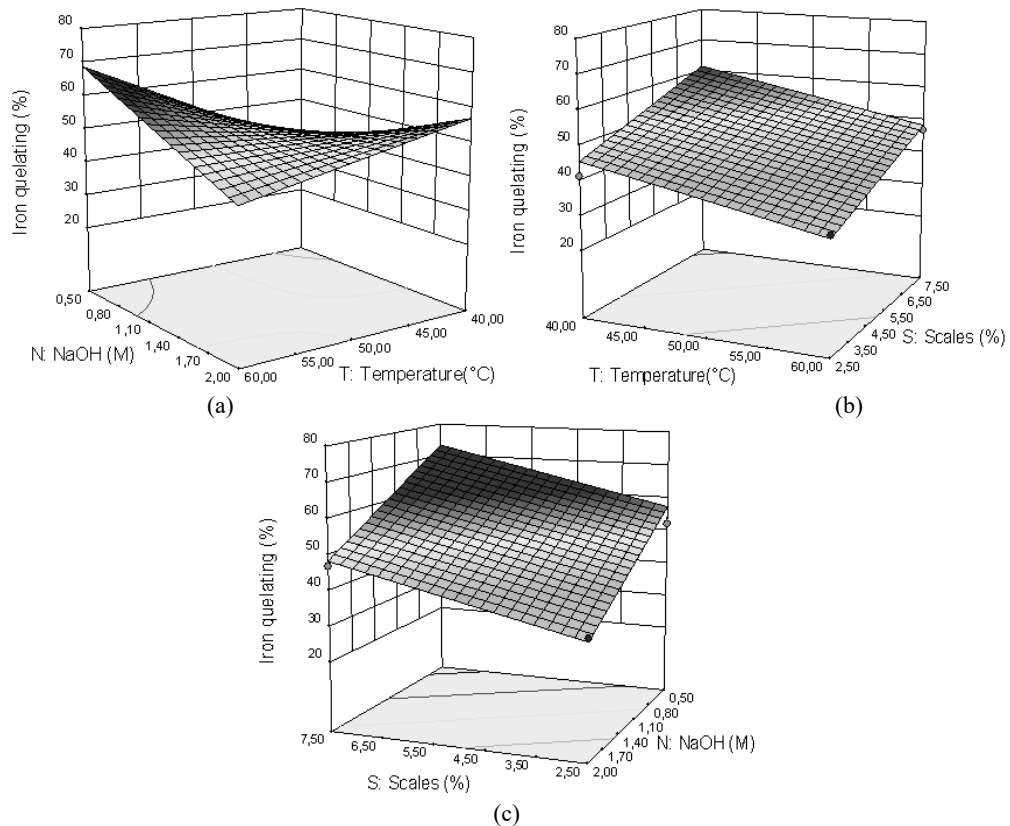


Fig. 4: Response surface for iron chelating; a) %Scales: 7,5; b) NaOH:2M; c) Temperature: 60°C

Table 3: Response variables on optimal conditions

Variable	Predicted	Experimental
Protein(g/L)	22,99	20.8±1
ABTS (µM ET/L)	1581,43	1966.6±190.7
FRAP (µM ET/L)	416,97	324.1±8.9
ICH (%)	48,42	60±1

Table 4: Results of other authors for antioxidant from fish waste

Results of this study	Process	Other results	Reference
288 µmolTrolox/g (ABTS)	Alkaline and enzymatic hydrolysis from scales	400 µmolTrolox/g (ABTS)	Wangtueai <i>et al.</i> (2016)
15,87 µmolTrolox/g (FRAP)		9 µmolTrolox/g (FRAP)	
60% iron chelating activity with 7,6 g/L of protein	Enzymatic hydrolysis with complete tilapia	80 µmolTrolox/g (ABTS)	Chuaychan and Benjakul (2016)
		107 µmolTrolox/g (ABTS)	Mosquera <i>et al.</i> (2014)
		102 µmolTrolox/g (ABTS)	Chuaychan <i>et al.</i> (2017)
		3 µmolTrolox/g (FRAP)	
60% iron chelating activity with 7,6 g/L of protein	Enzymatic hydrolysis skin from tilapia	82.5% of activity with 5 g/L of protein	Foh <i>et al.</i> (2011)
		77,27 % of activity with 10 g/L of protein	Choonpicharn <i>et al.</i> (2015)
		70% of activity with 1,7 g/L of protein	Farvin <i>et al.</i> (2014)

Table 5: Evaluation on time for response variables

Time (h)	Protein (g/L)	ABTSµM ET/L)	FRAP (µM ET/L)	ICH (%)
2	25.9±0.23 ^a	1722,61±197,25 ^a	178,986± 10,34 ^a	61±8.14 ^a
4	22.8±0.84 ^a	1862,1±383,785 ^a	200,236± 37,56 ^{ab}	53±10.14 ^a
8	23.6±0.83 ^a	1824,06±63,35 ^a	228,847±7,12 ^b	58± 1.52 ^a

Different letters indicate statistically significant differences between groups

of protein and bioactivities. The results of the process gave 60°C, NaOH 2M and 7,5% of scales, as the optimum condition. Table 3 presents the results of the experimental verification of this process, which agreed with Nolsøe and Undeland (2008), who reported that the solubilization of protein starts at pH 10 and higher temperature.

The predicted values are close to experimental, in the case of protein and FRAP those are higher, but ABTS and ICH are lower, though these could be accepted in this process. Besides this process has a yield of 98%, because there were 37,5 gr of scales in the beginning and in the final, there were only 6,4 gr.

On the other hand, other authors have studied the protein extraction using two combined processes such as alkaline and enzymatic hydrolysis, a summary of these results is present in Table 4 in contrast to the results of this study.

These works obtain protein with bioactivity using the alkaline and enzymatic hydrolysis coupled (Wangtueai *et al.*, 2016; Chuaychan *et al.*, 2017), whereas in the present study it is shown that with a single process it is possible to recover the protein without cause a chemical damage to the biological activity of the peptides. Besides, it is important to note that no reports are showing the use of fish scales for the generation of peptides with iron chelating capacity. According to Sanmartin *et al.* (2009), the alkaline process is faster and inexpensive process than enzymatic. Therefore, these results show the potential for converting the wastes from tilapia industry by alkaline hydrolysis into useful bioactive compounds.

Evaluation protein extraction on the time: Table 5 presents the time effect on variable response, which displayed that in 2 h is enough to extract high quantity of protein with antioxidant and iron chelating activity, because using more time for the process doesn't affect statistically protein, ABTS and iron chelating, however for FRAP there is a difference at 4 h. This process behavior could be possible because during alkaline hydrolysis occurs a generation of polypeptides rapidly and later a degradation a slower rate (Ingadottir and Kristinsson, 2010).

These results are higher than other processes which require more than 4 h to obtain hydrolysates with less activity (Chuaychan and Benjakul, 2016; Mosquera *et al.*, 2014; Chuaychan *et al.*, 2017).

CONCLUSION

The protein and bioactivities are affected by temperature, sodium hydroxide concentration and substrate concentration and not by the time.

Under the conditions of the present study, optimal conditions to extract protein with high biological activities were temperature 60°C, hydroxide sodium concentration 2M, level of the substrate of 7,5% and time 2 h.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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