
Water ratio effect on the proteins hydrolysis tongue sole by-products (*Cynoglossus senegalensis*)

Effet de l'eau sur l'hydrolyse enzymatique des protéines de coproduits de la sole tropicale (*Cynoglossus senegalensis*)

Sylla K.S.B.^{1,*} ; Musabyemariya B¹. ; Berge J.P.² ; Seydi Mg¹

¹ Service d'Hygiène et Industrie des Denrées Alimentaires d'Origine Animale (HIDAOA). Ecole Inter-Etats des Sciences et Médecine Vétérinaires de Dakar (EISMV). B.P. 5077 Dakar-Fann. Tél./ Fax: (221) 834 33 54

² IFREMER, BP 21105, 44311 Nantes, France

*: Corresponding author : Sylla K.S.B., email address : khsylla@refer.sn

Abstract:

In this study the effectiveness of four different water ratio to hydrolyze sole by-products by using Protamex® were compared. Average proteins yields and degree of hydrolysis (DH %) of different hydrolysates were measured, and their peptide profiles were analysed by using gel filtration chromatography. It was found that by changing the hydrolysis conditions it was possible to produce hydrolysates with the same proportion of proteins (60%) and very different in peptide composition (from 294 to 13700 Da).

Keywords: By-products ; water ; enzymatic hydrolysis ; proteins

Résumé:

Dans cette étude les effets de la variation du volume d'eau (quatre pourcentages) au cours de l'hydrolyse enzymatique de coproduits de la sole tropicale ont été comparés. Les teneurs moyennes en protéines et le degré d'hydrolyse (DH) ont été mesurés d'une part, et d'autre part, les profils peptidiques ont été analysés en utilisant la méthode chromatographie gel filtration. Il a été trouvé que le changement des conditions d'hydrolyse, a permis de produire des hydrolysats avec la même proportion de protéines (60%) et ayant une composition peptidique différente (tailles des peptides entre 294 et 13700 Da).

Mots clés: Coproduits ; eau ; hydrolyse enzymatique ; protéines

1. Introduction

There has been an increasing interest in fish by-products during the past years. Today it is seen as a potential resource instead of a waste. Much research is being done in order to explore the possible uses of different by-products. Today overexploitation of fish resources is a large problem: only about 50-60% of the catch is used for human consumption. Globally more than 91 million tons of fish and shellfish are caught each year. Some of the by-products are utilized today, but huge amounts are wasted. Annual discard from the world fisheries were (FAO) estimated to be approximately 20 million tons (25%) per year. Therefore it is a great potential for the fishing industry to utilize more of what is landed. This includes “waste” or by-products or what should really be called rest raw materials [16] .

In Senegal, fishing is the first national industrial sector with a sales turnover which rises 185 billion francs CFA in 2008. Composed by 50 companies (threading, refrigeration, frozen), it is the first industrial employer with more than 600 000 paid.

The volume of organic tongue sole byproducts resulting from these companies was estimated at 45 000 tons in 2008. Valorization of these byproducts represents today a quadruple stake for these halieutic sector. Initially it's the environmental aspect within the framework of the sustainable development in terms of solutions to propose in order to find the ways of respectful valorization of the environment. The second stake is economic, it is a question of knowing how to improve the productivity of these companies by a better control of the ways of valorization and at which cost they can treat their waste. Thirdly, with technical dimensions which consists in placing modern practice according to generated volumes of by-products. Finally the fourth stake is about regulation because the normative and law constraints are reinforced more and more and the companies must prepare themselves.

Recent research showed that the fish byproducts can be a significant source of proteins with very high biological value. Fish proteins have long been known for their nutritional and technological value. Proteins are important constituents of the human diet, since they comprise a principal source of nitrogen and essential amino acids. Fish proteins have high nutritional value compared to other proteins because of their relatively high content of essential amino acids and good digestibility [5, 2] .

In order to assess the effect of the water in the proteins level, a number of replicated small-scale laboratory experiments were produced from tongue sole byproducts hydrolysates with addition of 10%, 25%, 50% and 100% water.

The aim of the present study was to monitor addition effects of water during sole byproducts hydrolysis on chemical composition in order to minimize the cost of the water when trials will be proceeded in large industry scale.

2. Material and methods

I-1) Raw material

Tongue sole by-products (*Cynoglossus senegalensis*) were provided by Sénégal Pêche, a seafood processing company (Dakar, Senegal). Mature individuals (500 kg) were caught during November 2007 through the FAO fishing area number 34.

Upon capture, the specimens were transported to the fish plant by a frozen fish boat where they were stored into a negative freezing room. The collected by-products were realized during the processing fillet operation. They were immediately frozen at - 20°C until used.

By-products are composed to the head, the backbone and the viscera (liver, caecums).

The collected samples were mixed and homogenized into the IFREMER (France) laboratory.

I-2) Enzyme

The enzyme used for the hydrolysis of tongue sole byproducts were provided by Novozymes A/S (Bagsvaerd, Denmark). Protamex is a *Bacillus* protease complex; It is a bacterial serine endopeptidase prepared from a strain of *Bacillus licheniformis*. The rates used in this study was at 0,1%.

I-3) Enzymatic hydrolysis

Hydrolysis was carried out in a thermostatted 4 l vessel (batch) equipped with a stirrer. Sole by-products (750 g) were mixed with distilled water. The trials were performed by the ratios of 100%, 50%, 25% and 10% distilled water.

All hydrolysis reactions were performed at pH 6 and 40°C for optimal activities of Protamex. Before adding enzyme, water is mixing with by-products during 15 min in order to obtain the desired temperature. Then hydrolysis was initiated by adding 0.1% w/w for Protamex. The mixtures were continuously stirred at 300 rpm for 6 h. Protamex can be inactivated in 10 minutes at 85°C.

Afterwards hydrolysates was then centrifuged at 3,000 x g at 20°C for 15 minutes. At the end of the centrifugation two different fractions were obtained: fish protein hydrolysate and sludge fraction. The fish protein hydrolysate was a clear yellow liquid and the sludge fraction was insoluble bottom fraction. These fractions were then freeze-dried. After drying, the dried fish protein hydrolysates were light yellow powders (soluble protein powders) and the sludge was a brown, grey powders (insoluble protein powders).

For the need of the following up to the soluble proteins liberation and level, several samples were taken according to this sequence :

Table 1 : Frequency of the sampling

Number	Sampling (minutes)	time	Quantity
1.	0		50 ml
2.	2		50 ml
3.	4		50 ml
4.	6		50 ml
5.	8		50 ml
6.	10		50 ml
7.	20		50 ml
8.	30		50 ml
9.	40		50 ml
10.	50		50 ml
11.	60		50 ml
12.	90		50 ml
13.	120		50 ml
14.	150		50 ml
15.	180		50 ml
16.	210		50 ml

17.	240	50 ml
18.	270	50 ml
19.	300	50 ml
20.	330	50 ml

I-4) Analytical methods

Dry matter content in the raw materials and in the produced fractions from hydrolysis was determined gravimetrically after drying at 105⁰C.

Total nitrogen content was determined by the Kjeldahl method [4, 9], crude protein was estimated by multiplying total nitrogen (%N) by the factor 6.25.

The degree of the hydrolysis (DH) was determined by measuring the liberation of free amino groups using the dinitrofluorobenzene (DNFB) reagent referred to as Sanger'reagent (Sanger, 1949). After centrifugation, the liquid protein hydrolysate was diluted by 200. One milliliter of diluted solution was added to 1ml of the sodium tetraborate (2%). After mixing, 0.25 ml of solution of 2,4- Dinitrofluorobenzene (DNFB/ethanol: 0,013/1, v/v) was added. Samples were heated 10 minutes at 60⁰C. After cooling, 2 ml of hydrochloric acid was added in order to stop the reaction. A calibration curve was established by using glycine (SIGMA, France) and by reading the absorbance at 410 nm. Results were expressed in equivalent glycine.

The molecular weight distribution of peptides in the hydrolysates was analyzed by gel filtration chromatography. The molecular weight fractions were separated using a high performance liquid chromatography (HPLC) system equipped with a size exclusion column (Superdex 75 HR 10/30). The mobile phase consisted of water with trifluoroacetic 0.1% and acetonitrile 0.5% (70:30) the flow rate was 0.5ml/min. The chromatography was monitored by measuring the absorbance at 214 nm. The column was calibrated with standards: Ribonuclease A (13700 Da), Aprotinin (6500 Da), Renin (1760 Da), Vasopresine (1084 Da) and Leucine (294 Da).

I-5) Statistical analysis

The tests were done in triplicates. The ANOVA test was used for data processing and statistical analysis. Mean were accepted as significantly different at 95% level (p<0.05).

3. Results

II-1) Dry matter distribution

The proximate dry matter composition of tongue sole by-products was given in figure 1. The results indicated that dry matter content of by-products was variable according to water ratio the maximum was: 72.26%, 67.52%, 65.54% and 57.73% for the 10%, 25%, 50% and 100% respectively.

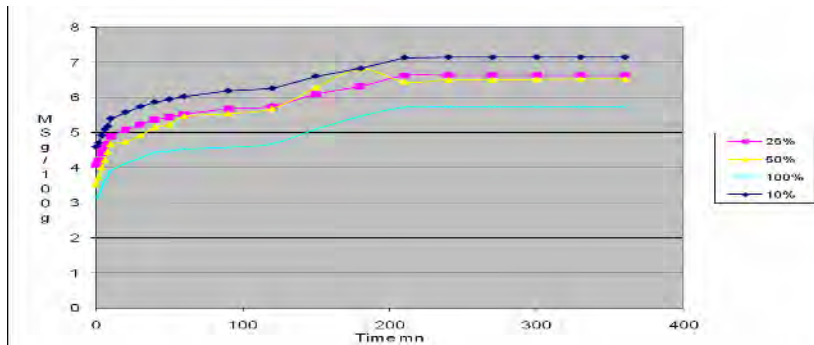


Figure 1: Kinetics of dry matter in relation to water rate

II-2) Proteins distribution

Figure 2 shows the chemical composition of soluble protein from the tongue sole by-products after 330 mn of hydrolysis. The protein contents of the dried soluble protein powders obtained were high: 57.39%, 59.02%, 60.03 and 60.58% respectively.

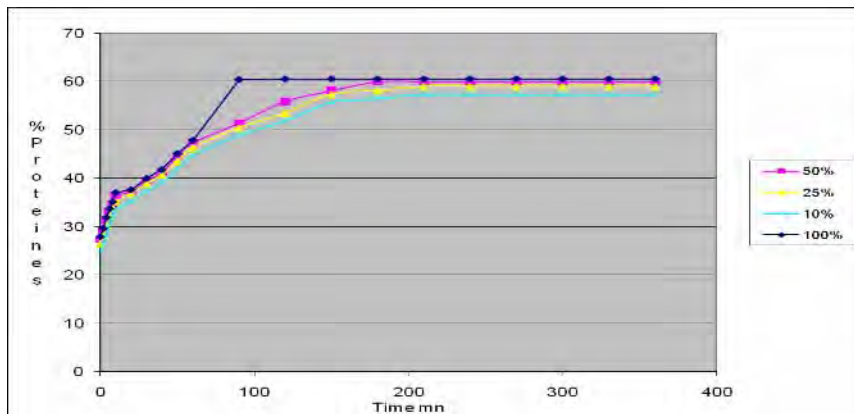


Figure 2: Kinetics of proteins in relation to water rate

II-3) Degree of hydrolysis

The degree of hydrolysis obtained by using Protamex enzyme according to the different water rates increased with hydrolysis time. The rapid hydrolysis was observed within the first period. Thereafter, a lower rate of hydrolysis found up to 2 hours.

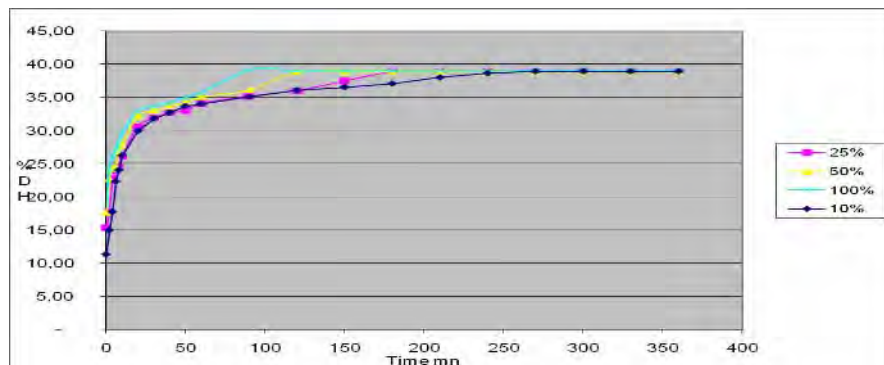


Figure 3: Kinetics of DH in relation to water rate

II-4) Peptides molecular weight distribution

Gel filtration chromatograms showed the distribution of sole protein hydrolysate fraction molecular weight. Molecular profiles of tongue sole hydrolysates at different water rates are displayed in figure 4.

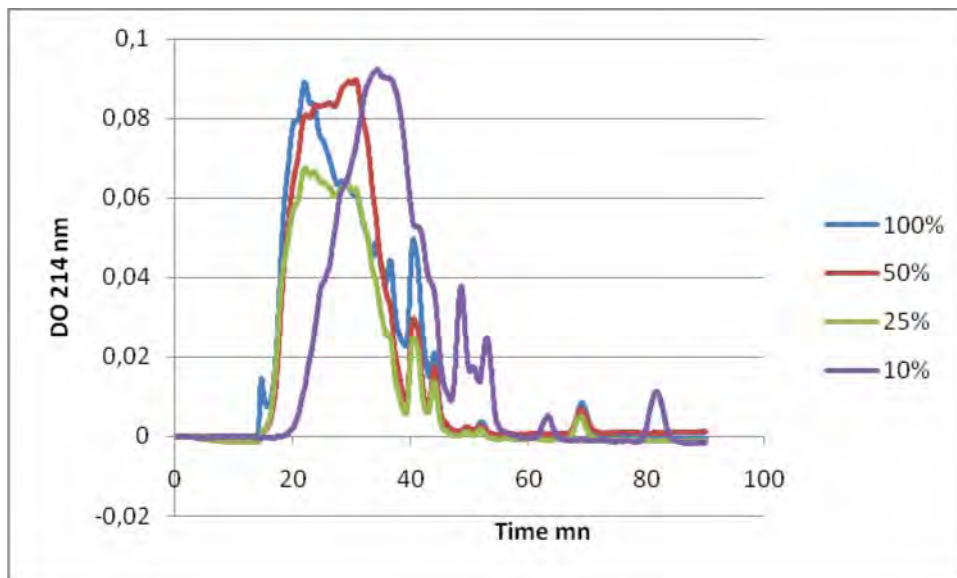


Figure 4: Peptides molecular weight distribution in relation with water rates

4. Discussion

➤ Dry matter distribution

Dry matter distribution in the soluble fraction was growing up similarly at the beginning of the hydrolysis (from 2 to 180 mn). After 180 mn the concentration was on the maximum level whatever the water rate. The proportion of dry matter in the soluble fraction was increased, this means that the dry matter was solubilized during hydrolysis under the action of water and the enzyme Protamex.

These results were similar to those showed for sardine viscera [4]. These authors indicated that 80% of the dry matter was located in the soluble fraction after 24 h of hydrolysis compared to 46% for the non-hydrolysed viscera. **ASPMO et al. (2005)** also showed high solubilization of the dry matter from the cod viscera after 24 h of hydrolysis with Protamex at 55°C without pH control.

KECHAOU et al. (2009) obtained in comparison between cuttlefish and sardina byproducts 32% and 30 % respectively in aqueous phase. However, she showed that after 24 h of hydrolysis, the major percentage of dry matter was contained in the supernatant, regardless of the enzyme and substrate. This indicates that proteolysis by using water improved

solubilization of dry matter into the soluble fraction. Similar results were found in recent studies on cod (*Gadus morhua*) viscera [15, 3] showing sufficient solubilization of dry matter under similar conditions.

As can be seen, solubilization of dry matter by using various water ratio seemed to be efficient process. Almost all tested water rates reached highest average yield within 210 mn hydrolysis. However only 10% water addition showed substantially increase of average.

➤ Protein contents

The protein contents of these hydrolysates were similar to those in other reports, ranging from 50.3 to 91.6% [13, 10, 15, 11].

KECHAOU et al. 2009 reported protein recoveries from the soluble fraction of sardine hydrolysates ranged between 57.4 and 61.2%, which is in comparable to the recovery levels we had in this study. The sample from 100% water hydrolysates contained higher protein content and low dry matter content, which might significantly contribute to concentration of soluble proteins into the soluble fraction.

The hydrolysis of the sole protein was characterized by an initial rapid phase, during which a large number of peptides bonds were hydrolyzed in relation to water rate. Then the rate of enzymatic hydrolysis subsequently decreased, and the enzymatic reaction reached the steady-state phase when the undigested or partially digested sole proteins took place.

As can be seen, solubilization of proteins by using various water rates seemed to be efficient process. Almost all tested rates reached highest average yield within 3 hours hydrolysis. Only 100%, showed substantially increase of average yield after 1h 30 mn hydrolysis. In the case of 50% water addition the increase was 52%, while in the case 25% the yield increase was 40% when average yields after hydrolysis of 3 hours was compared. Previous studies have likewise indicated that the solubilization of proteins could be achieved quickly by enzymatic hydrolysis [8, 7].

Meanwhile prolonged hydrolysis from 1 hour to 6 hours seemed to have a slight increasing effect to average yield with Protamex. However, degradation of proteins into shorter peptides still continued after 3 hours hydrolysis. This observed increase in average yield may be attributed to formation of hydrophobic peptides, which have weaker solubilization ability into hydrolysis buffer, during proteins degradation. This finding is in accordance with previous studies. **GUÉRARD (2001)** postulated that the hydrolysis of tuna by-products proceeded in two stages: in the first stage ovomucin is solubilized, and in the second stage it is hydrolyzed more extensively to peptides having shorter chain lengths.

➤ Degree of hydrolysis

This result was in accordance with **SHAHIDI et al. (1995)** who studied on the capelin hydrolysis with Alcalase and Neutrase. In the initial period of hydrolysis, a large number of peptide bonds were cleaved, after a period of hydrolysis the concentration of soluble peptides in the reaction mixture increased, which recovery the maximum level after 3 hours of hydrolysis.

The shape of hydrolysis curves was similar to those previously published for yellowfin tuna stomach (**Guérard et al, 2001**), herring (**Liceaga-Gesualdo and Li-Chan, 1999; Sathivel et al, 2005**), salmon muscle (**Kristinsson and Rasco, 2000**), salmon head (**Sathivel et al, 2005**).

Degree of hydrolysis with 100% water addition was higher than those for 50%, 25% and 10%. After the hydrolysis of 6 hours, the degree of hydrolysis was at the same value for all

the water proportion used, 39%, was obtained. The same degree of hydrolysis may be explained that the sole by-products is a complex substrate (with viscera, heads and backbones), it contain significant amount of nitrogen in the form of non-protein nitrogen (NPN). While the non-protein compounds needed long time of hydrolysis in order to realize proteolytic degradation and solubilization [1, 12] .

DUMAY *et al.* (2006), using Protamex 0.1% for sardine viscera hydrolysis at 50⁰C, reported a degree of hydrolysis of 31% after 24 hours. Low degree of hydrolysis from sardine viscera hydrolysis compared to that obtained from tongue sole viscera hydrolysis may due to the different pH. Tongue sole viscera hydrolysis was carried out at natural substrate pH (6.3 - 6.5), these values were within the optimal range for Protamex (pH 5.5 - 7.5), while the sardine viscera hydrolysis was carried out at pH 8.

In general the results showed that, protein content increased after hydrolysis so a relationship between DH and protein content was observed. The differences in protein content at the beginning of hydrolysis at different DH were due to the corresponding difference in non-protein nitrogen. The produced peptides can interact with unhydrolyzed protein via

hydrophobic interactions resulting in increase of the insoluble protein fraction. Also some protein/peptides were lost during the centrifugation prior to freeze-drying being also related to higher dry matters levels in the samples.

However, increasing the water rate increased the solubility and the DH of the total hydrolysates. High solubility of proteins is due to cleavage of proteins into smaller peptides that usually have increased DH. The difference in solubility observed in the beginning reaction among hydrolysates can be due to peptide length and the ratio of hydrophilic/hydrophobic peptides.

➤ Peptides profiles

The molecular weight of the peptides was below 13.700 Da. The molecular weight distribution of the tongue sole hydrolysates was between 294 Da and 13.700 Da. The results showed an increase of the low molecular weight peptides at 10% that is in correlation with the high concentration when the water is low during the hydrolysis.

The molecular weight distribution of the tongue sole hydrolysates with 25%, 50% and 100% was similar and the most peptides had molecular weight below 1.084 Da. No significant differences are found between these hydrolysates.

Conclusion

This study showed that by using different water ratio it was possible to produce very different kind of hydrolysates. It was found that Protamex® can work in smaller water concentration (10%). But the higher average yield of proteins was obtained after 3 hours of hydrolysis. Hydrolysates having DH values of 39 % were generated from sole by-products. Comparison of molecular peptides obtained, indicated that the rate 10% water improved the solubility of small peptides. This may be useful when peptides having different kind of bioactive properties are produced and further works should be done to isolate and characterize these peptides.

Bibliography

Aspmo SI, Horn SJ, Eijsink VGH, 2005. Enzymatic hydrolysis of Atlantic cod (*Gadus morhua* L.) viscera. *Process Biochemistry*; (40), 1957-1966.

Badal, C.S. and Kiyoshi H. 2001. Debittering of protein hydrolysates. *Biotechnol. Advanc.* 19(5):355-370.

Castillo-Yanez, F.J., Pacheca-Aguilar, R., Garcia-Carreno, F.L., and Navarrete-Del Toro M., 2004. Characterization of acidic proteolytic enzymes from Monterey sardine (*Sardinops sagax caerulea*) viscera. *Food Chem.*, (85), 343-350.

Dumay J, Donnay-Moreno C , Barnathan G, Jaouen P, Bergé JP, 2006. Improvement of lipid and phospholipid recoveries from sardine (*Sardina pilchardus*) viscera using industrial proteases. *Process Biochemistry*; 1-6.

Gildberg A., I. Batista, 1989, E. Strom, Preparation and characterization of peptones obtained by a two-step enzymatic hydrolysis of whole fish, *Biotechnol. Appl. Biochem.* 11 (1989) 413–423.

Guérard F, Dufossé L, De La Broise D, Binet A, 2001. Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacares*) wastes using Alcalase. *Journal of Molecular Catalysis B: Enzymatic*; (11), 1051-1059.

Hoylen NT, Merritt JH, 1994. Quality of fish protein hydrolysates from herring (*Clupea harengus*). *Journal of Food Science*; 59(1):76-79.

Huong N, Donnay-Moreno C, Bergé JP, 2007. Enzymatic hydrolysis of tuna by-products by using Protamex protease. *Proceeding WEFTA Annual Meeting*; Spain.1-6

Kechaou E. S., Dumay J., Donnay-Moreno C., Jaouen P. , Gouygou J-P., Bergé J-P. Ben Amar R., 2009. Enzymatic hydrolysis of cuttlefish (*Sepia officinalis*) and sardine (*Sardina pilchardus*) viscera using commercial proteases: Effects on lipid distribution and amino acid composition. *Journal of Bioscience and Bioengineering*, (107), Issue 2, 158-164

Kristinsson HG, Rasco BA, 2000. Biochemical and functional properties of Atlantic salmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. *Journal Agricultural and Food Chemistry*; (48), 657-666.

Liceaga-Gesualdo AM, Li-Chan ECY, 1999. Functional properties of fish protein hydrolysate from herring (*Clupea harengus*). *Journal of food science*; 64(6):1000-1004.

Métayer S., Seilliez I., Collin A., Duchêne S., Mercierc Y., Geraertc P-A., Tesseraud S., 2008. Mechanisms through which sulfur amino acids control protein metabolism and oxidative status, *Journal of Nutritional Biochemistry*, (19), 207-215.

Sathivel S, Smiley S, Prinyawiwatkul W, Bechtel PJ, 2005. Functional and nutritional properties of red salmon (*Oncorhynchus nerka*) enzymatic hydrolysates. *Journal of food science*;70(6):401-406.

Shahidi F, Han Xiao-Qing H, Synowieck J, 1995. Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). *Food Chem* 1995; 53:285-293.

Slizyte R, Dauksas E, Falch E, Storro I, Rustad T., 2005. Yield and composition of different fractions obtained after enzymatic hydrolysis of cod (*Gadus morhua*) by-products. *Process Biochemistry*; (40)1415-1424

Souissi Nabil, Ali Bougatef, Yousra Triki-Ellouz and Moncef Nasri, 2007. Biochemical and Functional Properties of Sardinella (*Sardinella aurita*) By-Product Hydrolysates, *Food Technol. Biotechnol.* 45 (2) 187–194