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ANTIOXIDANT EFFECT OF THE HYDROLYSATE DERIVED FROM FISH GELATIN ON THE SHELF LIFE OF RAINBOW TROUT

[*1,2,3]Carolina Cristina Colão Barcellos, [4]Ana Beatriz Monteiro Fonseca, [5]Marcos Aronovich, [5,6,]Ana Iraidy Santa Brígida, [7]Luiz Antonio Moura Keller and [8]Eliana de Fátima Marques de Mesquita

[1]Fellowship of Coordenação de Aperfeiçoamento Pessoal (CAPES); [2]Master of the Programa de Pós-graduação em Medicina Veterinária - Higiene Veterinária e Processamento Tecnológico de Produtos de Origem Animal, Departamento de Tecnologia dos Alimentos – MTA, Faculdade de Medicina Veterinária, Universidade Federal Fluinense(UFF), Niterói, Rio de Janeiro, Brazil; [3]Doctor of the Programa de Pós-graduação em Medicina Veterinária - Higiene Veterinária e Processamento Tecnológico de Produtos de Origem Animal, Departamento de Tecnologia dos Alimentos – MTA, Faculdade de Medicina Veterinária, Universidade Federal Fluinense (UFF), Niterói, Rio de Janeiro, Brazil; [4]Teacher of the Departamento de Estatística, Instituto de Matemática e Estatítica, Universidade Federal Fluminense(UFF), Niterói, Rio de Janeiro, Brazil. Rua Professor Marcos Waldemar de Freitas Reis, s/n, Bloco G, 3º andar, Campus do Gragoatá, São Domingos, Niterói, RJ, 24210-201; [5]Resarcher of Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro (PESAGRO-RJ), Niterói, Rio de Janeiro, Brazil. Alameda São Boaventura, nº 770, Niterói, RJ, 24120-191; [6]Resarcher of Embrapa Agroindústria de Alimentos, Rio de Janeiro, Rio de Janeiro, Brazil. Av. das Américas, nº 29.501, Guaratiba, Rio de Janeiro, RJ, 23020-470; [7]Resarcher of Embrapa Agroindútria Tropical, Fortaleza, Ceará, Brazil. Rua Dra. Sara Mesquita, nº 2270, Planalto do Pici, Fortaleza, CE, 60511-110; [8]Teacher of Programa de Pós-graduação em Medicina Veterinária - Higiene Veterinária e Processamento Tecnológico de Produtos de Origem Animal, Departamento de Tecnologia dos Alimentos – MTA, Faculdade de Medicina Veterinária, Universidade Federal Fluinense (UFF), Niterói, Rio de Janeiro, Brazil

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Corresponding author:
Carolina Cristina Colão Barcellos,

ABSTRACT

Hydrolysate proteins with antioxidant properties obtained from the enzymatic hydrolysis of fish gelatin enable the waste reintroduction into the production chain as natural food additives acting in the preservation of the food quality. In this context, this study evaluated the effects of a commercial fish gelatin bioactive protein hydrolysate on the preservation of rainbow trout fillets (*Oncorhynchus mykiss*) quality maintained under refrigeration. Fillet samples were distributed into three groups, control and hydrolyzate addition at 1:10 and 1:1 ratios, vacuum packed and refrigerated at temperatures between 2°C and 4°C for up to 96 hours. Several physico-chemical analyses were carried out, namely hydrogenionicpotential, peroxide, crude fat, thiobarbituric acid reactive substances, total volatile basic nitrogen, eicosapentenoic acid, docosahexaenoic acid, total protein, dry matter and mineral material. Peroxide, thiobarbituric acid reactive substances and total volatile basic nitrogen were considered the best biomarkers for antioxidant capacity and fish quality. Was verified the reduction of polyunsaturated fatty acids in all the studied groups ($p>0.05$), showing no relation with the addition of the hydrolysate.

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INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) (Walbaum, 1792) is a commercially prominent species, with significant production rates worldwide. Most of trout and salmon consumed in Brazil are imported from Chile (Costa *et al.*, 2014). Trout production corresponds to only 23% of the salmonidae consumption in the country, according to the Associação Brasileira de Truticultores (ABRAT) (Porto-Foresti *et al.*, 2002). Trout present significant omega-3 fatty acid concentrations, commonly associated with the prevention of cardiovascular

diseases (Igafa, 2015). According to FAO (2018), in 2016 the trout participates of the main groups species in the world fish trade and fish products, representing 7.4% in quantity, together with salmon and smelts, of the 79.8% of fish. In the same year the rainbow trout stood out among the main species produced in the world aquaculture, with production of 814 t, equivalent to 2% of annual production. The prospect until 2030 is that aquaculture production will continue to expand on all continents, with an emphasis on the production of higher-value species such as shrimps, salmon and trout.

Concerning fish waste, skin and scales are collagen-rich residues which can be converted into gelatin (Zhang *et al.*, 2012). Gelatin, due to its functionalities as a microencapsulating and emulsifying agent, is widely applied in pharmaceutical, food and cosmetic industries. In addition to direct collagen and fish gelatin applications, several studies have evaluated the production and application of protein hydrolysates (PH), that may display technological and biological functions, such as cryoprotectant activity (Wang *et al.*, 2009), antioxidant activity (Ngo *et al.*, 2010; Zhang *et al.*, 2012) and antihypertensive activity (Choonpicharn *et al.*, 2015), adding value to these compounds and leading to broad applications. From the bibliographical survey related to the thematic, it were evaluated that studies in a similar matrix and products with antioxidant potential some articles showed *in vitro* trials using buffers and processed meat. The proposal of this experiment was to add the PH directly to trout fillets to observe the effects on the matrix quality parameters. PH are food additives designated as "GRAS" or "Generally Recognized as Safe" in the United States (2010). Additives are used to preserve and, in some cases, improve, the safety and freshness of food products, and they assume different roles according to their inherent characteristics. For example, some PH act as preservatives, by preventing deterioration, protecting against oxidation, controlling microbial load growth, which are often pathogenic and associated with diseases, or maintaining the color of fresh cut fruits. With the increase of population awareness concerning proper nutrition and food quality, due to growing health concerns, studies on the deleterious effects of artificial preservatives and the benefits of natural additives for food preservation are now in the spotlight, leading to more frequent studies monitoring the action of these compounds in perishable food products. Nikoo *et al.* (2014), for example, analysed the efficacy Amur sturgeon skin gelatine hydrolysates applied to crushed Japanese sea bass (*Lateolabraxjaponicus*) regarding antioxidant action and crioprotector effect, and reported that the investigated compounds exhibited sequestering activity against 2,2-diphenyl-1-picrylhydracyl (DPPH), 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and hydroxyl radicals, as well as control over lipid oxidation, confirmed by hydroperoxide measurements. Other studies in this regard include PH from capelin (*Mallotusvillosus*) applied to cooked minced meat (Shahidi*et al.*, 1995); PH from mechanically separated Anchoita meat added to sausage prepared with Anchoita (*Engraulisanchoita*) surimi (Piotrowicz, 2012), and wheat gluten, soy protein, carrageenan and chitosan edible films and coatings applied to cooked meat burgers (Wu *et al.*, 2000).

According with the results of previous studies emphasizing the functional properties of bioactive peptides from several protein sources, especially in relation to the antioxidant activity in the preservation of polyunsaturated fatty acids (PUFAs), the objective of the present study was to make a PH, with the enzymatic hydrolysis of fish gelatina, applied on frozen rainbow trout (*Oncorhynchus mykiss*) fillets, due to the high lipid content of the species, aimed to assess the effects on the physico-chemical parameters and the possible extension of the shelf life of the matrix. In the present study, the choice of rainbow trout was linked to the important role of the product in both the Brazilian and international market, as it is significantly consumed and presentes high attributed value, is very palatable and presents high nutritional quality. Its rich composition is related to the predominance of PUFAs, which are more susceptible to oxidation, consequently leading to loss of functional lipid values for the species. Another interesting aspect of this study is the possibility of reintroducing fish gelatine in the production chain, emphasizing environmental and financial concerns.

MATERIAL AND METHODS

A total of 8 kg of commercial trout fillets, in packs containing two fillets each, weighing about 350 g, from the same lote, of aquaculture from Friburgo - Rio de Janeiro, Brazil. The samples were transported frozen in an isothermal container to the State Center for Research on Food Quality (CEPQA) belonging to the Rio de Janeiro State Agricultural Research Company (PESAGRO-RJ) for physicochemical analyses. The fillets were thawed under overnight refrigeration, discarding the tail and the liquid lost in the thawing process, homogenous divided in two samples of 150 g. Randomized and distributed into three groups: control and containing PH solution at 1:10 and 1:1 ratios. The evaluated Protein Hidrolyseted (PH) was obtained by extraction described and published by Nikoo *et al.* (2014) and modified by Lima *et al.* (2018), at Biochemistry Laboratory, Center of Technology and Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). The process comprises the hydrolysis of commercial fish gelatin (número do registro no Ministério da Saúde do Brasil: 6.6660.0006), which is composed of proteins derived from different fish species by-products. The gelatin was dissolved in water at 55°C, pH 6 and hydrolysis was performed using alcalase® 2.4 (donated by Novozymes Latin America Ltda.) in a 2.0 L jacketed reactor (working volume of 1.5 L) under controlled agitation, pH and temperature. The reaction was carried out for 90 min, at constant pH through the continuous addition of NaOH. The reaction was stopped by heating the reaction medium at 85°C for 10 min, followed by cooling. The PH was added with a pipette and vacuum-packaged maintained at 2 to 4°C throughout the experiment.

The analyses were carried out in accordance to the "Official analytical methods of physico-chemical control of hake and its derivatives" manual (AOAC, 1990; 2005).Assessments were carried out on day zero (1st analysis point – T_0), when the hydrolyzed product was applied to the samples, followed by an analysis every 24-hour intervals, totaling five sampling points (T_0, T_{24}, T_{48}, T_{72} and T_{96}) in 96 hours. All analyses were performed in duplicate. Peroxide levels and pH determinations were carried out using a potentiometer (TECNAL, SP, Brazil) in a suspension obtained by mixing 10 g of trout fillet and 10 mL deionized water for 60 seconds. Peroxide results were expressed as meq O_2/kg.

Thiobarbituric acid reactive substances (TBARS) determination followed the methodology reported by Benjakul

and Bauer (2001). The trout samples (1 g) were dissolved in 9 mL HCl 0.25 meq/L containing 0.375 g/100mL of TBA (Sigma-Aldrich, St Louis, USA) and then mixed in 15 g/100mL of tricloracetic (TCA) (Merck, Darmstadt, Germany), boiled for 10 minutes, made up with deionized water and centrifuged at 3,500 rpm for 15 min (MIKRO 20, HettichZentrifugan, Germany). The optical densities of the supernatants were determined at 532 nm on a UV-160 spectrophotometer (Thermo-Waltham, MA, USA) and results were expressed as mg of malonaldehyde (MDA)/kg sample.

Total volatile basic nitrogen (TVB-N) determination is often used as a spoilage indicator for fresh seafood maintained on ice (Haaland and Njaa, 1988). Ten grams of each sample were added to a Kjeldahl flask containing magnesium oxide (2 g) (Merck KGaA, Darmstadt, Germany) and distilled water (300 mL) was added before connecting the flask to the Kjeldahl system. The transferred fluid was collected in an Erlenmeyer flask containing boric acid (2%, 25 mL) (Merck KGaA, Darmstadt, Germany) and methyl-red indicator (Merck KGaA, Darmstadt, Germany) (Hasegawa, 1987; Hollingworth andWekell, 1997). The mixture was then boiled and 25 mL of the distillate were collected and measured by titrimetry using 0.1 N H_2SO_4 (Merck KGaA, Darmstadt), which changes the solution color from green to light red. TVN values were calculated multiplying the volume of sulfuric acid used by 14, and the results were reported for 100 g of each sample (Lyman and Couch, 1953). The lipid content determinations were based on the evaluation of the ethereal extract and total and saturated lipids. A total of 10 g of each sample were inserted into a *Goldfish* type extractor, using a filter paper. Petroleum ether was used as solvent and the reaction was maintained for 4 hours at 80°C. The samples were kept in a desiccator until cooling and reaching constant weight for subsequent gravimetric determinations.

EPA (Eicosapentenoic acid) and DHA (Docosahexaenoic acid) extractions followed the method reported by Bligh and Dyer (1959), and determinate high resolution gas chromatography (GC/HRMS).The analytical method was validated according to the International Conference on Harmonization (ICH) guidelines. Linearity was determined from different concentrations (n = 3) ranging from 0.1–1.0 μM. Limits of detection (LOD) and limits of quantification (LOQ) were determined from the standard calibration curve. The Brazilian legislation standard for "vitamin and mineral supplements and similars, in the form of powder, capsules, dragees and the like"(Brazil, 2001) was chosen to carry out comparisons to the product assessed herein, being established Coliforms at 45°C, *Staphylococcus aureus* and *Salmonella* spp. analyses. However, in the present study were also performed: *Escherichia coli*, Mesophilic Aerobic Heterotrophic Bacteria (CBHAM) and Heterotrophic Aerobic Psicotrophic Bacteria Counts (CBHAP). Descriptive statistics were applied to perform an exploratory analysis.

Kruskal-Wallis non-parametric tests were applied considering a significance level of 5% ($p \leq 0.05$) for comparisons between the three experimental groups, while the Mann Whitney test at a significance level of 2% ($p \leq 0.02$) was applied for comparisons between pairs. Pearson's linear correlation coefficient was used to assess potential associations among variables, at a significance level of 5% ($p \leq 0.05$). The data were processed using the IBM Statistical Package for the Social Sciences (SPSS), version 17.0.

RESULTS AND DISCUSSION

Fish fillets were 18.99 mg/100g crude protein, 6.82 mg/100g crude fat, 72.79% moisture and 1.04% dry matter content. The value of TVB-N was 10.95 mg/ 100 g flesh. In addition, lipid change as TBARS and peroxide values were 0.62 mg MDA/kg and 0.73 meq O_2/kg (AOAC, 1990; 2005). The results of the physico-chemical characterizations and freshness evaluations of the trout fillets, ratified of the PH safety. Physico-chemical characterizations: 17.40 mg/ 100g of total protein, 0.99 of water activity, 0.09 mg/kg EPA and 0.10 mg/kg of DHA; and freshness: 7.03 pH, 21.3 mg O_2/kg peroxide, "undetected" in mg/kg non-protein nitrogen and 0.87 ml H_2S. No pathogenic microorganisms were detected in the evaluated trout fillets, which were, thus, considered microbilogically safe for consumption. Thus, the PHwas confirmed as safe before being placed in contact with the fillets. The biomarker results concerning fish quality after PH addition are displayed in Tab. 1.

Evaluation of trout fillets with the addition of hydrolysed fish: Rainbow trout fillets comprise approximately 15 g of protein and 3 g of crude fat in 100 g (Igafa, 2015). This lipid content makes this product highly vulnerable to oxidative rancidity, emphasizing the importance of the use of an additive with antioxidant action. The antioxidant activity of the produced hydrolysate was assessed by pH, peroxide, crude fat, TBARS and TVB-N analyses in the three experimental groups (Fig. 1). According to the Brazilian legislation (Brazil, 2017), adequate fresh fish quality and identity standards are observed at pH values lower than 7.0 and TVB-N values of up to 3.0 mg/kg. Decreases in pH values were detected during the assessments, although all samples remained within the established standard (Brazil, 2017). Regarding peroxide, an expressive increase was noted, ranging from 12.50 mg O_2/kg (control in T_0) to 40.50 mg O_2/kg (1:1 in T_{96}), consequently leading to decreased in fatty extracts, varying from 23.64 mg/100g (control in T_0) at the beginning of the storage period to 11.24 mg/100g (1:1 in T_{96}) at the end of the experiment.

TBARS and TVB-N, although remaining within the established standards, were both higher on the last day of experiment, confirming progressive sample deterioration (Brazil, 2017). When comparing experimental groups, no influence of PH addition in relation to pH ($p=0.388$), TBARS ($p=0.644$) and TVB-N ($p=0.949$) was observed. No statistical differences were found for peroxide ($p=0.536$), despite the difference observed between samples from the control group (34.50) and the 1:1 group (40.50) at T_{96}, and also for crude fat ($p=0.384$), whose control (23.64) and 1:1 (18.11) samples were observed at T_0.Considering non-parametric tests, no statistically significant difference ($p>0.05$) was observed between the three groups. According to the results displayed in Fig. 1, the three sample groups maintained their characteristics within the recommended limits, i.e. pH ranging from 5.97 (1:1 in T_{96}) to 7.01 (control at T_0) and TVB-N at T_0 ranging from "Undetected" (nd, LOD≤0.001) to 1.59 mg/kg (1:1 at T_{96}). Thus, the final product remained suitable for consumption after PH addition and chilled storage for 96 hours. TBARS values ranging from 1 to 2 mg MDA/kg of fish have been stipulated as the maximum permissible values by the USDA (2005). In the present study, TBAR values at T_0 ranged from "nd" to 2.66 mg MDA/kg (1:1 at T_{96}), thus surpassing the USDA limit for 72 hours of chilled storage.

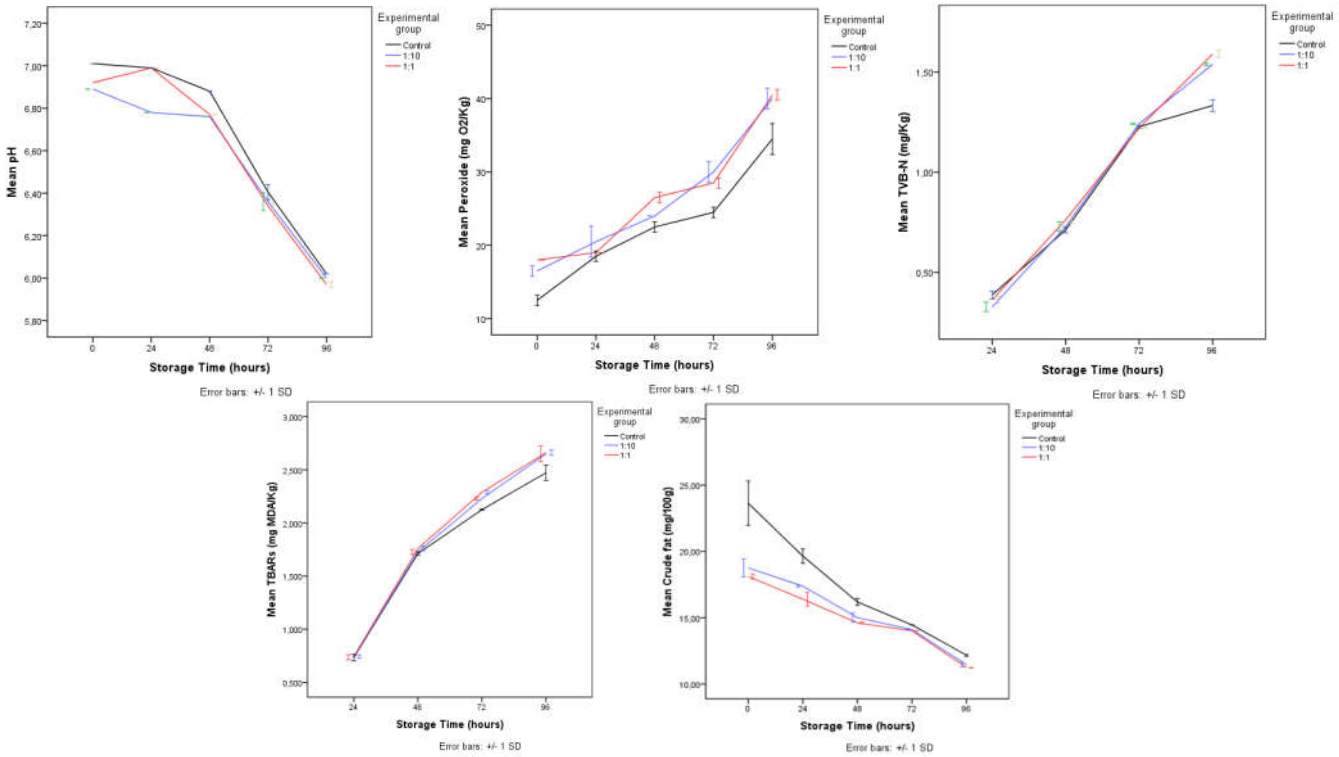

Figure 1. Alterations in the mean physico-chemical parameter values of rainbow trout fillets with and without PH addition and stored at 2 to 4°C for 96 hours: (A) pH; (B) peroxide; (C) TVB-N; (D) TBARS; (E) crude fat

Table 1. Physico-chemical parameters at T_0 (1st analysis point) (Mean± SD) concerning PH addition to rainbow trout fillets (*Oncorhynchus mykiss*).

Analyses[a,b]	Time T_0 (1st Point)[c]		
	Control	1:10	1:1
pH	7.01±0.00	6.89±0.00	6.92±0.00
TBARs	nd	nd	nd
TVB-N	nd	nd	nd
Peroxide	12.50±0.71	16.5±0.71	18.00±0.00
Crudefat	23.64±1.68	18.76±0.67	18.11±0.20
Crudeprotein	6.56±0.28	6.38±0.07	6.36±0.14
Dry matter	23.66±0.14	23.56±0.00	23.45±0.00
Mineral matter	3.28±0.09	3.44±0.01	3.39±0.08
Moisture	76.34±0.14	76.44±0.00	76.55±0.00
EPA	11.23±0.00	11.61±0.71	11.21±0.16
DHA	10.21±0.00	10.51±0.07	10.10±0.01

SD: Standard Deviation. [a]TVB-N: Total volatile basic-nitrogen, TBARS: Thiobarbituric acid reactive substances, EPA: Eicosapentaenoic acid, DHA: Docosahexaenoic acid. [b]The values are expressed in mg/kg, exception crude fat, crude protein and mineral matter (mg/100g); peroxide (mg O_2/kg) and TBARs (mg MDA/kg). [c]nd: Undetected; LOD: 0.001mg/100g; LOQ: 0.008mg/100g. Control; 1:10 and 1:1, no significant difference ($p>0.05$).

Table 2. Physico-chemical nutritional analysis parameters of rainbow trout (*Oncorhynchus mykiss*) chilled fillets according to storage time for 96 h (Mean± SD).

Time (hours)	Analyses[a]	Crudeprotein	Drymatter	Mineral matter	Moisture	EPA[b]	DHA[c]
T_0	Control	6.56±0.28	23.66±0.14	3.28±0.09	76.34±0.14	11.23±0.00	10.21±0.00
(1st Point)	1:10	6.38±0.07	23.56±0.00	3.44±0.01	76.44±0.00	11.61±0.71	10.51±0.07
	1:1	6.36±0.14	23.45±0.00	3.39±0.08	76.55±0.00	11.21±0.16	10.10±0.01
T_{24}	Control	6.44±0.00	23.29±0.23	3.34±0.16	76.72±0.23	10.23±0.16	10.01±0.01
(2st Point)	1:10	6.14±0.00	23.42±0.02	3.28±0.07	76.59±0.02	10.02±0.01	9.16±0.07
	1:1	6.33±0.01	23.12±0.00	3.22±0.01	76.88±0.00	10.06±0.06	8.96±0.06
T_{48}	Control	6.25±0.00	22.12±0.16	3.40±0.23	77.88±0.16	9.67±0.01	8.05±0.08
(3st Point)	1:10	6.23±0.00	22.12±0.00	3.28±0.09	77.88±0.00	8.84±0.21	7.01±0.01
	1:1	6.31±0.00	22.23±0.16	3.49±0.05	77.77±0.16	8.78±0.16	6.90±0.00
T_{72}	Control	6.15±0.00	22.12±0.16	3.39±0.40	77.88±0.16	9.28±0.09	8.11±0.01
(4st Point)	1:10	6.03±0.00	22.12±0.00	3.28±0.06	77.88±0.00	8.22±0.33	6.78±0.15
	1:1	6.01±0.01	22.23±0.16	3.55±0.14	77.77±0.16	8.05±0.23	6.39±0.22
T_{96}	Control	5.50±0.25	22.12±0.16	3.44±0.33	77.88±0.16	4.44±0.30	4.50±0.10
(5st Point)	1:10	5.03±0.02	22.12±0.00	3.27±0.08	77.88±0.0	2.67±0.47	2.00±0.01
	1:1	4.84±0.08	22.23±0.16	3.16±0.07	77.77±0.16	2.05±0.06	0.94±0.09

SD: Standard Deviation. [a] Crude protein and mineral matter (mg/100g), EPA and DHA values are expressed in mg/kg. [b]EPA: Eicosapentaenoicacid.

Although TBARS and TVB-N are the most indicated parameters to determine lipid oxidation, pH is the most representative product quality analysis for low lipid species. As described by Cruz-Casallas, Cruz-Casallas and Suárez-Mahecha (2014), in a study with frozen catfish meat (*Leiariusmarmoratus*), where TBA values did not exceed 0.1 mg MDA/kg, pH remained within the neutrality range, and the maximum detected value for TVB-N was of 1.53 ± 0.2 mg/kg. It is important to note that, since catfish (*Leiariusmarmoratus*) is a lean fish, lipid oxidation occurs more slowly than in rainbow trout, which contains high lipid content leading to more intense rancification. The comparison of oxidative parameters in fatty fish is only possible if the association of TBARS, TVB-N and peroxide tests is applied. According to a study carried out by Sathivel (2005), pH values were maintained within neutrality in fresh salmon fillets (*Oncorhynchusgorbuscha*) displaying different types of coatings. In addition, the coatings were effective in protecting against oxidative action during the storage period, since TBAR values in coated fillets ranged from 1.0 mg MDA/kg to 3.3 mg MDA/kg. Likewise, Tomita *et al.* (2006) reported different TBARS values in a study carried out with chilled marine fish, ranging from 0.28 to 4.27 mg MDA/kg of fish, according to the presentation form. Concerning whole fish, 41.94% of the samples presenting high TBARS levels were fatty species, due to the higher amount of PUFAS content. Nikoo *et al.* (2015) analyzed peroxide and TBARS to verify the antioxidant action of a cryoprotectant peptide derived from Amur sturgeon tetrapeptide Pro–Ala–Gly–Tyr (PAGT) gelatin applied to ground fish meat, and concluded that the byproduct used to obtain the antioxidant PAGT can be used to protect meat from the cold, since PAGT addition reduced TBARS levels when compared to controls ($p \leq 0.05$). In the present study, PH was shown to be inert, since no interference when comparing groups was observed for any of the analyzed parameters. In addition, the treated samples remained within the identity and quality standards recommended for consumption at the end of the storage period. As cited previously, pH, TVB-N and TBARS analyses are recommended for the antioxidant analyses in foods.

However, the particularities of each test must be taken into account, since pH can be influenced by the simple and immediate interaction of the matrix molecule with oxygen present in the environment, being a sensitive, but not very specific, assessment. While TVB-N, although less sensitive, may be influenced by factors indirectly related to lipid oxidation. For this reason, some authors have recently pointed to TBARS analyses as the most suitable for evaluating oxidation, suggesting greater specificity. In the present study, Pearson's correlation indicated no significant relationship between TBARS and TVB-N ($r=+0.142$; $p=0.507$) and also between peroxide and TBARS ($r=+0.059$; $p=0.785$), in constrast to that verified between peroxide and TVB-N values ($r=+0.909$; $p<0.001$). This indicates that a small variation in peroxide can be easily observed taking into account TVB-N values, since their correlation is stronger than in the case of TBARS. The low existing correlation between TBARS and TVB-N thus allows for the application of both parameters for lipid oxidation ratings. However, a global assessment indicates that, even if TVB-N can be altered by enzymatic actions and microbial metabolism, it is the most appropriate method, as recommend by Brazilian standards. Crude protein, dry matter, mineral matter and moisture parameters did not vary significantly (p-values are equal to 0.394, 0.908, 0.821, 0.908, respectively) when experimental groups were compared, all maintained within identity and quality standards (Tab. 2). Sample degradation in all groups was gradual and presented no other alteration than the action of fish fillet cell enzymes, since certain molecules capable of delaying or inhibiting enzymatic action (Erkan *et al.*, 2014) were not observed in the present study. Because it is rich in PUFAs, especially those of the omega-3 type, EPA and DHA concentrations were monitored in the rainbow trout fillets, and an abrupt reduction in the values of the two lipid fractions was observed. However, the falling was also observed in the control samples of each groups, more pronounced between T_{72} and T_{96}. No statistical difference was observed in EPA and DHA concentrations between experimental groups ($p=0.558$ and $p=0.475$, respectively), indicating that, although the hydrolyzate did not display antioxidant action preventing the degradation of the fatty compounds in question, it also did not accelerate the process. A growing number of studies concerned with additive development from a wide variety of food sources is now observed, and application to salmonids, such as rainbow trout and Atlantic salmon (*Salmo salar*), are justified by the high consumption of these species.

Conclusion

PH applied to rainbow trout fillets as an antioxidant agent did not interfere in product quality and did not prevent the degradation of crude fat. All samples were within the quality and identity standards of Brazilian and international legislation. The best biomarkers indicated for evaluation of the antioxidant activity were peroxide and TVB-N. Future studies should consider the application of concentrated PH in the glaze stage, with the purpose of evaluating the antioxidant capacity in fish.

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[1]*Abbreviations*: PH, proteinhydrolysate; TBARS, reactive substances to thiobarbituric acid; MDA, malonaldehyde; TVB-N, total volatilebasicnitrogen; EPA, Eicosapentaenoicacid; DHA, Docosahexaenoicacid; PUFAs, polyunsaturatedfattyacids

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