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AVALIAÇÃO DA HIDRÓLISE ENZIMÁTICA NA PRODUÇÃO DE HIDROLISADO PROTEICO DE PEIXE COM PROPRIEDADES BIOATIVAS E APLICAÇÃO EM MOLHO DE SALADA

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BIOGRAFIA

Thaysa Fernandes Moya Moreira nasceu na cidade de Paranavaí, Paraná. Possui graduação em Engenharia de Alimentos pela Universidade Tecnológica Federal do Paraná (UTFPR), campus Campo Mourão e mestrado em Tecnologia de Alimentos pelo Programa de Pós-Graduação em Tecnologia de Alimentos (PPGTA) da UTFPR campus Campo Mourão.

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APRESENTAÇÃO

Esta tese de doutorado está apresentada na forma de três artigos científicos.

No Capítulo 1 é apresentado o artigo de revisão bibliográfica de autoria de Thaysa Fernandes Moya Moreira, Odinei Hess Gonçalves, Fernanda Vitória Leimann e Ricardo Pereira Ribeiro intitulado “Fish protein hydrolysates: bioactive properties, encapsulation and new technologies for enhancing peptides bioavailability” submetido na revista Current Pharmaceutical Design.

No Capítulo 2 é apresentada a primeira fase do trabalho experimental de autoria de Thaysa Fernandes Moya Moreira, Luiz Gustavo Antunes Pessoa, Flavio Augusto Vicente Seixas, Rafael Porto Ineu, Odinei Hess Gonçalves, Fernanda Vitória Leimann e Ricardo Pereira Ribeiro, denominado “Chemometric evaluation of enzymatic hydrolysis in the production of fish protein hydrolysates with acetylcholinesterase inhibitory activity” publicado na revista Food Chemistry.

No Capítulo 3 são apresentados os últimos resultados obtidos no trabalho experimental de autoria de Thaysa Fernandes Moya Moreira, Anielle de Oliveira, Vanessa de Carvalho Rodrigues, Amarilis Santos de Carvalho, Ana Paula Peron, Odinei Hess Gonçalves, Angela Maria Gozzo, Fernanda Vitória Leimann e Ricardo Pereira Ribeiro, com título “Bioactive Fish Protein Hydrolysates (FPH) as antioxidant in salad dressing” submetido na revista Food Research International.

SUMÁRIO

GENERAL ABSTRACT

INTRODUCTION AND OBJECTIVES - Fish consumption is recommended for its nutritional diversity. Despite this, the fish is highly perishable, hindering processing and resulting in greater losses of raw material. Fish protein hydrolysates (FPHs) can be an alternative to reduce waste and assign value to fish by-products, featuring various bioactive activities and making essential amino acids more available in peptides. These hydrolysates are being researched in several applications as anti-inflammatory, anticancer, antimicrobial, antioxidant and enzymatic inhibitors. These bioactivities can be diversified according to hydrolysis conditions, as well as fish species and their part used as substrate. These conditions combined with adequate peptide fractionation and isolation can also potentiate some specific bioactivity. Due to the great potential of FPHs, recent work has sought to optimize their acquisition through new technologies for fish treatments, such as high-pressure processing, microwave, ultrasound and thermal treatments to achieve a better protein cleavage with lower losses. The objectives of this work were: Chapter 1 - To carry out a literature review on the important aspects in the production of FPHs and their peptides, the most explored bioactive properties and applied methods to obtain larger bioactivities and to ensure viable applications; Chapter 2 - Selecting the best enzymatic hydrolysis conditions in the production of FPHs obtained from Nile Tilapia residues to relate it to its bioactive ability to inhibit the enzyme acetylcholinesterase (AChE) as well as evaluate changes in the protein structure responsible for this property; Chapter 3 - Investigate whether the substrate pretreatments, by thermal heating by sterilization and homogenization by Ultra-turrax, could affect the antioxidant and functional properties of FPHs after enzymatic hydrolysis and, analyze the technological properties of a real food system, salad dressing, when incorporated with FPHs.

METHODS - For the review article, a survey was conducted to evaluate the publications related to FPH (production, bioactive activities, encapsulation methods, technologies applied to substrates) published from 1940 to 2022, in which only peer-reviewed articles published in journals from January 2015 to 2022 were included. For the experimental article described in Chapter 2, the byproducts of Nile Tilapia were first homogenized: viscera, carcass, skin and bones and evaluated their centesimal composition. Then the by-products were solubilized in distilled water (1:2, p:v), adjusted pH (7,2-8,8), added the alcalase enzyme (0,53-1,9, [E:S] % v/p), adjusted to temperature (42-58; °C) and the solution kept under agitation by 120 min, according to the experimental planning (DCCR, with complete planning 23, 6 axial trials and 3 repetitions at the central point, totaling 17 trials), in which, the response variables were the hydrolysis yield, hydrolysis degree (DH) and AchE inhibition (20, 30, 50 mg.mL-1; %). To evaluate the relationship between the FPHs characteristics when obtained under different experimental conditions, Principal Component Analysis (PCA) was performed. The selected FPHs were characterized by Fourier transform spectroscopy (FTIR) and reverse phase HPLC amino acid profile analysis. The AChE activity and inhibition kinetics assay was performed in three FPH concentrations (20, 30 and 50 $mg.mL^{-1}$) using electric eel (*Electrophorus electricus*) as a source of enzyme (expressed in enzymatic activity compared to control). Finally, a molecular Docking assay was performed to evaluate a possible interaction site of the

FPHs responsible for enzyme inhibition of AChE. For the experimental article described in Chapter 3, were homogenized Nile Tilapia muscles submitted to three pretreatments: Control (C, without treatment before hydrolysis), Sterilization (EST, thermal treatment in autoclave at 120 ºC for 15 min) and Ulta-turrax (UT, ultra-turrax homogenization at 20,000 rpm for 15 min). After pretreatment the substrates were submitted to enzymatic hydrolysis. For this, the samples were solubilized in distilled water (1:2 w/v), the pH was adjusted to 7.5 with NaOH 1 M or 0.1 M HCl, added alcalase enzyme (0.8 % v/p [E:S]) and, the temperature adjusted to 55 ºC. Hydrolysis was maintained by agitation for 120 min. FPHs were characterized by degree of hydrolysis (DH), yield, free amino acids, antioxidant ability (FRAP, DPPH and ABTS analyses), acetylconilesterase inhibition activity (AChE), solubility, emulsifying ability, Fourier transform spectroscopy (FTIR), cytotoxicity and genotoxicity. FPHs were applied in salad dressing to evaluate its action as an emulsifier and antioxidant for 0, 15 and 30 days. The analyses evaluated in the sauce were rheological behavior, texture analysis, color and oxidative stability.

RESULTS, DISCUSSIONS AND CONCLUSIONS - From the review of the literature, carried out in the review article (Chapter 1), it can be verified that the bioactive properties most found in FPHs were those with antioxidant, antimicrobial, anticancer and antihypertensive properties. These bioactivities are dependent on the conditions of hydrolysis, fish species and, fractionation and isolation of specific peptides. New technologies for treating by-products can reduce process losses and achieve better results by cleaving proteins. On the other hand, encapsulation and film application techniques can increase bioactivity, bioavailability, and control release when applied to food, resulting in improved health. In Chapter 2, hydrolysis adjusted to 55 °C, pH 7.5 and enzyme concentration of 0.8% (Enzyme:Substrate) was selected by Principal Component Analysis (PCA) because it has greater inhibition potential than other experiments. Molecular characteristics have shown that higher temperatures possibly result in wider amide A bands. The results of enzyme kinetic inhibition of acetylcholinesterase (AChE) demonstrated mixed type inhibition behavior of FPH. The amino acid profile was similar for all the FPHs evaluated, however, the FPH with higher Ache inhibition showed a higher amount of total and essential amino acids. In addition, according to the Docking Molecular analysis, it has been observed that arginine is the most likely amino acid to bind to Ache, demonstrating that basic amino acids can be a key factor for this bioactivity. In Chapter 3, an extensive characterization of the functional and bioactive properties of FPHs was performed, comprising antioxidant activity (DPPH, ABTS and FRAP), acetylcholinesterase inhibition and emulsifying properties. The degree of hydrolysis (DH) obtained was 37.9, 37.66 and 40.55 % for the Control (C), EST and UT samples, respectively. Treatment with UT resulted in a sample with less radical removal ability. Ache inhibition was evaluated at three concentrations (15, 45 and 60 $mg.mL^{-1}$) demonstrating to be a potential property of FPH. The cytotoxic assays in *Allium cepa* L. showed that toxicity to FPHs is not expected. As proof of concept, the FPHs were used as an emulsifying/antioxidant agent to prepare a salad dressing. The emulsifying activity index (EAI) and the emulsifying stability index (ESI) of FPH indicated better emulsifying capacity and stability in basic pH, probably due to the hydrophobic character of proteins. The FPH provided an increase in protein content, pseudoplastic behavior, characteristic color and texture. In addition, the FPHs helped in the oxidative stability of salad dressing, demonstrating potential application in emulsified foods, acting in the elimination of radicals generated in lipid oxidation. Finally, in general, we can conclude that FPHs have AChE inhibition properties, and this potential may be related to the binding to basic amino

acids, mainly arginine. We can also verify the antioxidant and emulsifying potential of FPHs and its ability to assist in the oxidative stability of emulsified foods such as salad dressing.

Key-words: Fish protein hydrolysates (FPHs); Ache inhibition; pretreatment; antioxidant property; emulsifying property.

RESUMO GERAL

INTRODUÇÃO E OBJETIVOS – O consumo de peixe é recomendado pela sua diversidade nutricional. Apesar disso, o peixe é altamente perecível, dificultando a transformação e resultando em maiores perdas de matéria-prima. Os hidrolisados de proteína de peixe (FPHs) podem ser uma alternativa para reduzir o desperdício e atribuir valor aos subprodutos de peixe, apresentando várias atividades bioativas e tornando os aminoácidos essenciais mais disponíveis em peptídeos. Estes hidrolisados estão sendo pesquisados em diversas aplicações como anti-inflamatórios, anticancerígenos, antimicrobianos, antioxidantes e inibidores enzimáticos. Estas bioatividades podem ser diversificadas de acordo com as condições de hidrólise, bem como as espécies de peixes e sua parte utilizada como substrato. Estas condições combinadas com o fracionamento e isolamento adequados do peptídeo também podem potencializar alguma bioatividade específica. Devido ao grande potencial dos FPHs, trabalhos recentes têm procurado otimizar sua aquisição através de novas tecnologias para tratamentos de peixes, como processamento de alta pressão, microondas, ultrassom e tratamentos térmicos para alcançar uma melhor clivagem de proteínas com menores perdas. Com isto, os objetivos deste trabalho foram: Capítulo 1 – Realizar uma revisão da literatura sobre os aspectos importantes na produção de FPHs e seus peptídeos, as propriedades bioativas mais exploradas e métodos aplicados para obter maiores bioatividades e garantir aplicações viáveis; Capítulo 2 – Selecionar as melhores condições de hidrólise enzimática na produção de FPHs obtidos de resíduos de Tilápia do Nilo para relacioná-la à sua capacidade bioativa de inibir a enzima acetilcolinesterase (AChE), bem como avaliar alterações na estrutura proteica responsável por essa propriedade; Capítulo 3 – Investigar se os pré-tratamentos do substrato, por aquecimento térmico por esterilização e homogeneização por Ultra-turrax, poderiam afetar as propriedades antioxidantes e funcionais dos FPHs após a hidrólise enzimática e, analisar as propriedades tecnológicas de um sistema alimentar real, o molho de salada, quando incorporado com FPHs.

METODOLOGIA – Para o artigo de revisão, foi realizada uma pesquisa para avaliar as publicações relacionadas a FPH (produção, atividades bioativas, métodos de encapsulamento, tecnologias aplicadas a substratos) publicado de 1940 a 2022, na qual, apenas artigos revisados por pares publicados em periódicos de janeiro de 2015 a 2022 foram incluídos. Para o artigo experimental descrito no Capítulo 2, primeiramente foram homogeneizados os subprodutos de Tilápia do Nilo: vísceras, carcaça, pele e ossos e avaliada sua composição centesimal. Em seguida os subprodutos foram solubilizados em água destilada (1:2, p:v), o pH ajustado (7,2-8,8), adicionada a enzima alcalase (0,53-1,9, [E:S] % v/p), ajustada a temperatura (42-58 °C) e a solução mantida sob agitação por 120 min, conforme o planejamento experimental (DCCR, com planejamento completo 2^3, 6 ensaios axiais e 3 repetições no ponto central, totalizando 17 ensaios), na qual, as variáveis respostas foram o rendimento da hidrólise, grau de hidrólise (DH) e inibição de AChE (20, 30, 50 $mg.mL^{-1}$; %). Para avaliar a relação entre as características dos FPHs quando obtidos em diferentes condições experimentais foi realizada Análise de Componentes Principais (PCA). Os FPHs selecionados foram caracterizados por análise de espectroscopia no infravermelho por transformada de Fourier (FTIR) e Perfil de aminoácidos por HPLC em fase reversa. O ensaio de atividade e cinética de inibição de AChE foi realizada em três concentrações de FPH (20, 30 e 50 $mg.mL^{-1}$) utilizando enguia elétrica (*Electrophorus electricus)* como fonte de enzima (expresso em atividade

enzimática em comparação com o controle). Por fim, foi realizado ensaio de Docking molecular para avaliar um possível local de interação dos FPHs responsável pela inibição enzimática de AChE. Para o artigo experimental descrito no Capítulo 3, foram homogeneizados músculos de Tilápia do Nilo submetido à três pré-tratamentos: Controle (C, sem tratamento antes da hidrólise), Esterilização (EST, tratamento térmico em autoclave à 120 ºC por 15 min) e Ulta-turrax (UT, homogeneização em Ultra-turrax à 20,000 rpm por 15 min). Após os pré-tratamentos os substratos foram submetidos à hidrólise enzimática. Para isto, as amostras foram solubilizadas em água destilada (1:2 p/v), o pH foi ajustado a 7,5 com NaOH 1 M ou HCl 0,1 M, adicionada enzima alcalase (0,8 % v/p [E:S]) e, a temperatura ajustada a 55 ºC. A hidrólise foi mantida por agitação por 120 min. Os FPHs foram caracterizados quanto ao grau de hidrólise (DH), rendimento, aminoácidos livres, capacidade antioxidante (análises de FRAP, DPPH e ABTS), atividade de inibição de acetilconilesterase (AChE), solubilidade, capacidade emulsificante, análise de espectroscopia no infravermelho por transformada de Fourier (FTIR), citotoxicidade e genotoxicidade. Os FPHs foram aplicados em molho de salada para avaliar sua ação como emulsificante e antioxidante por 0, 15 e 30 dias. As análises avaliadas no molho foram comportamento reológico, análise de textura, cor e estabilidade oxidativa.

RESULTADOS, DISCUSSÕES E CONCLUSÕES – A partir da revisão da literatura, realizada no artigo de revisão (Capítulo 1), pode se verificar que as propriedades bioativas mais encontradas em FPHs foram as com propriedades antioxidantes, antimicrobianas, anticâncer e anti-hipertensiva. Estas bioatividades são dependentes das condições de hidrólise, espécie de peixe e, fracionamento e isolamento de peptídeos específicos. Novas tecnologias para o tratamento de subprodutos podem reduzir as perdas no processo e alcançar melhores resultados por clivagem das proteínas. Por outro lado, técnicas de encapsulação e aplicação em filmes podem aumentar a bioatividade, biodisponibilidade e, controlar a liberação quando aplicado em alimentos, resultando em melhoria da saúde. No Capítulo 2, a hidrólise ajustada a 55 °C, pH 7,5 e concentração de enzima de 0,8 % (Enzima:Substrato), foi selecionada por Análise de Componente Principal (PCA) por apresentar maior potencial de inibição que os demais experimentos. As características moleculares mostraram que as maiores temperaturas possivelmente resultam em bandas de amida A mais amplas. Os resultados de inibição cinética enzimática de acetilcolinesterase (AChE) demonstraram um comportamento de inibição tipo mista dos FPH. O perfil de aminoácidos foi similar para todos os FPHs avaliados, no entanto, o FPH com maior inibição de AChE apresentou maior quantidade de aminoácidos totais e essenciais. Além disso, de acordo com a análise de Docking Molecular, foi observado que a arginina é o aminoácido mais provável de ligar-se à AChE, demonstrando que os aminoácidos básicos podem ser um fator chave para esta bioatividade. No Capítulo 3 foi realizada extensa caracterização das propriedades funcionais e bioativas das FPHs, compreendendo atividade antioxidante (DPPH, ABTS e FRAP), inibição da acetilcolinesterase e propriedades emulsificantes. O grau de hidrólise (DH) obtido foi de 37,9, 37,66 e 40,55% para as amostras Controle (C), EST e UT, respectivamente. O tratamento com UT resultou em uma amostra com menor capacidade de remoção de radicais. A inibição de AChE foi avaliada em três concentrações (15, 45 e 60 $mg.mL^{-1}$) demonstrando ser uma propriedade potencial de FPH. Os ensaios citotóxicos em *Allium cepa* L. mostraram que não se espera toxicidade para as FPHs. Como prova de conceito, as FPHs foram usadas como agente emulsificante/antioxidante para preparar um molho para salada. O índice de atividade emulsionante (EAI) e o índice de estabilidade emulsionante (ESI) de FPH indicaram melhor capacidade emulsionante e estabilidade em

pH básico, provavelmente devido ao caráter hidrofóbico das proteínas. A FPH proporcionou um aumento no conteúdo de proteínas, comportamento pseudoplástico, cor característica e textura. Além disso, as FPHs auxiliaram na estabilidade oxidativa do molho de salada, demonstrando potencial de aplicação em alimentos emulsionados, atuando na eliminação de radicais gerados na oxidação lipídica. Por fim, de modo geral, podemos concluir que as FPHs apresentam propriedades de inibição de AChE, podendo este potencial estar relacionado à ligação com aminoácidos básicos, principalmente a arginina. Também podemos verificar o potencial antioxidante e emulsificante de FPHs e sua capacidade de auxiliar na estabilidade oxidativa de alimentos emulsionados como molho de salada.

Palavras-chave: hidrolisados de proteína de peixe (FPHs); inibição de AChE; pré-tratamento; propriedade antioxidante; propriedade emulsificante.

CAPÍTULO 1

FISH PROTEIN HYDROLYSATES: BIOACTIVE PROPERTIES, ENCAPSULATION AND NEW TECHNOLOGIES FOR ENHANCING PEPTIDES BIOAVAILABILITY

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ABSTRACT

Fish consumption is recommended for its nutritional diversity. Despite this, fish are highly perishable, making processing difficult and resulting in greater losses of raw material. Fish protein hydrolysates (FPHs) can be an alternative to reduce waste and assign value to fish by-products by presenting several bioactive activities and making essential amino acids more available in peptides. This review addresses the production of FPHs and the main bioactive activities found recently and emphasises the methods of encapsulation, application in films, and treatment of substrates by high-pressure processing, microwave, ultrasound, and thermal treatments to achieve better bioactivities. The bioactive properties most found in FPHs were antioxidants, antimicrobials, anticancer, and antihypertensive. These bioactivities may vary depending on the conditions of hydrolysis, fish species, and fractionation and isolation of specific peptides. New technologies for the treatment of by-products can reduce process losses and achieve better results by cleavage of proteins. Conversely, encapsulation and film utilisation can improve bioactivity, bioavailability, and controlled release when applied to foods, resulting in improved health.

Keywords: protein hydrolysates, bioactive compounds, bioactive peptides, hydrophobic amino acids, encapsulation, new technologies.

1 INTRODUCTION

The aquaculture sector is on the rise, with expectations of stable growth in global fish production annually. In 2019, production totalled 177.8 million tonnes. In contrast to aquaculture, wild catches showed an estimated 3.4% decrease, reinforcing the value of captive production [1]. Fish consumption is recommended for its diverse nutritional benefits that make it an alternative protein in a healthy diet [2]. This is because fish are composed of fatty acids, minerals, proteins, and vitamins [3]. Fish proteins have high nutritional value because they are rich in essential amino acids [4].

Aiming to reduce waste and attribute value to sub-products, several studies have focused on the production of fish protein hydrolysates (FPHs) and their peptides [5]. FPH are products of the enzymatic conversion of native proteins into small peptides, usually containing 2–20 amino acid residues. With hydrolysis, essential amino acids become more available in peptides, so FPHs can exhibit bioactive properties and act on various physiological functions of the human body [6,7]. Among the bioactive properties most frequently identified are antioxidants [8–10], anticancer [11,12], antimicrobial [13–15] and antihypertensive properties [16–18].

In this perspective, fish-derived bioactive peptides have great potential for application as functional foods and are consumed in foods and beverages, as well as in the form of tablets, capsules, powders, and liquids [19]. Substrate treatment technologies, encapsulation, and the application of hydrolysates and their peptides in bioactive films are being employed to masking the bitter taste, reduction of hygroscopicity, as well as to increase their bioavailability and stability [20–22].

Therefore, this review article addressed important aspects in the production of FPHs and their peptides, the most explored bioactive properties, and methods applied to obtain increased bioactivities and guarantee viable applications.

2 LITERATURE SEARCH

A search on the SCOPUS Database Platform [23] (www.scopus.com) was used to evaluate the number of publications related to FPHs (production, bioactive activities, encapsulation methods, technologies applied to substrates) published from 1940 to 2021. The search considered all types of documents (article, review, book, book chapter, conference article, conference review, letter, editorial, note, brief research, business article, erratum and data paper) and was conducted using three different sets of keywords, exploring the title, abstract and keywords of published works. The results of this research are shown in Figure 1, where a total of 1634 publications on FPHs as the main approach, with considerable growth in the number of publications since 2015. Among the researched subjects, Figure 1 (B) shows that the terms “bioactive” and “FPHs” has increased in recent years, reaching 110 publications. The same relevance can be emphasised in terms of encapsulation techniques and new technologies related to FPHs, reaching 97 publications since 2015. In addition, terms that relate encapsulation methods and new technologies aimed at improving the bioactive properties of FPHs can be highlighted with 72 publications as of 2015 and therefore have also been addressed. Only peer-reviewed articles published in English-language peer-reviewed journals from January 2015 to 2022 were included.

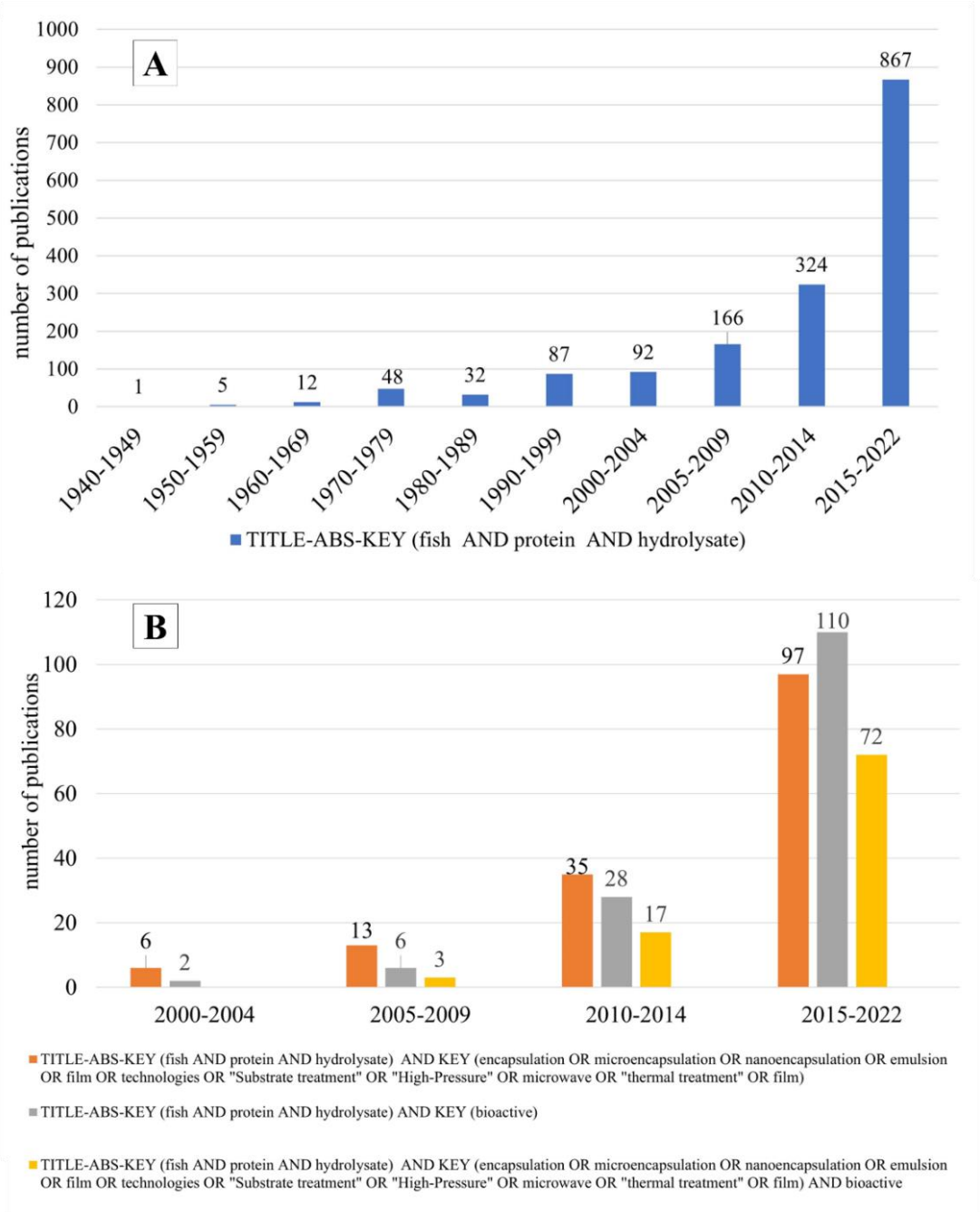

Figure 1. Number of published works about FPHs (A), FPH AND bioactive; FPH AND encapsulation OR microencapsulation OR nanoencapsulation OR emulsion OR film OR technologies OR "substrate treatment" OR "High-Pressure" OR microwave OR "thermal treatment" OR film; fish protein hydrolysate AND encapsulation OR microencapsulation OR nanoencapsulation OR emulsion OR film OR technologies OR "substrate treatment" OR "High-Pressure" OR microwave OR "thermal treatment" OR film AND bioactive (B), published from 1940 to 2021 according to SCOPUS Database Platform [23].

3 PRODUCTION OF FPH

The production of FPHs can be performed from various substrates obtained from fish, the most common being muscle, skin and by-products [5]. The sub-products such as viscera, head, trimmings and frames, have the advantage of having greater added value to the parts of the fish that would be discarded industrially as waste, which has been highlighted in several studies [9,24,25].

The chemical hydrolysis (acid or basic substances) or enzymatic hydrolysis (proteolytic enzymes or microbial fermentation) are used for FPH production, being the enzymatic hydrolysis the most employed, for its better performance and facilities in reaction control to obtain reproducible bioactive protein hydrolysates [26]. Based on the recent literature, the main procedures for enzymatic hydrolysis, fractionation, and identification of bioactive peptides in FPHs are illustrated in Figure 2 [5,27–29].

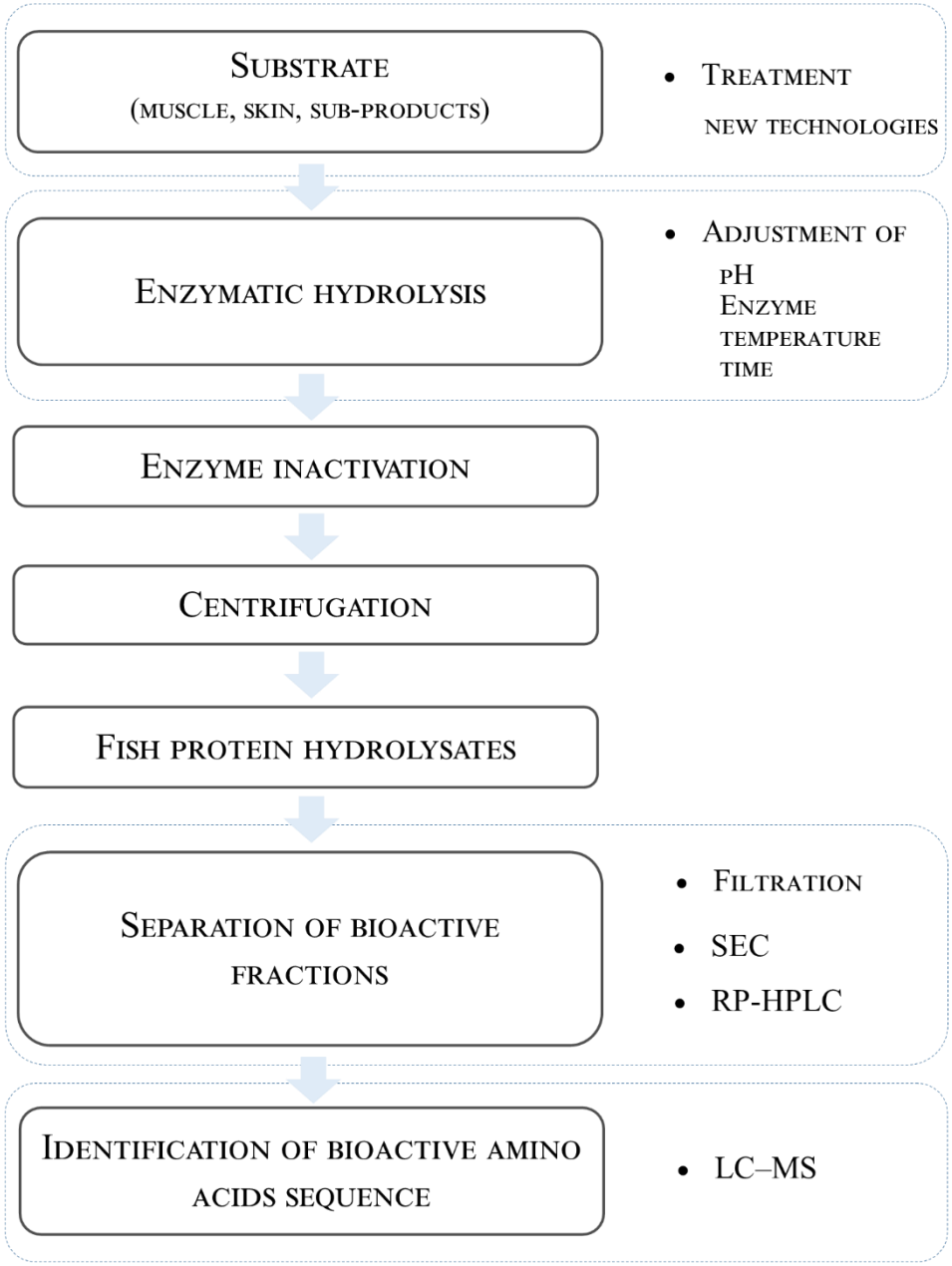

Figure 2. Production steps of bioactive protein hydrolysates from fish by an enzymatic process, fractionation, and identification.

The enzymes generally cited in the works are: alcalase 2.4 L, neutrase HT, neutrase PF, alcalase PAL, Protamex, papain, bromelain, ficin, novo-proD, thermolysin and flavorzymes [12,14,30–34]. Other determining parameters in the production of FPH by enzymatic hydrolysis are pH, temperature and hydrolysis time [6,7,35].

4 BIOACTIVE PROPERTIES

An overview of the bioactive properties and peptides found in FPH described in recent studies is presented in Table 1. Each bioactivity is described in detail on the subsequent topic.

Table 1. Fish species, substrate used in FPH production, bioactive activity in FPH, and identified bioactive peptide.

Fish species	Substrate	Bioactive activity	Identified bioactive peptide	Reference
Salmon (*Salmo salar*)	By-products (skin and trimmings)	Antidiabetic	Gln-Met, Gln-Ser, Leu-Gln, Gln-Leu, Gly-Gly-Leu-Gln, Gly-Pro-Ser-Gln, Gln-Pro-Pro-Glu, Gln-Glu, Glu-Gln, Gln-Ser, Phe-Gln, Ala-Leu-Gln and Glu-Leu-Gln	Harnedy et al. [25]
Salmon (*Salmo salar*)	By-product (trimmings)	Antihypertensive (ACE inhibitor), antidiabetic (peptidase IV inhibitor) and antioxidant	Ile/Leu-His, Phe-Phe, Gly-Pro-Ala-Val, Val-Pro, Val-Cys, Tyr-Pro, Phe-Phe, Pro-Pro, Asp-Pro, Ile/Leu-Asp and Ile/Leu-His	Neves et al. [36]
Bighead carp *(Hypophthalmichthys nobilis)*	Gill	Cryoprotective and antioxidant	n.e.*	Lin et al. [37]
Carp *(Cyprinus carpio)*	Skin gelatin	Antioxidant	n.e.*	Tkaczewska et al. [38]
Eel *(Anguilla marmorata)*	Muscle	Antioxidant	n.e.*	Cheng et al. [10]
Eel *(Monopterus sp.)*	Muscle	Antioxidant and human breast cancer (MCF-7) cell line inhibition	n.e.*	Halim et al. [11]
Argentine croaker *(Umbrina canosai)*	Muscle	Anti-inflammatory, antioxidant, and antimicrobial	n.e.*	Da Rocha et al. [14]
Miiuy croaker *(Miichthys miiuy)*	Swim bladder	Antioxidant	FYKWP, FTGMD, GFEPY, YLPYA, FPPYERRQ, GFYAA, FSGLR, FPYLRH, VPDDD and GIEWA	Zhao et al. [28]

Blue whiting *(Micromesistius poutassou)*	Muscle	Antidiabetic	Ala-Glu-Arg-Glu, Glu-Gln-Glu-Glu, Ser-Gln, Glu-Glu, Glu-Ala, Glu-Ala-Glu, Glu-Ser-Ala, Ala-Glu-Ala -Glu, Glu-Ser-Leu/Ile-Lys and Glu-Ala-Val	Harnedy et al. [39]
Tuna *(Katsuwonus pelamis)*	Muscle	Anti-hyperucemic and xanthine oxidase inhibitory properties	FH, FK, FR, FW, LR, LW, VW, YH, ALW, FVR, GVW, LFW, LGW and Allopurinol	He et al. [40]
Raibow trout *(Oncorhynchus mykiss)*	By-product (frames)	Antioxidant	Asp-Gly-Arg-Leu-Gly-Tyr-Ser-Glu-Gly-Val-Met or Gly-Asp-Arg-Leu-Gly-Tyr-Ser-Trp-Asp-Asp (1182.65 Da); Iso-Arg-Gly-Pro-Glu-Glu-His-Met-Arg or Arg-Val-Ala-Pro-Glu-Glu-His-Met-Arg (1261.77 Da) and Ser-Ala-Gly-Val-Pro-Arg-His-Lys or Ser-Ala-Arg-Pro-Arg-His-Lys (962.63 Da)	Ketnawa; Wickramathilaka; Liceaga [41]
Zebra blenny *(Salaria basilisca)*	Muscle	Antioxidant, antidiadetic, and anticoagulant	n.e.*	Ktari et al. [42]
Atlantic mackerel *(Scomber scombrus)*	Muscle	Antimicrobial	KVEIVAINDPFIDL, LILLILLLLKLLLLLI, LLILLLLKLLLLLI and LLILLLLLLILLLILLPF	Offret et al. [13]
Red tilapia *(Oreochromis spp.)*	By-product (viscera)	Antioxidant and cytoprotective	n.e.*	Gómez et al. [12]
Tilapia (*Oreochromis niloticus*)	By-products (head, tail and fins)	Antioxidant	n.e.*	Hemker et al. [43]
Tilapia *(Oreochromis niloticus)*	Skin gelatin	Antihypertensive (ACE inhibitor)	Leu-Ser-Gly-Tyr-Gly-Pro (592.26 Da)	Chen et al. [16]

Tilapia *(Oreochromis niloticus)*	Skin gelatin	Antihypertensive (ACE inhibitor)	VGLPNSR (741.4133 Da) and QAGLSPVR (826.4661 Da)	Ling; Liping; Yongliang [44]
Stripped weakfish (*Cynoscion guatucupa*)	Muscle	Antimicrobial and antioxidant	IELIEKPMGIF (1288.71 Da) and RADLSRELEEISERL (1814.95 Da)	Lima et al. [15]
Pacific hake *(Merluccius productus)*	Muscle	Cryoprotection	n.e.*	Jenkelunas; Li-Chan [45]
Smooth-hound (*M. mustelus*)	By-product (viscera)	Antihypertensive (ACE inhibitor)	IAGPPGSAGPAG, VVPFEGAV, PLPKRE and PTVPKRPSPT	Abdelhedi et al. [46]
Stone fish (Actinopyga lecanora)	Muscle	Antihypertensive (ACE inhibitor)	Ala-Leu-Gly-Pro-Gln-Phe-Tyr (794.44 Da), Lys-Val-Pro-Pro-Lys-Ala (638.88 Da), Leu-Ala-Pro-Pro-Thr-Met (628.85 Da), Glu-Val-Leu-Ile-Gln (600.77 Da) and Glu-His-Pro-Val-Leu (593.74 Da)	Auwal et al. [32]
Sea cucumber (*Acaudina molpadioidea*)	Muscle	Antihypertensive (ACE inhibitor)	PNVA and PNLG	Li et al. [47]
Marine Cobia *(Rachycentron canadum)*	Skin	Antihypertensive (ACE inhibitor)	Trp-Ala-Ala, Ala-Trp-Trp, Ile-Trp-Trp and Trp-Leu	Lin et al. [48]
Sardine *(S. pilchardus)*	Muscle	Antihypertensive (ACE inhibitor) and antioxidant	n.e.*	Rivero-Pino; Espejo-Carpio; Guadix [22]
Kawakawa *(Euthynnus affinis)*	Muscle	Antihypertensive (ACE inhibitor) and antioxidant	n.e.*	Taheri; Bakhshizadeh [49]
Turbot *(Scophthalmus maximus)*	By-products (head, trimmings, frames and viscera)	Antihypertensive (ACE inhibitor) and antioxidant	n.e.*	Vázquez et al. [9]

Blue-spotted stingray *(D. kuhlii)*	Muscle	Antioxidant	Trp-Ala-Phe-Ala-Pro-Ala (661.3224 Da) and Met-Tyr-Pro-Gly-Leu-Ala (650.3098 Da)	Wong et al. [50]
Flathead (*Platycephalus fuscus*)	By-products (heads, backbones and frames)	Antioxidant and anticancer	Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg	Nurdiani et al. [51]
Rainbow trout (*Oncorhynchus mykiss*)	By-product (skin)	Antioxidant and anticancer	n.e.*	Yaghoubzadeh et al. [52]
Atlantic mackerel *(Scomber scombrus)*	Muscle	Antimicrobial	KVEIVAINDPFIDL	Offret et al. [13]
Yellowfin tuna (*Thunnus albacores)*	By-product (viscera)	Antimicrobial	n.e.*	Pezeshk et al. [53]
Nile Tilapia (*Oreochromis niloticus*)	By-products (bones, carcass and fins)	Acetylcholinesterase inhibition	n.e.*	Moreira et al. [54]
Rainbow trout (*Oncorhynchus mykiss*)	By-product (skin)	Human colon cancer (HCT-116) cell line inhibition	n.e.*	Yaghoubzadeh et al. [52]
Atlantic cod (*Gadus morhua*)	Salt-cured cod skin	Antihypertensive (ACE inhibitor) and antioxidant	n.e.*	Coscueta et al. [55]
Atlantic codfish (*Gadus morhua*)	By-product (frames)	Antihypertensive (ACE inhibitor) and antioxidant	n.e.*	Rodrigues et al. [56]
Whitemouth croaker *(Micropogonias furnieri)* and Banded croaker (*Paralonchurus brasiliensis*)	Muscle and skin	Antioxidant	n.e.*	Camargo et al. [8]

Gurnard (*Trigla* spp.), atlantic horse mackerel (*Thachurus trachurus*), blue whiting (*Micromesistius poutassou*), red scorpionfish (*Scorpaena scrofa*), pouting (*Trisoreptus luscus*) and fourspot megrim (*Lepidorhombus boscii*)	Whole fish and By-products (heads, skins and bones)	Antihypertensive (ACE inhibitor),antioxidant, α-amylase and α-glucosidase inhibitions	n.e.*	Henriques et al. [57]
Mesopelagic fish (*Maurolicus muelleri* and *Meganyctiphanes norvegica*)	Whole fish	Acetylcholinesterase, ACE (antihypertensive) and dipeptidyl peptidase IV (antidiabetic) inhibitions	n.e.*	Naik et al. [58]

n.e.* not evaluated

SHR ** Spontaneously hypertensive rats

The diversity of bioactivity found in FPH is due to several factors such as fish or its residue, type of hydrolysis used, type of enzyme (in the case of enzymatic hydrolysis), degree of hydrolysis, molecular weight, amino acid sequence, hydrophobicity, peptide loading [6,59].

Antimicrobial peptides are generally positively charged and have less than 50 amino acid residues, of which almost 50% are hydrophobic and amphipathic [60]. The main mechanism of microbial action is membrane permeabilization, but mechanisms such as membrane destabilization, inhibition of macromolecular synthesis, peptide translocation and inhibition of DNA/RNA/protein synthesis have also been identified [61,62].

Cancer is a disease resulting from abnormal growth and proliferation of cells in the body. Under normal conditions, apoptosis constantly maintains the balance between proliferating cells and programmed cell death. With an imbalance resulting from cancer, mutations occur in DNA, and normal cells are transformed into cancer cells [63]. Bioactive nutrients may be allied to alternative therapies for cancer prevention and control, with the main advantage of reducing unwanted side effects [64].

According to Chalamaiah [63] the enzymatic hydrolysis of food proteins is the most frequently used process to obtain bioactive hydrolysates, and among the enzymes, pepsin has been shown to be one of the most efficient in the production of anticancer peptides. Since pepsin hydrolyzes mainly the peptide bonds (preferentially those containing hydrophobic amino acids, especially aromatic amino acid residues such as phenylalanine, tryptophan and tyrosine), there is the release of bioactive hydrophobic peptides, which are hidden in the inner core of the parent proteins. These hydrolysates and peptides are capable of inhibiting the growth of cancer cell lines, inducing apoptosis and inhibiting cell cycle.

Hypertension is a disease caused by high blood pressure, and about 45% of adults have been diagnosed in the United States. This disease can be promoted by a diet rich in salt, sugars, and fats. When left untreated, hypertension becomes a risk factor for the increase of cardiovascular and renal diseases [65].

Most studies that evaluated the antihypertensive action of FPH, analyzed its efficacy through the inhibition of the angiotensin converting enzyme (ACE), which acts in the renin-angiotensin system (RAS) [48,49]. ACE converts Angiotensin-I into Angiotensin-II, which is responsible for increasing blood pressure. Therefore, the function of ACE inhibitors is to decrease the formation of Angiotensin-II [66]. Several

synthetic drugs are used to treat hypertension by ingesting ACE inhibitors, but there are limitations that can result in symptoms ranging from mild to severe, making the search for natural substances essential [67].

The anti-hypertensive activity of FPHs depends mainly on the chain length, molecular weight, and molecular interaction of peptides [68]. Studies have mentioned the use of several fish species and their by-products in the evaluation of ACE inhibition, and tilapia *(Oreochromis niloticus)* is a substrate widely used by the authors due to the large volume of residues with a high nutritional value [16,17,69]. ACE inhibitors bind to the catalytic site at the C-terminal, but only hydrophobic inhibitors bind and block the catalytic site of the N-terminal. Therefore, most peptides (and new peptides and isolated fractions [16,32]) obtained from ACE-inhibiting FPHs have hydrophobic amino acids at their end [46,70,71]. In addition, the two terminals (C and N) have an active site binding to the zinc cofactor, allowing ACE to be inhibited by metal chelating agents [72].

Recently, antioxidant properties have been among the most evaluated bioactive properties in FPH and its peptides [9,10,42]. Antioxidants are compounds that have the potential to prevent, intercept, or repair the formation of free radicals and their metabolites, known as reactive oxygen species (ROS). ROS excess is responsible for oxidative stress, which causes the modification of lipids, proteins and DNA in cells and can contribute to the development of several chronic diseases such as Parkinson's, Alzheimer's, Huntington's disease, amyotrophic lateral sclerosis, cardiovascular and inflammatory diseases, diabetes, cataracts and cancer [73,74].

In foods, the antioxidant activity of peptides is related to the chemical and physical properties of their composition and, therefore, are higher than those of their free amino acids [75]. However, the antioxidant activity of hydrolysates depends on their molecular weight, composition and amino acid sequence [76]. Smaller peptides (2 to 10 amino acid residues) show greater potential for radical elimination because they are more accessible to the active radicals of the reactions than the native proteins [77]. In addition, in these peptides, hydrophobic amino acids and one or more residues of histidine, proline, methionine, cysteine, tyrosine, tryptophan and phenylalanine can significantly improve antioxidant activities and help access hydrophobic targets. The sequence of hydrophobic amino acids in peptides can cause them to interact with lipid molecules and donate protons to lipid-derived radicals. In addition to these, other aromatic and charged amino acids have also played a role in the antioxidant efficiency of peptides [6,77].

5 STRATEGIES USED FOR ENHANCING PEPTIDES BIOAVAILABILITY

5.1 *Substrate treatment methods for hydrolysis*

New methods and technologies are being used to treat fish before hydrolysis is performed with the aim of obtaining hydrolysates and their peptides with higher bioactive properties. Generally, these higher properties are due to the methods providing greater structural changes due to the ability to bind enzymes to the protein [78].

Hemker et al. [43] used various combinations of retention time (6–35 min) and high-pressure processing (HPP) (38–462 MPa) during the hydrolysis of tilapia by-product. The results were promising, with the highest antioxidant activity observed using 250 MPa for 20 min with a higher IC50 (304 μg.mL^{-1}) than the control (653 μg. mL^{-1}). The pressure aided in the hydrolysis and cleavage of proteins, thus increasing the ability of FPH to interact and donate electrons to the ferric ion.

Ketnawa, Wickramathilaka and Liceaga [41] evaluated the changes on degree of hydrolysis, antioxidant activity, molecular weight, and amino acid composition between undigested and after gastrointestinal digestion of peptides produced by two different microwave treatments (1- microwave assisted hydrolysis (55°C for 2 min); 2- microwave pre-treatment (90ºC for 5 min), followed by conventional hydrolysis in a water bath (55°C for 4 min)). Both treatments resulted in peptides with increased antioxidant activity after gastrointestinal digestion. However, the authors found that the microwave pre-treatment enhanced gastrointestinal hydrolysis of initial protein hydrolysate, when compared to the sample hydrolyzed by the microwave assisted method.

Li et al. [79] evaluated the effect of the following treatments on the structural and hydrolysis properties of golden threadfin bream (*Nemipterus virgatus*) myofibrillar proteins: microwave (100, 200, and 300 W), ultrasound (100, 200, 300, and 400 W) and combined microwave (100 W) and ultrasound (100, 200, 300, and 400 W). The peptides produced by the combined method demonstrated better bioactive properties (antioxidant, anti-inflammatory, and ACE inhibitor), being the most effective association, 100 W for microwave and 300 W for ultrasound treatments, produced bioactive peptides that allowed the protection of cellular apoptosis induced by ROS. This was due to the moderate microwave field-oriented structural modifications that suppressed the excessive denaturation and reaggregation of sonicated myofibrillar proteins.

Korczek et al. [80] evaluated the thermal treatments of boiling (85–90°C for 10–15 min), roasting (200°C for 20 min), frying (150–180°C) in mackerel fillets (*Scomber scombrus*) and sterilised canned products on the antioxidant and antihypertensive properties of protein hydrolysates. The frying treatment provided the best hydrolysates with better biological properties. For the antioxidant properties, the highest activity of hydrolysates treated by frying may be the result of lipidation or Maillard reactions between proteins and reducing sugars, in which free radicals are captured and stable intermediate radicals with antioxidant properties are formed. This antioxidant activity was lower in boiling because of the loss in the aqueous solution of endogenous compounds and the thermoxidation of muscle components.

In contrast to the results of Korczek et al. [80], Rivero-Pino, Espejo-Carpio and Guadix [22] observed that heat treatment by boiling (90 and 100°C for 1 h) caused higher ACE inhibition activity compared to the treatment by sterilisation (121°C for 20 min) in sardine hydrolysates (*S. pilchardus*). However, the authors also attributed this bioactivity to Maillard compounds.

Rivero-Pino, Espejo-Carpio and Guadix [22] also evaluated the effect of high pressure processing (from 50 to 1200 bar) and ultrasound (500 W with variations up to 15 min). The samples treated with 800 and 1200 bar by high pressure homogenization showed the best inhibition of ACE, possibly due to the conformational changes of the peptides during the high-pressure processing, which allowed the exposure of larger hydrophobic groups in comparison to the control. As well as the high-pressure treatment, the ultrasound also increased surface hydrophobicity and, consequently, obtained better ACE inhibition.

5.2 *Encapsulation*

Despite the high potential for treating various diseases, biologically active hydrolysates and peptides present some aspects that need to be improved for a viable application, such as low bioavailability and biostability, physicochemical incompatibility, high degree of bitterness and high hygroscopicity. These characteristics can hinder the storage, stability, and application of these compounds in functional foods and food supplements [81].

For these reasons, the encapsulation of FPHs and peptides have been studied recently mainly to protect these compounds against inadequate environmental conditions,

improving biocompatibility and bioavailability, as well as providing controlled release [82,83]. The purposes of encapsulating FPH described in the literature can be observed in Figure 3.

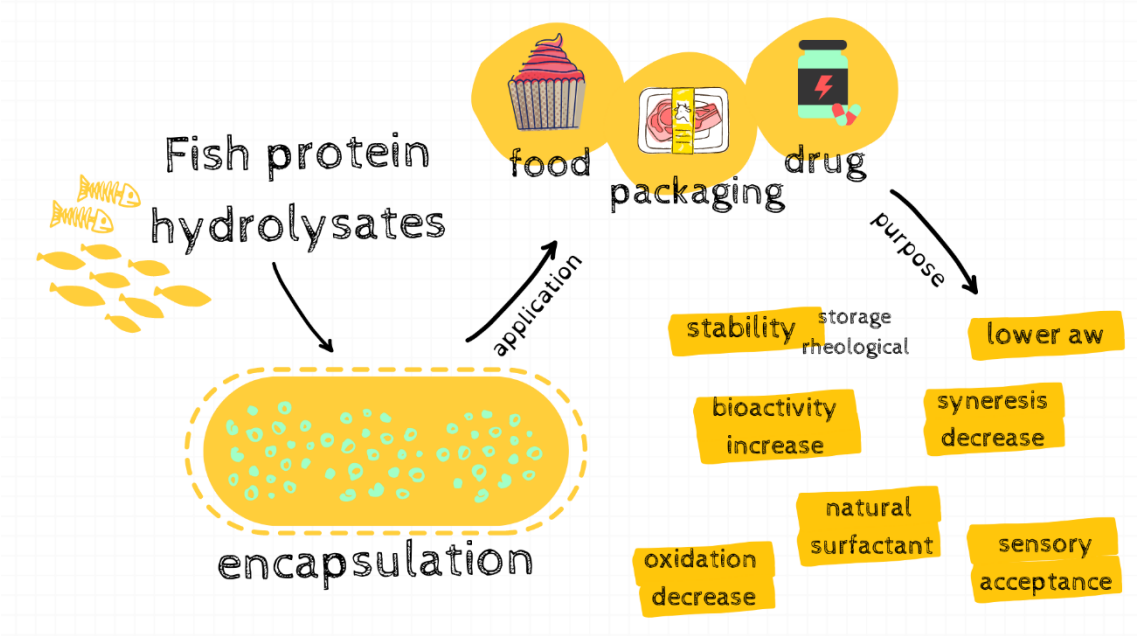

Figure 3. Applications and purposes of FPH encapsulation.

To meet these requirements, the choice of encapsulation method is a key factor because there is great difficulty in choosing materials that allow efficient encapsulation [84].

The methods evaluated for encapsulating FPHs, as described in Table 2, where spray-drying, electrospinning, lipid film hydration, thin film hydration, ionic gelation, double emulsions, hydrothermal method, freeze-dried empty liposome, and film hydration.

Table 2. Fish species, hydrolysate evaluated, bioactive or functional activity, encapsulation technique, and encapsulating materials used to encapsulate fish hydrolysates.

Fish species	Hydrolysate	Bioactive/functional activity	Encapsulation technique	Encapsulating materials	Reference
Whitecheek shark (*Carcharhinus dussumieri*)	Fish protein hydrolysates	Antioxidant	Spray-drying	-	Alinejad et al. [85]
Common kilka (*Clupeonella cultriventris caspia*)	Fish protein hydrolysates	Antioxidant	Electrospinning	Chitosan and Polyvinyl Alcohol (PVA)	Hosseini; Nahvi; Zandi [20]
Common kilka (*Clupeonella cultriventris caspia*)	Fish protein hydrolysates	Antioxidant	Ionic gelation	Tripolyphosphate cross linked chitosan	Hosseini; Soleimani and Nikkhah [86]
Bigeye Ilisha (*Ilisha megaloptera*)	Fish protein hydrolysates	Oxidative stability in yogurt	Double emulsions (W_1/O and $W_1/O/W_2$)	Complexes of whey protein concentrate with inulin and fucoidan	Jamshidi et al. [87]
Bigeye Ilisha (*Ilisha megaloptera*)	Fish protein hydrolysates	Oxidative stability	Double emulsions (W_1/O and $W_1/O/W_2$)	Gum, maltodextrin, whey protein concentrates and inulin	Jamshidi et al. [21]

Tilapia (*Oreochromis* spp.)	Fish collagen	Skin regeneration and antibacterial	Electrospinning	Triblock copolymer of polyethylene oxide–polypropylene oxide–polyethylene oxide, tetraethyl orthosilicate, $Ca(NO_3)_2 \cdot 4H_2O$, triethyl phosphate and HCl	Zhou et al. [88]
Anchovy (*Setipinna taty*)	Half-fin anchovy hydrolysates	Antibacterial	Hydrothermal method	Zinc acetate	Song et al. [89]
Rainbow trout (*Onchorhynchus mykiss*)	Fish gelatin hydrolisate fractions	Antioxidant	Freeze-dried empty liposome	1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol	Hosseini; Ramezanzade and Nikkhah [90]
Rainbow trout (*Oncorhynchus mykiss*)	Skin gelatin hydrolysates fractions	Antioxidant	Freeze-dried empty liposome	Chitosan-coated	Ramezanzade; Hosseini and Nikkhah [91]
Carp (*Cyprinus carpio*)	Carp skin gelatin hydrolysate	Antioxidant *in vivo*	Solid dispersion	Furcellaran (FUR) and glycerin	Tkaczewska et al. [92]
Sardine (*S. pilchardus*)	Fish protein hydrolysate	Antioxidant and ACE inhibition	Spray-drying	-	Rivero-Pino; Espejo-Carpio; Guadix [22]
Giant squid (*Dosidicus gigas*)	Squid collagen hydrolysate	Antioxidant	Film hydration	Partially purified phosphatidylcholine	Marín et al. [93]

Mussel (*Perna perna*)	Mussel protein hydrolysate	Food flavoring agent	Spray-drying	-	Breternitz et al. [94]
Goby Fish (*Zosterisessor ophiocephalus*)	Fish protein hydrolysate	Antioxidant	Ionic gelation	Chitosan	Nasri et al. [95]
Smooth hound (*Mustelus mustelus*)	Fish protein hydrolysate	Prolyl endopeptidase inhibition, Angiotensin-I-converting enzyme inhibition	Emulsification/ internal gelation	Alginate-whey protein isolate	Lajmi et al. [96]
Phytophagous carp (*Hypophthalmichthys molitrix*)	Fish protein hydrolysate fraction	Bioactive non cytotoxic	Coacervation	Chitosan and inulin	Grigore-Gurgu et al. [97]
Yellowfin tuna (*Thunnus albacares*)	Fish protein hydrolysate	Antioxidant	Spray drying	Maltodextrin, gum Arabic and sodium caseinate	Unnikrishnan et al. [98]
Tilapia (*Oreochromis* spp.)	viscera hydrolysate	Antioxidant	Sonication	Soy lecithin and rapeseed lecithin	Sepúlveda et al. [99]
Shrimp (*Penaeus notialis*)	Shrimp peptide fractions	Antioxidant, dipeptidyl-peptidase-IV and ACE inhibition	Film hydration	Soy phosphatidylcholine nanoliposomes	Montero et al. [100]
White shrimp (*Litopenaeus vannamei*)	Shrimp peptide fractions	Antioxidant and antihypertensive	Film hydration	Soy phosphatidylcholine nanoliposomes	Latorres et al. [82]

Whitemouth croaker (*Micropogonias furnieri*) and Banded croaker (*Paralonchurus brasiliensis*)	Fish protein hydrolysate	Antioxidant	Spray drying	Maltodextrin	Camargo et al. [101]
Tilapia (*Oreochromis* spp.)	Gelatin hydrolysate	Bioaccessibility of Co-enzyme Q_{10} and emulsifying agent	Spray drying	-	Cao et al. [102]
Stripped weakfish (*Cynoscion guatucupa*)	Fish protein hydrolysate	Antioxidant, ACE inhibition and bioactivity in nematoid *C. elegans*	Spray drying	Maltodextrin	Lima et al. [103]
whiteleg shrimp (*Litopenaeus vannamei*)	Shrimp waste hydrolysate	Improve fish (rainbow trout) growth performance and resistance against bacterial infections	Ionic gelation and thin film hydration	Chitosan and soy lecithin	Rashidian et al. [104]
Asian seabass (*Lates calcarifer*)	Gelatin skins hydrolysate	Antioxidant	Thin film hydration	Soy phosphatidylcholine	Chotphruethipong et al. [105]
Common kilka (*Clupeonella cultriventris caspia*)	peptide fraction	Antioxidant	Freeze-dried empty liposome	Chitosan cross-linked with sodium tripolyphosphate	Ramezanzade et al. [83]

n.e.* not evaluated

The materials used in encapsulation also contribute to the protection of bioactive properties. Lima et al. [103] observed that maltodextrin used as an encapsulant in the spray drying method can act to protect peptides against thermal damage and Maillad reaction products. Camargo et al. [101] found that pea protein can act as a protector of bioactive FPHs against high temperature during the spray drying microencapsulation process. Pea protein probably also helps in the exposure of the hydrophobic amino acids of FPHs, consequently increasing their antioxidant potential.

Despite this, it is necessary to carefully investigate the type of material used and the effects of its interaction with the peptide, since recent studies have shown a negative relationship between some of these components.

Chotphruethipong et al. [105] point to the flocculation of liposomes containing FPHs and aggregation in the bilayers of loaded peptides as the cause of the decrease of antioxidant activity of these particles during storage. This decrease in antioxidant capacity occurred due to the oxidation of lipids from unsaturated fatty acids of phospholipid bilayers from liposomes, resulting in modifications in the encapsulated structure and lower release of bioactive peptides.

Tkaczewska et al. [92] also observed that the microencapsulation of carp skin gelatin hydrolysate in furcellaran reduced the antioxidant activity of hydrolysates by DPPH after *in vitro* digestion. Thus, for the animal studies, the authors incorporated more microcapsules to the diet of the animals from the microcapsule group (1.5%) compared to the group fed with pure hydrolysate (1%). Even under this condition there was a reduction on antioxidant activity after *in vivo* digestion for the encapsulated peptides. The authors presumed that this behavior may be associated with the low digestibility of furcellaran in the small intestine of the tested animals, thus, the hydrolysates were not released from the microcapsules.

In the study developed by Hosseini, Nahvi and Zandi [20], the result was positive when incorporating three fractions of Common kilka antioxidant hydrolysates according to molecular weight (10–30 kDa, 3–10 kDa, <3 kDa) in electrospun nanofibers composed by chitosan and poly(vinyl alcohol).The incorporation of the peptides made the produced material more hydrophobic, reducing water vapor permeation and increasing the contact angle with water, as well as increasing material's tensile strength (3-fold higher). The method was efficient for encapsulating the peptides (> 94% efficiency) and when evaluating their release kinetics in a hydrophilic medium, it occurred in a prolonged way, demonstrating the potential application in food packaging. In addition, due to the need for

a longer release time, electrospun nanofibers presented lower antioxidant activity than pure fractions.

Besides being used as encapsulated bioactive components, FPH is also being studied as encapsulating material for fish and corn oils. The results so far have demonstrated great potential of FPH to act as alternative agents for the food and pharmaceutical industry, because they are natural antioxidants and improve the oxidative stability of the oily microcapsules during storage [106–108].

In emulsions, protein hydrolysates have helped to avoid lipid oxidation. This stability is reached mainly by the action of hydrophilic and hydrophobic amino acids, that are located at emulsion's interface acting as antioxidants and natural surfactants as well [109].

The application of FPHs in food matrices with emulsion characteristics, as in yogurts, has shown good results. Jamshidi et al. [87], for instance, encapsulated FPH and fish oil in microcapsules by double emulsion ($W_1/O/W_2$) using Whey Protein Concentrate (WPC) with inulin and fucoidan as wall material complexes and evaluated its effect on yogurts. The results were positive, demonstrating that the presence of fucoidan helped oxidative stability and nutritional quality. In addition, the microcapsule produced with WPC and inulin masked the taste of fish in yoghurts, demonstrating the potential for application as a functional ingredient of fortified products.

In the work developed by Lima et al. [110], the application of encapsulated FPH (with maltodextrin) in yogurt provided greater storage stability with lower values of syneresis and increased cohesiveness, in addition to providing greater sensory acceptance by masking the taste of fish hydrolysate.

The use of High Pressure Homogenisation to aid in the formation of double emulsions ($W_1/O/W_2$) of FPH and encapsulated fish oil has also proven to be an interesting alternative. Jamishidi et al. [21] found that microcapsules submitted to the treatment with high pressure presented higher yield, total oil, encapsulated oil, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and emulsion stability in relation to emulsion without this treatment. This improvement in microcapsules can be attributed to the fact that homogenization at high emulsion pressure decreases and standardizes the size of oil droplets, breaking their aggregates, and spreading them evenly.

5.3 *Bioactive Films*

FPH has been evaluated as a renewable and biodegradable alternative compound for incorporation into bioactive films. Although this application enables better bioactive properties, it can also result in physical and mechanical property modifications of the films [111].

Lima et al. [112] developed films based on chitosan, xanthan gum, and FPH. The addition of FPH was able to increase the antioxidant activity of films, but high concentrations tend to increase humidity and decrease tensile strength. Hydrophobicity and mechanical properties also decreased in gelatin films enriched with cuttlefish protein hydrolysates in the study conducted by Kchau et al. [113]. Despite this, hydrolysates attributed greater antioxidant and UV barrier activities to films, suggesting their application as an active packaging against food oxidation.

Silver carp FPH was evaluated by Rostami et al. [114] as a possible fish gelatin film plasticizer with antioxidant properties. The application of FPH in the films resulted in greater antioxidant activity, elongation at break, color difference, water vapor permeability, and opacity. However, as in the works cited above, the mechanical properties of tensile strength, elastic modulus, and contact angle decreased considerably.

Da Rocha et al. [115] incorporated FPHs from Argentine croaker into an agar film for application in sole fillets. FPH provided to films increased water solubility, water vapor permeability, elongation at break, yellowing, and antimicrobial activity. In sole fillets, the films helped to prolong the shelf life by improving biochemical and microbiological parameters during storage.

6 FOOD APPLICATION AND HEALTH EFFECTS

Studies have shown, by animal models and study in humans, the health effects of FPH consumption [116]. Among these, there are effects such as control of body composition [117] and improvement in vascular functions. In the latter effect, Benomar et al. [118] and Nasri et al. [119] observed a reduction of hyperlipidemia in rats, while Oliveira et al. [120] observed an improvement in flow-mediated dilation and in parameters of desaturation and restocking of muscle O_2 in people at high risk of cardiovascular disease.

In addition, to the possibility of directly assisting in the reduction of cardiovascular diseases, FPH has also been applied to fried foods, which are related to hypertension and obesity, as they act in reducing the fat content of these foods [121–123].

FPH were also added in other foods to perform different functions, e.g., in mayonnaise as a fortifying and stabilizing agent [124], in biscuit as a fortifying agent [125], gluten-free noodles with antioxidant agent [126], natural antioxidants for meat preservation [127] and in fish soup and apple juice as antioxidant and antihypertensive agents [128].

7 CONCLUSION

Several studies have evaluated the production of FPHs with bioactive capacity. This bioactivity can be diversified according to the hydrolysis conditions, as well as the fish species and its part used as a substrate. These conditions combined with the appropriate fractionation and isolation of the peptide can also lead to specific bioactivity, with the antioxidant property being the most evaluated. Generally, peptides of lower molecular masses, of smaller peptide sequences, and with hydrophobic character at the C and N terminals, have greater bioactive properties. Due to the great potential of FPHs, recent works have sought to optimise their acquisition through new technologies for fish treatments, such as high-pressure processing, microwave, ultrasound, and thermal treatments for achieving better cleavage of proteins with lower losses. Encapsulation and use in films have also been approached to improve biocompatibility and bioavailability and provide controlled release, enabling application in foods with health effects.

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CAPÍTULO 2

CHEMOMETRIC EVALUATION OF ENZYMATIC HYDROLYSIS IN THE PRODUCTION OF FISH PROTEIN HYDROLYSATES WITH ACETYLCHOLINESTERASE INHIBITORY ACTIVITY

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ABSTRACT

Fish protein hydrolysates (FPH) obtained from industrial processing residues are sources of bioactive peptides. The enzymatic hydrolysis process is essential in obtaining specific bioactivities such as inhibition of the enzyme acetylcholinesterase (AChE). In this study the effect of different hydrolysis conditions on the properties of FPH to inhibit the enzyme acetylcholinesterase. A chemometric evaluation, based on a central composite rotatable design and principal component analysis, was applied to select hydrolysis conditions with best yield, degree of hydrolysis and acetylcholinesterase inhibition. Experimental design results for AChE inhibition were between 10.51 - 40.45% (20, 30 and 50 $mg.mL^{-1}$ of FPH), and three hydrolysis conditions were selected based on PCA evaluation. The amino acids profile, FTIR and AChE inhibition kinetics were evaluated. Results showed a mixed type of inhibition behavior and, the docking molecular analyzes suggest that the inhibition AChE occurred due to the basic amino acids, mainly by arginine.

Key-words: Fish protein hydrolysates; enzymatic hydrolysis; experimental design; acetylcholinesterase.

1 INTRODUCTION

The 2019 estimate for global fish production is an increasingly stable growth, totaling 177.8 million tons. Despite this, among this scenario, the aquaculture sector stands out for presenting an increase of around 3.9% when producing in nurseries, mainly, salmon, tilapia and pangasius species (Food and Agriculture Organization [FAO], 2020). With this increase in processing, concerns about the generation and reuse of waste are also intensified. In aquaculture farms, the by-products generated in the filleting process, such as heads, trimmings, frames and viscera, are considered the new sources of waste and constitute about 60 - 70% of the weight of live fish (Ananey-Obiri & Tahergorabi, 2018; Vázquez et al., 2020). According to Food and Agriculture Organization (FAO, 2016), the decrease in post-catch losses could add 15 million tons of fish to the food chain.

An alternative to reduce these losses may be the re-use of filleting by-products for the production of high aggregate value products such as fish protein hydrolysates (FPH), which represent peptides and short chain amino acids resulting from partial hydrolysis (Chalamaiah et al., 2012). FPH are known as the most important sources of bioactive proteins and peptides, showing potential in several studies for presenting antioxidant activities (Hemker et al., 2020; Wong et al., 2019); antihypertensive (Vázquez et al., 2020); angiotensin converting enzyme (ACE) inhibitory agent (Nasir & Sarbon, 2019); anti-inflammatory (Ahn et al., 2015) and antimicrobial (Lima et al., 2019).

There has been an effort in the last decades to find compounds from natural sources that could act as acetylcholinesterase (AChE) inhibitors. The main reason is that there is evidence that the damage to the cholinergic system is closely related to brain dysfunctions such as Parkinson's disease (Grella Miranda et al., 2020; Prasasty et al., 2018; Ventura et al., 2010). This relationship is recognized for Alzheimer's disease (AD), in which the loss of cholinergic function contributes to the decrease in cognitive activity associated with AD (Tan et al., 2018). To our knowledge, FPH was not yet evaluated for obtained from by-products and little explored for food-derived peptides is the inhibitory activity of acetylcholinesterase. The possibility of obtaining low cost, low toxicity, natural substances that could act in the inhibitory mechanism of acetylcholinesterase could be of great importance to the food industry.

The objective of this study was to select the best conditions for enzymatic hydrolysis to relate it to its bioactive capacity to inhibit the acetylcholinesterase enzyme, as well as evaluating changes in the protein structure responsible for this property.

2 MATERIAL AND METHODS

2.1 *Materials*

Nile Tilapia (*Oreochromis niloticus*) waste (bones, carcass and fins) were obtained in a local market in 2019 (Campo Mourão, state of Paraná, Brazil). The enzymatic hydrolysis was started using alcalase 2.4 L enzyme (≥ 2.4 U/g, P4860, Sigma-Aldrich). The concentration of soluble proteins was determined by bovine albumin standard curve (A7030, Sigma-Aldrich). The reagents used for the acetylcholinesterase (AChE) activity were trihydroxymethyl aminomethane (Tris-HCl, Dinâmica), acetylcholinesterase enzyme from *Electrophorus electricus* (electric eel, Sigma-Aldrich), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB, 98%, Sigma-Aldrich), acetylthiocholine iodide (ASCh) (Sigma-Aldrich, 99%), for the preparation of potassium phosphate buffer (TFK) were used monobasic potassium phosphate (Dinâmica) and dibasic potassium phosphate (Neon). To identify and quantify the aminoacids (Asp, Glu, Ser, Gly, His, Arg, Thr, Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Phe and Lys) were used an external standard (Standard H, Pierce, P/N 20088), and an internal standard (alpha-aminobutyric acid - Aldrich, Milwawkee-USA).

2.2 *Treatment of Nile Tilapia waste*

Fish waste (bones, carcass and fins) were separated from the carcass, ground, homogenized and dried at 180 °C in a convection oven (Cienlab) for one hour to remove the excess of water. After this period, the waste was stored at -80 °C until its use for the production of fish protein hydrolysates (FPH).

2.3 *Proximate composition of Nile Tilapia waste*

After drying as described above, the proximate composition of the fish waste was carried out as described by Association of Official Agricultural Chemists (AOAC, 2005). The gravimetric method was used to determine the moisture at 105 ºC until obtaining

constant weight. In order to evaluate the ash content, the fish waste was incinerated in muffle at 550 °C. The lipids were determined by Bligh & Dyer method and the protein content was obtained by MicroKjeldahl method with correction nitrogen-to-protein factor of 6.25.

2.4 *Obtaining of fish protein hydolysates (FPH)*

The production of protein hydrolysates was performed according to Alvares et al. (2018), with some adaptations. Briefly, fish waste was partially thawed at 10 °C for 8 h, solubilized in distilled water (1:2, wt:v), the pH adjusted with NaOH 1M or HCl 0.1M (7.2 – 8.8), after that the alcalase enzyme was added (0.53 – 1.9, [E:S] % v/wt), the temperature was adjusted (42 – 58 °C) and the mixture was kept under agitation for 120 min, as per experimental design. The enzymatic reaction was ended by heating the mixture at 90 °C for 15 min. After that, the mixture was cooled and centrifuged at 6,000 rpm (4,850 xg) for 20 min. The supernatant was collected and filtered with a 0.45 μm cellulose acetate membrane filter (11106-47-N, Sartorius) with the aid of a vacuum pump. The filtrate FPH was frozen in ultra-freezer (-80 °C) for 24 h and freeze-dried.

2.5 *Degree of hydrolysis (DH)*

The FPH samples degree of hydrolysis (DH) was determined by modifying the methodologies described by Hoyle and Merrltt (1994) and Baek and Cadwallader (1995). After hydrolysis, 6 mL were removed from the hydrolysates and inactivated with 4 mL trichloroacetic acid (TCA) 6.25%, followed by rest for 15 min and centrifuged at 6,000 rpm for 20 min. The concentration of soluble and proteins was determined by the modified Lowry method (Lowry et al., 1951). For this, a bovine albumin standard curve ($y = 16.541x + 0.0238$; $R^2 = 0.9915$) was obtained to compare absorbance readings at 750 nm that were performed in UV-Vis spectrophotometer (Ocean Optics USB650UV, USA). The degree of hydrolysis (GH) was calculated by Equation 1.

$$DH(\%) = \left(\frac{\text{6.25\% TCA soluble protein in the sample (mg)}}{\text{total protein in the sample (mg)}}\right) x100\% \quad (1)$$

Total protein (29.01%) was determined by the MicroKjeldahl method (AOAC, 2005) and the conversion factor used was 6.25.

2.6 *Yield*

After the thermal inactivation of the enzymatic hydrolysis samples were cooled at room temperature. The samples were weighed and then fractionated into a centrifuge under conditions of 6,000 rpm for 20 min, in which the aqueous fraction (containing the protein hydrolysates) was transferred, weighed, and stored at -80 ºC. The yield was calculated according to Equation 2.

$$\text{Yield } (\%) = \frac{\text{aqueous fraction weight } (g)}{\text{hydrolyzed weight before fractionation } (g)} x100\% \quad (2)$$

2.7 *AChE activity assay*

The AChE activity of FPH was measured as described by Ellman et al. (1961). The analysis was performed in duplicate using in the reaction medium: 90 µL of potassium phosphate buffer (TFK, 50 mM, pH 7.5), 45 µL of water, 15 µL of the enzyme from electric eel (1.25 $U.mL^{-1}$ in Tris-HCl buffer (20 mM, pH 7.5)) and 10 µL of FPH (at final concentrations of 20, 30, and 50 $mg.mL^{-1}$) in addition to control (without addition of FPH). The medium was incubated at 25 °C for 10 minutes and then 20 µL of DTNB (2 mM) and 20 µL of acetylthiocholine iodide (ASCh, 0.8 mM) were added to AChE test in the dark. Reading was performed every minute (for 4 minutes) in a plate reader (Thermo-Plate Reader) at a wavelength of 405 nm. The experiment was performed in quadruplicate. The inhibition rate was determined using Equation (3), where 13.6 is the molar extinction coefficient, 0.01 is the volume of FPH solution and Δ absorbance is the absorbance variation per minute. The enzymatic activity was expressed in percentage of activity relative to the control group (100 %).

$$Reaction\ rate\ \left(\frac{mol}{L.h}\right) = \frac{\Delta absorbance}{13.6 \times 0.01} \quad (3)$$

2.8 *Experimental design*

The FPH were obtained according to the experimental conditions determined by a central composite rotatable design (CCRD) generated by the software Statistica 7.1 (StatSoft Incorporation, Tulsa, OK). The objective was the evaluate the influence of experimental conditions used to obtain the FPH on its inhibitory action and other properties. Seventeen experimental points including 8 factorial, 6 axial and 3 replicates at the central point (Table 1) were used. The selected dependent variables (X_1, X_2 and X_3) were:

X_1 = Temperature (T, °C)

X_2 = pH

X_3 = Enzyme, proportion to the substrate (E, [E:S] %)

The analytical range was determined based on preliminary experiments and literature reports (Halim et al., 2016). The responses evalua,ted from the proposed experimental design were:

Y_1 = yield (Y, %)

Y_2 = degree of hydrolysis (DH, %)

Y_3 = AChE activity inhibition (AChE 20 $mg.mL^{-1}$, %)

Y_4 = AChE activity inhibition (AChE 30 $mg.mL^{-1}$, %)

Y_5 = AChE activity inhibition (AChE 50 $mg.mL^{-1}$, %)

2.9 *Principal component analysis (PCA)*

A principal component analysis (PCA) was performed using MATLAB R2008b (MathWorks Inc., Natick, MA) in order to explore the relation between the FPH characteristics when obtained under different experimental conditions. The results obtained for Yield (Y), degree of hydrolysis (DH) and inhibition of AChE activity (AChE 20 $mg.mL^{-1}$, AChE 30 $mg.mL^{-1}$ and AChE 50 $mg.mL^{-1}$) were placed in columns and the experimental runs were used as rows. Before analysis, each column was mean centered and divided by its variance, resulting in a scaled matrix. The first principal components with eigenvalues higher than 1.0 were used to evaluate the samples distribution in the new projection space.

2.9.1 *FPH characterization*

For the determination of the molecular characteristics of FPH samples, spectra were collected with an Infrared Spectrophotometer with Fourier Transform (IR AFFINITY-1, Shimadzu), in the range of 4000 to 600 cm^{-1}, using 32 accumulations and 4 cm^{-1} resolutions. The samples were previously conditioned in a desiccator containing anhydrous calcium chloride ($CaCl_2$) for 7 days to remove moisture and spectra bands were normalized for spectrum comparison.

The method for amino acid analysis was based on White et al. (1986) and Hagen et al. (1989). The samples were subjected to acid hydrolysis and pre-column derivatization with phenyl isothiocyanate (PITC). The separation of amino acids was performed by reverse phase liquid chromatography (SHIMADZU Corporation, Tokyo, Japan) equipped with C18 reverse phase column (50 ºC) (LUNA C18, 100 Å, 5 µm, 250 x 4.6 mm, Phenomenex Inc., Torrance, USA), with DAD detector at 254 nm. The mobile phase used was a gradient composed by (A) Sodium acetate buffer 94 % (0.036 M, pH 6.4) + 5.7 % acetonitrile and (B) Acetonitrile 40 %. Gradient time (45 minutes): In the concentration of the mobile phase A and time (min) respectively (95%, 0-5), (77.5%, 5-10), (68%, 10-12), (40%, 12-20), (0%, 20-36), (95%, 36-45), and flow rate of 1 mL min^{-1}, volume injected: 50 µL.

2.10 *AChE Reaction kinetics*

Kinetics parameter determination was carried out using five acetylthiocholine iodide final concentrations (0.8, 0.6, 0.4, 0.2, 0.1, and 0 mM) and three concentrations (20 $mg.mL^{-1}$, 30 $mg.mL^{-1}$ and 50 $mg.mL^{-1}$) from the FPH assays (2, 12 and 14). The inhibition constant of the enzyme substrate inhibitor complex (Ki) was obtained through the Lineweaver- Burk methodology using Prism GraphPad 5.0 software.

2.11 *Molecular docking studies*

Molecular docking was carried out in order to give insight on the possible interaction site responsible for the enzyme inhibition. The crystallographic structure of choline-linked acetylcholinesterase (pdbid: 2ha3) with 2.25 Å resolution was chosen for

docking studies. The library with the three-dimensional structure of amino acids present in the FPH was obtained from the *.sdf. The programs and protocols used in the docking simulations were defined by redocking the choline ligand (pubchem cid: 305). The protocols were considered validated when the mean square root of the distance (rmsd) from the overlapping choline ligand in the crystallographic complex was less than 2.0 Å. The program Autodock-4.2.3 (Morris et al., 2009) used the graphical interface Pyrx-0.9.8 (Dallakyan & Olson, 2015) and had as protocol the standard algorithm for search and ranking, number of runs = 50, energy adjusted for Medium, 30-dimensional search box on the three axes and centered on 26, 20 and 14 on the x, y and z axes respectively.

The Gold-2020.2 program (Jones et al., 1997) used the Goldscore search method with 200% efficiency, a search radius of 8 Å centered on coordinates 26, 20 and 14, on the x, y and z axes respectively. The water molecules present in the structure were kept in the simulations. Due to the different ranking methods used by each program, Equation 4 was applied to the mean scores provided by the programs to select the most likely ligand to bind to AChE.

$$\text{Mean relative score } = \frac{1}{2}\left(\frac{Gold}{Gold_{max}} + \frac{Autodock}{Autodock_{max}}\right) \quad (4)$$

Where Gold represents the score provided by the Gold program for each ligand and $Gold_{max}$ the score of the highest rated ligand. Same goes for t18he Autodock variables.

3 RESULTS AND DISCUSSION

3.1 *Proximate composition of Nile Tilapia waste*

The proximate composition of the Nile Tilapia waste (Table S1, Supplementary Material) presented compatible values to those reported in the literature. These proportions of the proximate composition may vary according to the residues used for processing. The Nile Tilapia by-products used by Silva et al. (2014) for the production of FPH showed 42.3 % protein while the by-products used by Roslan et al. (2014) showed 14.6 %. Therefore, the proportion of protein (29.01%) obtained this work enables its use to produce protein hydrolysates and justifies the importance of the waste reusing.

3.2 *Experimental design and Principal Component Analysis*

Experimental conditions used in the CCRD, as well as the experimental results for the five evaluated responses o FPH production, are presented in Table 1.

Table 1. Coded levels (and real values in parentheses) for the experimental design (X_1, temperature; X_2, pH; X_3, Alcalase 2.4 L enzyme) and the obtained responses: Y_1, Yield (Y, %); Y_2, degree hydrolysis (DH, %); Y_3, Y_4 and Y_5, AChE activity inhibition by 20 (AChE 20), 30 (AChE 30) and 50 (AChE 50) $mg.mL^{-1}$, respectively.

run n°	coded levels (real values)			responses				
	X_1 T (°C)	X_2 pH	X_3 E (%)	Y_1	Y_2	Y_3	Y_4	Y_5
				Y (%)	DH (%)	AChE activity inhibition (%) ($mg.mL^{-1}$)		
						AChE 20	AChE 30	AChE 50
1	-1 (45)	-1 (7.5)	-1 (0.8)	76.49	14.05 ± 0.11	20.93 ± 4.72	22.00 ± 4.44	29.30 ± 3.48
2	1 (55)	-1 (7.5)	-1 (0.8)	86.33	18.54 ± 2.15	21.53 ± 2.66	26.19 ± 1.41	40.45 ± 3.63
3	-1 (45)	1 (8.5)	-1 (0.8)	83.66	13.74 ± 0.61	15.77 ± 0.96	22.98 ± 2.41	37.00 ± 1.75
4	1 (55)	1 (8.5)	-1 (0.8)	74.75	16.22 ± 0.53	19.98 ± 1.83	20.63 ± 2.05	34.64 ± 1.56
5	-1 (45)	-1 (7.5)	1 (1.6)	85.83	15.20 ± 0.27	22.21 ± 1.17	26.70 ± 2.31	30.75 ± 2.57
6	1 (55)	-1 (7.5)	1 (1.6)	82.48	23.37 ± 1.17	17.85 ± 2.24	22.49 ± 1.48	31.69 ± 2.33
7	-1 (45)	1 (8.5)	1 (1.6)	81.99	18.06 ± 0.61	10.51 ± 2.04	13.71 ± 1.03	18.11 ± 1.07
8	1 (55)	1 (8.5)	1 (1.6)	88.48	18.98 ± 0.58	17.54 ± 1.86	32.94 ± 3.34	34.45 ± 2.52
9	0 (50)	0 (8)	0 (1.2)	76.46	51.31 ± 2.57	14.30 ± 2.70	22.36 ± 2.49	35.40 ± 3.62
10	0 (50)	0 (8)	0 (1.2)	77.96	50.49 ± 8.07	22.34 ± 2.15	27.76 ± 1.86	37.64 ± 1.08
11	0 (50)	0 (8)	0 (1.2)	79.65	47.41 ± 1.70	20.96 ± 0.62	27.79 ± 2.82	36.38 ± 2.49
12	-1.68 (42)	0 (8)	0 (1.2)	79.73	48.80 ± 3.09	14.53 ± 3.24	21.27 ± 3.24	29.60 ± 1.61
13	1.68 (58)	0 (8)	0 (1.2)	87.14	63.42 ± 3.01	20.68 ± 1.88	21.22 ± 2.39	33.25 ± 0.50
14	0 (50)	-1.68 (7.2)	0 (1.2)	75.43	54.16 ± 0.72	12.15 ± 4.47	17.96 ± 4.46	30.94 ± 5.49
15	0 (50)	1.68 (8.8)	0 (1.2)	78.41	64.40 ± 1.42	17.49 ± 2.51	27.53 ± 2.76	32.57 ± 4.58
16	0 (50)	0 (8)	-1.68 (0.53)	73.25	59.52 ± 2.68	16.64 ± 2.06	22.35 ± 1.37	31.54 ± 1.61
17	0 (50)	0 (8)	1.68 (1.9)	77.70	64.33 ± 2.14	13.65 ± 0.81	19.97 ± 1.54	31.80 ± 1.57

The response values ranged between 73.25 - 88.48% for yield; 13.74 - 64.40% for degree of hydrolysis and 10.51 - 40.45% for AChE activity inhibition with different concentrations (20, 30 and 50 $mg.mL^{-1}$). Nevertheless, the experimental models evaluated, based on the experimental data, did not present significant regression for all evaluated variables (Table S2, Supplementary Material), demonstrating inappropriate for predicting the response in the production of FPH. Therefore, the responses evaluated the experimental design (yield, DH, AChE 20, AChE 30 and AChE 50, Table 1) were submitted to a principal component analysis (PCA) in order to extract more information about the relation between these variables. The obtained PCA graphs are presented in Figure 1.

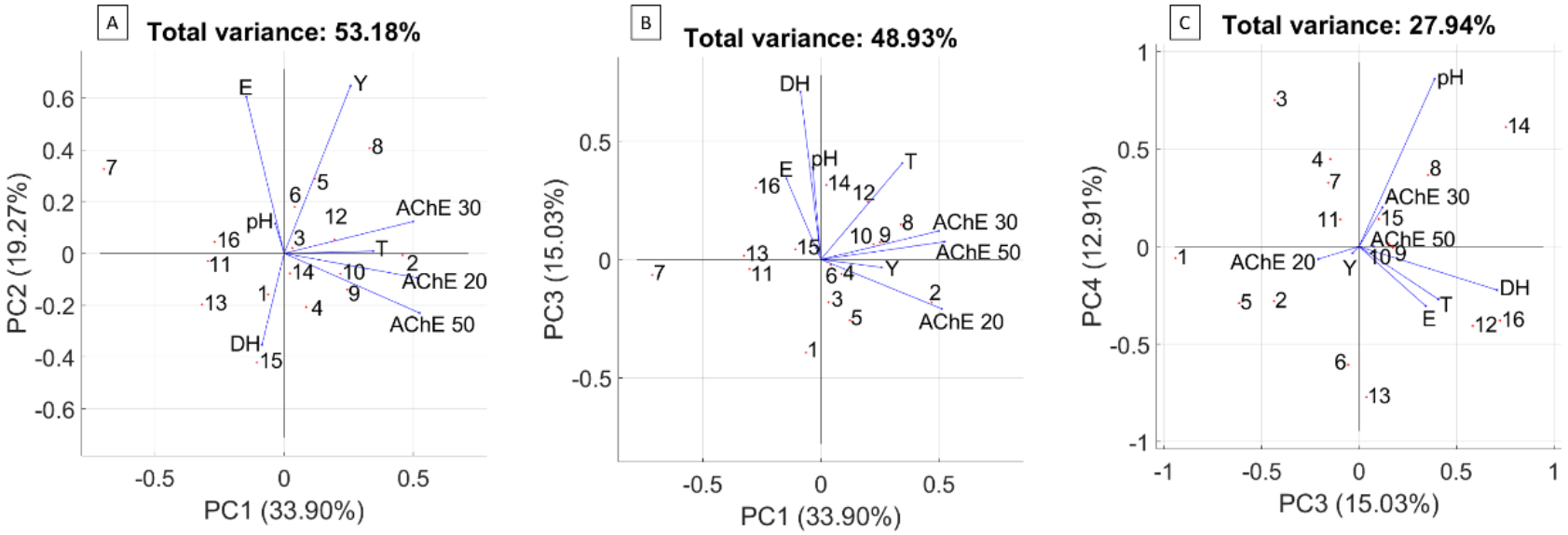

Figure 1. Principal Component Analysis (PCA) for Yield (Y), degree of hydrolysis (DH) and AChE activity inhibition by 20 (AChE 20), 30 (AChE 30) and 50 (AChE 50) mg.mL^{-1}, respectively. PC1 versus PC2 (A), PC1 versus PC3 (B) and PC3 versus PC4 (C).

The scree plot of eigenvalues, as well as the Mahalanobis distance obtained from the evaluated data set are presented in Figure S1 (Supplementary Material). It was possible to select PCs with eigenvalues higher than 1. Also, the Mahalanobis distance plot did not show any outlier among the evaluated experimental point. Therefore, the loadings obtained to the selected PCs, which show the importance of each PC on each variable, are presented in Figure S2 (Supplementary Material). The variables Enzyme (E) and Yield (Y) were better described by PC2, while the variables Temperature (T), Degree of hydrolysis (DH) and pH could be represented by PC3 and PC4. Regarding to acetylcholinesterase inhibitions (AChE 20, AChE 30 and AChE 50), all were better represented by PC1.

In Figure 1 (A), where are presented the scores from PC1 versus PC2 (53.17% of total variance explained), it is possible to note that all AChE concentrations, as well as the temperature are highly positively correlated, since the corresponding vectors are aligned at the same direction. Thus, it is possible to conclude that the higher hydrolysis temperature, the higher inhibitory action of the final hydrolysate on acetylcholinesterase enzyme. Also, the enzyme concentration and the yield of hydrolysis presented a positive correlation.

For the scores plot related to PC1 versus PC3 (Figure 1 (B), 49.93% of total variance explained) the same relation between temperature and AChE inhibition to all considered FPH concentrations was detected. In Figure 1 (B) it was also verified that the relation between the DH (that represents the cleavage of the peptide bonds), pH and enzyme concentration is positive with high correlation. Therefore, as high the pH and enzyme concentration applied, the higher DH obtained for FPH. This result corroborates that described in the literature, in which Mohammad et al. (2015) verified that the higher concentration of the alcalase enzyme was responsible for cleaving more peptide bonds available in the substrate. Cao et al. (2009) noticed a significant interaction between pH and alcalase 2.4 L enzyme in the optimization of the shrimp protein hydrolysates, as well, they observed the optimum points with higher DH at pH 8 and temperature equal to 57 °C. In this sense, when evaluating Figure 1, could be verified that the experimental points 12, 14 and 16 represented the best hydrolysis conditions because they were closer to the positive correlations. At these points, although the mechanisms performed for hydrolysis are different, the DH and AChE activity inhibition results were similar.

Analyzing the scores plot from PC3 versus PC4 (Figure 1 (C), 27.94% of total variance explained) the same relation between the degree of hydrolysis, temperature and enzyme concentration can be observed.

Considering the scores plot evaluation, three experimental points to further characterization were selected. The points presenting higher inhibitory action to AChE according to Figures 1 (A) and (B) were 2, 12, 9 and 10. Among these points, it can be observed that point 2 presented greater inhibition potential than the other experimental conditions. On the other hand, even with reasonable AChE inhibitory results, points 12 and 14 were also selected since they represent results with greater contribution of enzymatic hydrolysis. The selected experimental runs were: 2 (T: 55 ºC, pH: 7.5, E: 0.8 %), 12 (T: 42 ºC, pH: 8, E: 1.2 %) and 14 (T: 50 ºC, pH: 7.2, E: 1.2 %).

3.3 *AChE activity inhibition*

The results of AChE activity inhibition (Table 1) ranged from 10.51 - 40.45% at different FPHs concentrations (20, 30 and 50 $mg.mL^{-1}$) obtained by the different hydrolysis conditions. As expected, inhibition results increased with concentration. Also, it could be verified that the evaluated concentrations and their respective inhibitions were adequate as reported by other studies.

Su et al. (2016) evaluated the AChE inhibition by anchovy protein hydrolysates, at higher concentrations when compared to the present study, between 100 and 400 $mg.mL^{-1}$, which resulted at 10 - 60% inhibition. On the other hand, Naik et al. (2020), evaluated the inhibition of mussel by-products hydrolysates at significantly lower concentration than reported in this study (1 $mg.mL^{-1}$) and obtained similar AChE inhibition (29.59%). This variation in the hydrolysates' concentration for AChE inhibition may be due to several factors such as enzyme specificity, DH, molecular weight, amino acid sequence, hydrophobicity and peptide loading (Gao, Yu, Shen, Chu, Chen, Fen, Yang, Yuan, McClements, et al., 2021). Malomo and Aluko (2016) indicate that AChE inhibitory activity occurs mainly by the type and sequence of amino acids in the peptide chain. However, the exact mechanism of the FPHs action in the AChE inhibition is not yet fully understood.

In this work, the hydrolysis temperature influenced the AChE activity (Figure 1 (A)). This may have occurred due to possible changes in the structure, which may result

in modifications on peptides and at their amino acids (Zhao et al., 2018). In addition, AChE inhibition was not directly related to peptide size, represented by DH (*i.e*. the higher the DH the smaller the peptide size), since the FPHs with very different DH values showed close inhibitions. For instance, points 2 and 10 with DH of 18.54% and 50.49% presented AChE inhibition values of 40.45% and 37.64%, respectively. This feature was also verified by Zent et al. (2021) and Malomo and Aluko (2016) when evaluating protein hydrolysates obtained from plants.

3.4 *FPH Characterization*

3.4.1 *Fourier transform infrared spectroscopy (FTIR)*

In Figure 2 are presented the FTIR spectra of the FPH obtained with experimental points 2, 12 and 14.

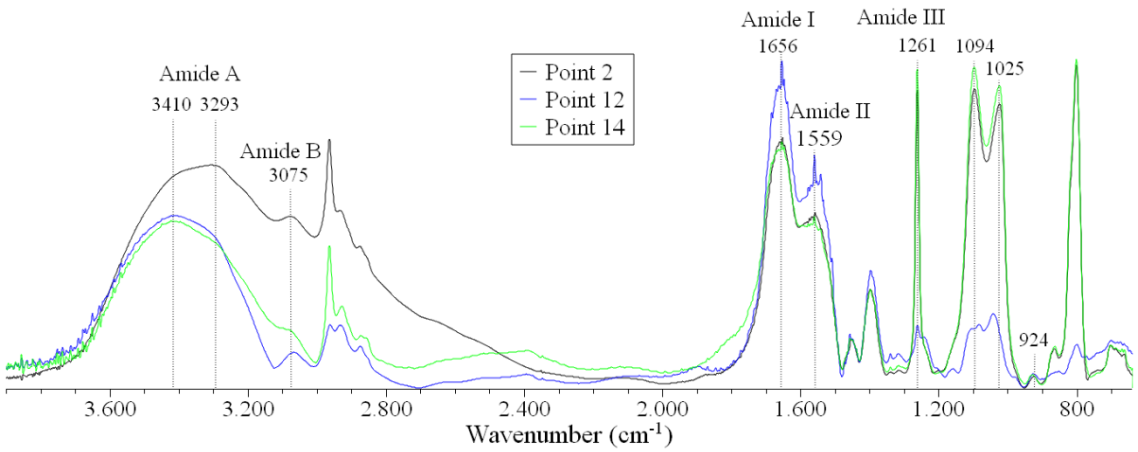

Figure 2. FTIR spectra of the FPH obtained in Points 2, 12 and 14.

FTIR is a method used to evaluate the structure modification of proteins and peptides submitted to enzymatic hydrolysis. This analysis is useful because after cleavage of peptide bonds structural changes can occur and C-terminal carboxylate (COO^-) and N-terminal amino (NH_3^+) groups can be formed, which can be identified in the infrared absorption bands (Böcker et al., 2017). Therefore, the characteristic bands of protein hydrolysates identified in Figure 2 for all analyzed FPHs were: amide A (3410 and 3293 cm^{-1}); amide B (3075 cm^{-1}); amide I (1656 cm^{-1}); amide II (1559 cm^{-1}) and amide III (1261 cm^{-1}) (Elavarasan et al., 2016; Noman et al., 2020).

In amide A, the N-H stretching vibration was observed for points 12 and 14 at 3410 cm^{-1} as described in the literature (3400-3440 cm^{-1}). However, for point 2 this position was shifted to a wavenumber of 3293 cm^{-1}, possibly because peptide O–H and N–H stretching is involved by a hydrogen bond (Elavarasan et al., 2016).

The Amide I band occurs at 1700-1600 cm^{-1} due to stretching vibrations of C=O coupled weakly with C–N stretch and N–H bending. In this region, there was only one band at 1656 cm^{-1} for all FPHs, which can be attributed to the existence of α-helical structures and/or more complex structures, such as an α-helix superimposed on a random coil. Amide II, observed at 1559 cm^{-1}, probably was due to C–N stretch along with N–H in-plane bending, and, amide III (at 1261 cm^{-1}), resulted from N–H bending and C–N stretching with deformation vibrations of C–H and N–H (Glassford et al., 2013).

These amides (I, II and III), are related to changes in the secondary structure of proteins (Yang et al., 2020). This is corroborated by the observed amide bands I and II, which presented lower intensity at points 2 and 14 compared to point 12. Possibly, this decrease in the intensity of the bands occurred due to temperature influencing the enzymatic hydrolysis process by modifying the protein structure (Noman et al., 2020).
In addition, the presence of phosphate, widely found in fish bones, can also be verified in the spectra. The characteristic bands of the PO_4^{3-} group were identified in three regions. The first one is represented by the bands located at 1094 cm^{-1}, 1025 cm^{-1} corresponding to *v3* stretching mode and 924 cm^{-1} associated to *v1* stretching mode (Boskey & Pleshkocamacho, 2007; Nawaz et al., 2020; Pal et al., 2017).

3.4.2 *Amino acid compositions*

The amino acid compositions of FPH (points 2, 12 and 14) are summarized in Table 2.

Amino acids are indicated as one of the key factors in the bioactive capacity of fish hydrolysates (Chalamaiah et al., 2012). Among the non-essential amino acids presented in Table 2, glutamic acid, that has a significant effect on the regulation of the immune system (Rajabzadeh et al., 2017), presented the highest concentration. Fish hydrolysates and peptides possess potent immunological activity in both cultured cells and mice (Gao, Yu, Shen, Chu, Chen, Fen, Yang, Yuan, Mcclements, et al., 2021).

Table 2. Amino acid compositions of FPH

Amino acids	P2	P12	P14
	g/100 g of dry matter		
Aspartic acid (Asp)	9.45	8.71	9.12
Glutamic acid (Glu)	14.83	13.56	13.94
Serine (Ser)	3.63	3.49	3.61
Glycine (Gly)	9.18	9.58	8.88
Histidine (His)	1.89	1.87	1.93
Arginine (Arg)	6.32	5.55	6.29
Threonine (Thr)	3.62	3.33	3.55
Alanine (Ala)	6.82	6.83	6.58
Proline (Pro)	5.56	5.68	5.41
Tyrosine (Tyr)	2.22	1.96	2.25
Valine (Val)	4.10	3.92	3.97
Methionine (Met)	2.59	2.49	2.50
Cysteine (Cys)	0.49	0.43	0.37
Isoleucine (Ile)	3.26	3.13	3.13
Leucine (Leu)	6.30	6.22	6.10
Phenylalanine (Phe)	2.73	2.89	2.64
Lysine (Lys)	7.40	7.59	7.02
PCAA	15.61	15.01	15.24
EAA	38.21	36.99	37.13
HAA	42.76	42.70	41.46
TAA	90.39	87.23	87.29

PCAA (positively charged amino acids): His, Arg and Lys.
EAA (essential amino acids): Ile, Leu, Lys, Met, Phe, Thr, Val, His and Arg.
HAA (hydrophobic amino acids): Gly, Ala, Pro, Tyr, Met, Val, Phe, Ile, and Leu.
TAA (total amino acids).

In general, the amino acid profile (Table 2) was similar for the hydrolysates evaluated, indicating that the peptides recovered in the supernatant presented average similarity on amino acid composition. This result was expected since all samples were produced with the same enzyme, which is capable to cleave a broad spectrum of peptide bonds. However, point 2 presented greater amount of total and essential amino acids. In comparison with the hydrolysates of tilapia residues, the values of the amino acids obtained were lower than those found by Silva et al. (2014) and higher than those obtained by Roslan et al. (2014). These differences in amino acid composition may be due to several factors such as raw material, enzyme used and hydrolysis conditions (mainly the combination of time, temperature, pH and enzyme concentration) (Halim et al., 2016).

3.5 *Reaction kinetics*

Figure 3 presents the Michaelis-Menten substrate competition assays for FPH: points 2 (A), 12 (B) and 14 (C).

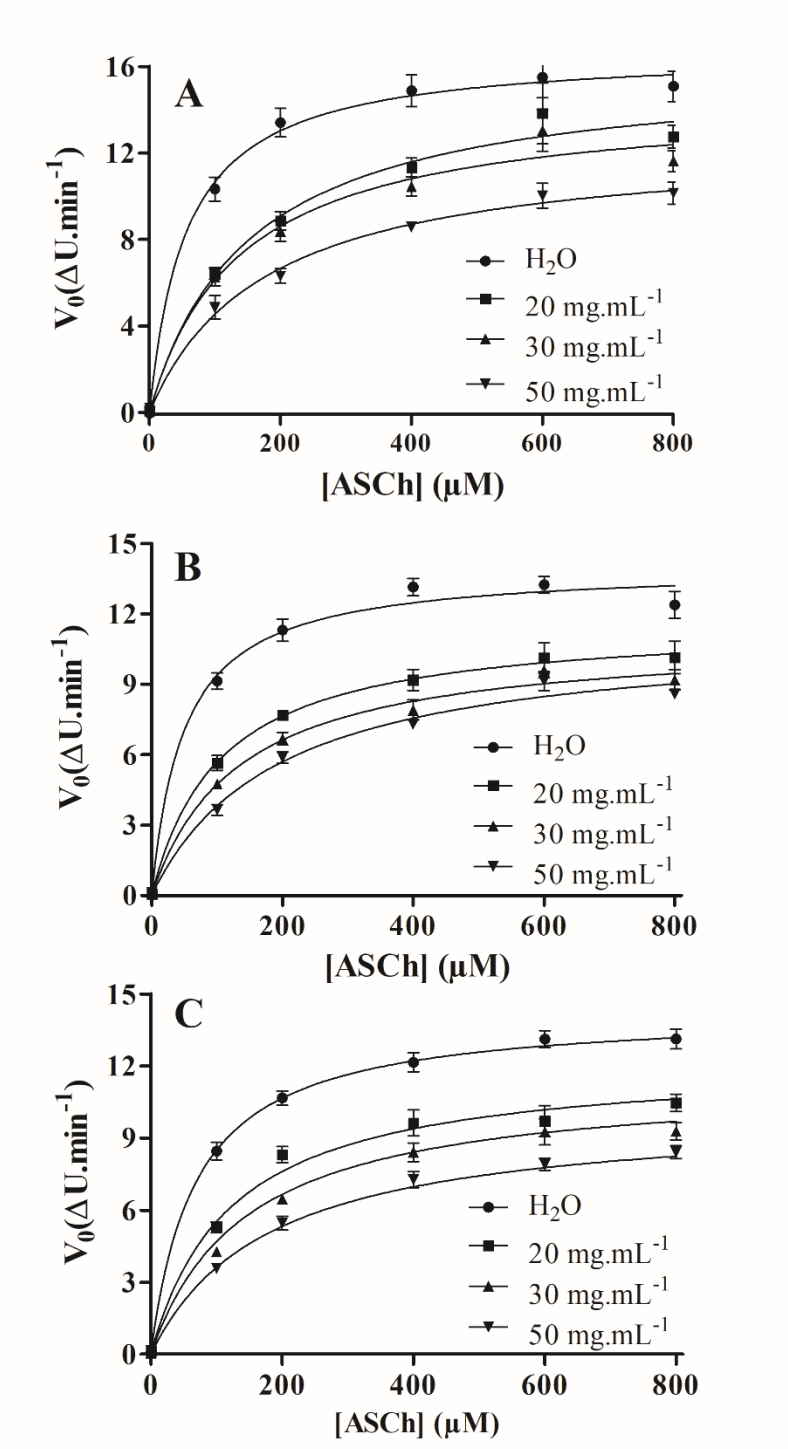

Figure 3. Michaelis-Menten substrate competition assays for FPH: Points 2 (A), 12 (B) and 14 (C).

The Michaelis-Menten assays demonstrated that the FPH obtained in points 2, 12 and 14 were able to decrease the activity of AChE according to the concentration (20, 30 and 50 $mg.mL^{-1}$) when compared to the control (H_2O curve). The Lineweaver-Burk methodology (Figure S3) indicated that all samples presented inhibition of competitive mixed-type, with Ki values of 15.75, 11.72 and 18.45 mg to points 2, 12 and 14, respectively. The competitive mixed-type model was also observed by Zhao et al. (2017) (at concentrations of 1 – 20 mM) and Malomo and Aluko (2019) (at concentrations of 0 – 0.05 $mg.mL^{-1}$) when evaluating the AChE inhibition kinetics of pure lysine and hemp seed protein-derived peptides, respectively.

3.5.1 *Molecular docking studies*

The structure of AChE enzyme from *Mus musculus* was used for having tertiary structure similar to that of AChE enzyme from *Electrophorus electricus* and identity of 60.065 %. Similar studies available in the literature also report the use of rat structure in simulations (Grella Miranda et al., 2020).

The visual inspection of the AChE crystallographic structure used in the simulations shows that choline appears bound in two sites of the active site in the enzyme, one at the bottom of the site (further inside the molecule) and another at the entrance of the active site (further on the surface of the molecule). The overlap of other AChE structures in the presence of ligands suggests that the site of choline binding with the greatest affinity should be the one inside the molecule. Binding of the other choline probably occurred due to the high amount of this compound in the structure crystallization solution, resulting in a lower affinity bond at another enzyme site. Thus, the site defined for the fitting of amino acids in the docking simulations was the innermost.

In addition, the structure of *Mus musculus* AChE, linked to choline (pdbid: 2HA3) was chosen for docking studies because choline has the approximate size of a medium amino acid, which were used in docking simulations.

The result shown in Figure S4 (Supplementary Material) indicates that the amino acid L-arginine (6322) is most likely to bind to AChE, followed by L-cystine (67678), L-tyrosine (6057), L-lysine (5962), L-phenylalanine (6140) and L-histidine (6274). This classification suggests that the basic amino acids prefer to bind to AChE in relation to the others.

Therefore, Figure 4 (A) and (B) show the overlap of the choline crystallographic ligand used as reference, (C) poses and arginine overlap of docking simulations and, (D) interactions between arginine and residues of the AChE active site.

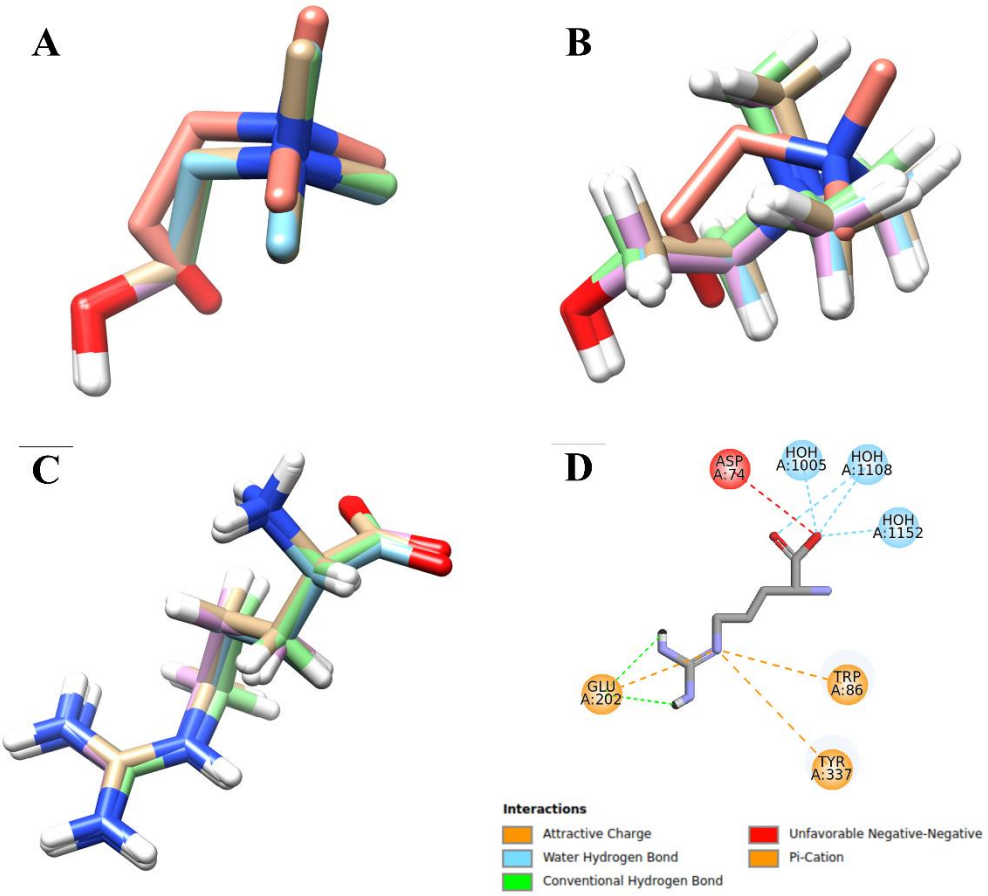

Figure 4. Overlap the best poses obtained with choline redocking using the protocols defined for the Autodock (A) and Gold (B) programs. In pink, the pose of the crystallographic ligand, the others are the best poses of four simulations. (C) Arginine poses obtained with repetitions of docking simulations. The overlap of the same pose in all simulations suggests a pattern for the connection. (D) Interactions between arginine and Ache active site residues generated by the Virtual Studio program.

The validation of the Autodock and Gold programs by redocking shows a good overlap of the choline crystallographic ligand used as a reference in this study (Figure 4, A-B).

Figure 4 (C) shows that the L-arginine poses obtained in the simulations are very similar, indicating bond stability. L-arginine was selected as the library binder most likely to bind to AChE. This fact is corroborated by evidence from the literature that indicate positively charged amino acids as the major responsible for AChE inhibition. These positively charged amino acids that exist in the peptides of FPH probably bind to one of the active sites of AChE: the peripheral anionic site (PAS), forming a stable complex and

preventing the entry of substrates into the enzyme active site (Malomo & Aluko, 2016; Zhao et al., 2018).

L-arginine makes several load-charge and charge-dipole interactions with residues of the active site (Figure 4 (D)), but the interaction of carboxyl oxygen from arginine with carboxyl oxygen from the Asp74 residue draws attention because it is unfavorable (negative charge interaction with negative charge). However, it is worth remembering that the enzyme structure used in the simulations is static and there are several water molecules in the cavity of the active site of AChE and many of them close to this interaction (Figure 4 (D)), which suggests that these water molecules can form a bridge (water-mediated interaction) between the carboxyl of the arginine ligand and the carboxyl of the Asp74 residue, which would make the interaction even more stable and favorable in the real environment.

4 CONCLUSIONS

Enzymatic hydrolysis may interfere with the bioactive properties of fish protein hydrolysates. Different hydrolysis conditions, varying temperature, pH and enzyme concentration, were used according to the composite rotatable design to evaluate the interference in the bioactive property of AChE inhibition. The hydrolysis adjusted at 55 °C, pH 7.5 and enzyme concentration of 0.8 % (Enzyme : Substrate), selected by principal component analysis, presented greater inhibition potential than the other experimental conditions. Hydrolysis temperature influenced AChE inhibition, but this inhibition cannot be directly related to the peptide size (represented by DH). The results of the kinetic study showed that all FPH evaluated had a mixed-type inhibition of the AChE activity. The amino acid profile was similar for the hydrolysates evaluated, however, the hydrolysate with higher AChE inhibition presented greater amount of total and essential amino acids. In addition, according to molecular docking analysis, it was found that arginine is the amino acid most likely to bind to AChE, demonstrating that basic amino acids can be a key factor for this bioactivity. Finally, it is expected as future perspectives that these FPH could be applied in functional food formulations and pharmaceutical products as AChE inhibitors.

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CAPÍTULO 3

BIOACTIVE FISH PROTEIN HYDROLYSATES (FPH) AS ANTIOXIDANT IN SALAD DRESSING

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ABSTRACT

Fish protein hydrolysates (FPH) are a product of the food industry that presents several bioactive properties. However, efficiently tunning the technological properties of FPH is a challenge and may impact its applicability and also bioactivity. Thermal and mechanical treatments may affect the structure of proteins to be hydrolyzed and thus influence in obtaining peptides with improved bioactivity. In this work, Nile Tilapia muscle was treated by thermal sterilization (EST) or homogenization with Ultra-turrax (UT) and then hydrolyzed to obtain FPHs with antioxidant properties to be used in salad dressing. An extensive characterization of the functional and bioactive FPHs properties was carried out comprising antioxidant activity (DPPH, ABTS, and FRAP), acetylcholinesterase inhibition, and emulsifying properties. The degree of hydrolysis (DH) obtained was 37.9, 37.66, and 40.55%, for the control FPH (C), EST, and UT samples, respectively. The UT treatment resulted in a sample with a smaller radical scavenging capacity. The AChE inhibition was evaluated in three concentrations (15, 45, and 60 $mg.mL^{-1}$) demonstrating to be a potential property of FPHs and cytotoxic assays in *Allium cepa* L. showed that no toxicity is expected for the FPHs. As a proof of concept, FPHs were used as an emulsifying/antioxidant agent to prepare a salad dressing. Emulsifying activity index (EAI) and emulsifying stability index (ESI) of FPHs indicated better emulsifying capacity and stability in basic pH, probably due to the hydrophobic character of the proteins. FPH provided an increase in protein content, pseudoplastic behavior, characteristic color, and texture. In addition, FPHs aided oxidative stability of salad dressing, demonstrating potential application in emulsified foods by acting on the elimination of radicals generated in lipid oxidation.

Keywords: Sterilization; pretreatment; homogenization; emulsion; food application; enzymatic hydrolysis; Nile Tilapia muscle.

1 INTRODUCTION

Fish protein hydrolysates (FPH) are products of the hydrolysis of abundant and high-quality native proteins that have been demonstrated to be an excellent source of diverse bioactive properties such as anti-inflammatory (Rocha et al., 2018), anticancer (Yaghoubzadeh et al., 2020), antimicrobial (Jemil et al., 2016) and antioxidant (Bashir et al., 2020; Cheng et al., 2020). This broad bioactivity is directly linked to smaller peptides and free amino acids formed during the hydrolysis which in turn may be affected by pH, temperature, enzyme, time, and substrate properties (Halim et al., 2016).

Recent studies have evaluated the use of pretreatments before hydrolysis of the substrate to modify the protein structure and improve the enzyme access, facilitating the exposure of the N and C terminals of peptides (Noman, Ali, et al., 2020; Noman, Qixing, et al., 2020). Among the technologies used in pretreatment are the microwave (Ketnawa et al., 2018), ultrasound (Z. Li et al., 2020), high-pressure processing (Hemker et al., 2020), and heat treatments (Korczek et al., 2020; Rivero-Pino et al., 2020). By altering the structure of proteins, pretreatments can also aid in modifying functional properties while maintaining high nutritional quality (Rivero-Pino et al., 2020). This allows FPH to be applied in the formulation of food products for better physical, functional, and nutraceutical properties (Noman et al., 2018). In the case of salad dressings, which are oil-in-water emulsions with high-fat levels (Tekin-Cakmak et al., 2021), FPH can provide better formation and stability of emulsions by favoring the adsorption of peptides at the oil/water interface (Ruiz-Álvarez et al., 2022). In addition, during storage, antioxidant peptides can be released to interact with other ingredients and retard lipid oxidation (García-Moreno et al., 2016; Ghorbani Gorji et al., 2016). However, it is worth noting that key aspects of the effects of the pretreatment on Nile Tilapia muscle technological and bioactive properties remain to be investigated.

The objective of this study was to investigate if the substrate pretreatments, thermal heating method by sterilization, and homogenization by Ultra-turrax, could affect FPH's antioxidant and functional properties after enzymatic hydrolysis and, analyze the technological properties of a real food system, the salad dressing when incorporated with FPHs.

2 MATERIAL AND METHODS

2.1 *Material*

Nile Tilapia muscle was obtained in a local market in 2020 (Campo Mourão, state of Paraná, Brazil). The enzymatic hydrolysis was started using alcalase 2.4 L enzyme (≥ 2.4 U/g, P4860, Sigma-Aldrich). The concentration of soluble proteins was determined by the bovine albumin standard curve (A7030, Sigma-Aldrich). The antioxidant reagents were 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ, Sigma-Aldrich) and Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid). The acetylcholinesterase (AChE) activity assay reagents were tris hydroxymethyl aminomethane (Tris-HCl, Dinâmica), for the preparation of potassium phosphate buffer (TFK) were used monobasic potassium phosphate (Dinâmica) and dibasic potassium phosphate (Neon), acetylcholinesterase enzyme from *Electrophorus electricus* (electric eel, Sigma-Aldrich) (1.25 $U.mL^{-1}$ in Tris-HCl buffer (20 mM, pH 7.5)), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB, 98%, Sigma-Aldrich), acetylthiocholine iodide (ASCh) (Sigma-Aldrich, 99%). The emulsifying capacity reagents were soybean oil (Coamo) and sodium dodecyl sulfate (SDS, Isofar). Cytotoxicity and genotoxicity methyl reagents were methyl methanesulfonate (MMS, CAS 66-27-3, Sigma-Aldrich), ethanol (99%, Dinâmica), and acetic acid (Dinâmica). French salad dressing formulations reagents were Butil Hidroxi Toluol (BHT) (commercial antioxidant, Êxodo Cientifica) and antimicrobial potassium sorbate (Casa dos Químicos). Oxidative stability reagent was isooctane solvent (Neon).

2.2 *Proximate composition of Nile Tilapia muscle (Oreochromis niloticus)*

Fish muscles were grounded, homogenized, and stored at -80 °C until their use for the substrate treatment. The proximate composition of the fish muscle was carried out as described by Instituto Adolfo Lutz (2008). The gravimetric method was used to determine the moisture at 105 ºC until obtaining constant weight. To evaluate the ash content, the fish waste was incinerated in muffle at 550 °C. The lipids were determined by Bligh &

Dyer method and the protein content was obtained by the MicroKjeldahl method with a correction factor of 6.25.

2.3 *Substrate treatment*

The muscles of the ground and stored fish were thawed and separated into three groups: Control (C, no treatment before hydrolysis), Sterilization (EST), and Ultra-turrax (UT). In the group EST, the substrate was sterilized in an autoclave (Phoenix, AV, 18 L) at 120 °C under 1 $kgf.cm^{-2}$ for 15 min. In the group UT, the substrate was homogenized in an Ultra-turrax (IKA, T25) at 20,000 rpm for 15 min.

2.4 *Production of fish protein hydrolysates (FPH)*

The production of protein hydrolysates was performed according to Alvares et al. (2018), with minor adaptations. The substrate samples (C, EST, and UT) were solubilized in distilled water (1:2, wt:v), the pH adjusted to 7.5 with NaOH 1 M or HCl 0.1 M, and after that, the alcalase enzyme was added (0.8 %v/wt, [E:S]). The temperature was adjusted to 55 °C and the mixture was kept under gentle agitation for 120 min. The enzymatic reaction was halted by raising the temperature to 90 °C for 15 min. After that, the mixture was cooled and centrifuged at 6,000 rpm for 20 min. The supernatant was collected and filtered with a cellulose filter with the aid of a vacuum pump. The filtrate FPH was frozen in an ultra-freezer (-80 °C) for 24 h and freeze-dried (L101, Liotop, Liobrás).

2.5 *FPH characterization*

2.5.1 *Degree of hydrolysis (DH)*

The degree of hydrolysis (DH) was determined using the methodologies described by Hoyle and Merrltt (1994), and Baek and Cadwallader (1995) with modifications. 6 mL were removed from the hydrolysates and inactivated with 4 mL trichloroacetic acid

(TCA) 6.25%, followed by rest for 15 min and centrifuged at 6,000 rpm for 20 min. The concentration of soluble proteins was determined by the modified Lowry method (Lowry et al., 1951). For this, a bovine albumin standard curve (y = 16.541x + 0.0238; R^2 = 0.9915) was obtained to compare absorbance readings at 750 nm that were performed in UV-Vis spectrophotometer (Ocean Optics USB650UV, USA). The degree of hydrolysis (DH) was calculated by Equation 1.

$$DH(\%) = \left(\frac{\text{6.25\% TCA soluble protein in the sample (mg)}}{\text{total protein in the sample (mg)}}\right) x100\% \quad (1)$$

Total protein was determined by the Microkjeldahl method (Instituto Adolfo Lutz, 2008) and the conversion factor used was 6.25.

2.5.2 *Yield (%)*

After the thermal inactivation of the enzymatic hydrolysis samples were cooled at room temperature. The samples were weighed and then fractionated into a centrifuge under conditions of 6,000 rpm for 20 min, in which the aqueous fraction (containing the protein hydrolysates) was transferred, weighed, and stored at -80 ºC. The yield was calculated according to Equation 2.

$$\text{Yield } (\%) = \frac{\text{aqueous fraction weight } (g)}{\text{hydrolyzed weight before fractionation } (g)} x100\% \quad (2)$$

2.5.3 *Free amino acid*

Free amino acid analysis of the FPHs was performed through the ninhydrin test, as indicated by Moore; Stein (1954) with adaptations. For this, 1 mL of 2% ninhydrin solution (Sigma-Aldrich) was added in 1 mL FPH samples (50 mg.mL^{-1}, or distilled water as blank) and kept in a bath at 100 °C for 15 min. After this time, 15 mL of ethanol (50 %v/v) was added to all samples. The absorbance at 570 nm was determined using a UV-Vis spectrophotometer (Ocean Optics USB650UV, USA). The quantification was carried

out using a Glycine amino acid (Sigma-Aldrich) calibration curve ($y = 0.4543x - 1.6067$; $R^2 = 0.9925$). The results were reported as μmol of Glycine equivalents ($\mu mol_{Gly\ E}/g_{sample}$).

2.5.4 *Antioxidant capacity of FPHs*

Antioxidant capacity assays (DPPH, ABTS, and FRAP) of FPHs were performed according to the methods described by Pires et al. (2017), Thaipong et al. (2006), and Urrea-Victoria (2016), respectively, with some modifications. FPHs were dissolved in distilled water, obtaining different concentrations (1.25 – 45 $mg.mL^{-1}$).

DPPH assay was performed by adding 20 μL of the FPH solutions with 280 μL of 80 μM DPPH methanol solution (or pure methanol as the control) in a test tube. After 20 min in the dark, it was subjected to 10 min centrifugation (Korczek et al., 2020). The supernatant was collected, and the absorbance was determined at 517 nm using a microplate reader (Thermo Plate, TP-Reader).

ABTS assay was based on the preparation of a stock solution of equal volumes of ABTS (7.4 mM) and sodium persulphate (2.6 mM) maintained for 12 hours in the dark. After this time, the stock solution was diluted in methanol until an absorbance of 1.10 ± 0.01 at 734 nm to obtain the working solution. In short, 150 μL FPHs solution (or methanol as the control) and 2850 μL working solution were mixed and kept in the dark for 2 h, then, the absorbance at 734 nm was determined using a UV-Vis spectrophotometer (Ocean Optics USB650UV, USA).

FRAP assay was based on the preparation of a stock solution of FRAP (in ratio 1:1:10, v/v/v): 5 mL 2,4,6-tripyridyl-s-triazine (TPTZ) solution (10 mM in 40 mM HCl), 5 mL ferric chloride hexahydrate aqueous solution (20 mM) and 50 mL acetate buffer (300 mM). Then, 100 μL FPHs solution (or distilled water as the control), 370 μL distilled water, and 3000 μL stock solution of FRAP were mixed and kept in a bath at 37°C for 30 min. The absorbance at 595 nm was determined using a UV-Vis spectrophotometer (Ocean Optics USB650UV, USA). Each essay and sample were analyzed in triplicate.

The results were reported as μmol of Trolox equivalents ($\mu mol_{TE}/g_{sample}$).

2.5.5 *AChE activity assay of FPHs*

AChE activity was performed according to the methodology described by Ellman et al. (1961) with some adaptations. The analysis was performed in triplicate and the experimental conditions were conducted as described by Moreira et al. (2022) and the FPHs were evaluated at concentrations of 15, 45, and 60 $mg.mL^{-1}$.

2.5.6 *Protein solubility*

Protein solubility of the FPHs was determined according to Chalamaiah et al. (2010) with modifications. For this, 300 mg of the sample were diluted in 30 mL of distilled water and solutions had the pH adjusted to 3, 5, 7, 9, and 11 with HCl 0.5 M or NaOH 0.5 M. Each solution was magnetically stirred at room temperature (25 ± 2 °C) for 30 min and centrifuged at 6,000 rpm for 30 min at 4 °C. The protein content in the supernatant was determined using the Biuret method and the total protein was determined using the MicroKjeldahl method (Instituto Adolfo Lutz, 2008). The solubility was calculated according to Equation 3.

$$\text{Solubility } (\%) = \frac{protein\ in\ the\ supernatant}{total\ protein\ in\ the\ sample} x100 \quad (3)$$

2.5.7 *Emulsifying capacity*

The emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined according to the method described by Pearce and Kinsella (1978) with some modifications. The pH values of 90 mL of the FPH samples (1 $mg.mL^{-1}$) were adjusted to 3, 5, 7, 9, and 11 after adding 30 mL of soybean oil, respectively. Each mixture was homogenized at a speed of 20,000 rpm per 1 min and then 100 μL of the emulsion were pipetted from the bottom of the mixture and diluted in 10 mL of a 0.1%wt/v SDS solution and 10 min after homogenization. The absorbance values were measured at 500 nm, just after the emulsion dilution (A_0) and after 10 min (A_{10}). The absorbance values were used to calculate the EAI and ESI, as shown in Equations 4 and 5.

$$EAI(m^2/g) = \frac{2 \times 2.303 \times DF \times A_0}{C \times 2.5 \times 10000} \quad (4)$$

$$ESI(\text{min}) = \frac{A_0}{A_0 - A_{10}} \times 10 \quad (5)$$

DF is a dilution factor (100), C is the initial concentration of FPH ($mg.mL^{-1}$) in the first emulsion, 0.25 is the fraction of soybean oil used to form the emulsion, A_0 and A_{10} are the absorbances of the emulsion diluted determined at 0 and 10 min after dilution in the SDS solution. Each sample was measured in triplicate.

2.5.8 *FTIR*

For the determination of the molecular characteristics of FPHs samples, spectra were collected with an Infrared Spectrophotometer with Fourier Transform (IR AFFINITY-1, Shimadzu), in the range of 4000 to 400 cm^{-1}, using 32 accumulations and a resolution of 4 cm^{-1}. The samples were previously conditioned in a desiccator containing anhydrous calcium chloride ($CaCl_2$) for 7 days before the analysis and spectra bands were normalized for spectrum comparison.

2.5.9 *Cytotoxicity and genotoxicity test in A. cepa root meristems*

For the evaluation of cytotoxicity and genotoxicity of FPH, five onion bulbs (beta crystal variety, from an organic vegetable) were placed for each sample, in containers with distilled water, constantly aerated, and germinated in dark environments until roots of 2.0 cm in length were obtained. Some roots were collected before undergoing treatment, to control the bulb itself (Co - 0 h). Then, the roots were placed in their respective treatments for 24 and 48 hours, being collected every 24 hours. Two controls were prepared: a negative control (treated only with distilled water) and a positive control (treated with methyl methanesulfonate (MMS), a substance known to be cytotoxic and genotoxic for the *A. cepa* test system at $4x10^{-4}$ $mol.L^{-1}$). The collected roots were fixed in

Carnoy 3:1 solution (ethanol:acetic acid) for up to 24 h. Slides were analyzed in an optical microscope (Nikon Eclipse E200) with a 40× objective lens.

The cytotoxic potential was determined by the Mitotic index (MI), in which the cells in interphase, prophase, metaphase, aphase, and telophase were counted and calculated according to Equation 6.

$$MI\ (\%) = \frac{total\ number\ of\ dividing\ cells}{total\ number\ of\ cells} \times 100 \quad (6)$$

The genotoxic potential was evaluated using micronucleus frequency, colchicine metaphases, anaphase and telophase bridges, gene amplifications, adhering cells, nuclear buttons, and multipolar anaphases, among other changes.

2.6 *Production and characterization of French salad dressing with FPHs*

Five French salad dressing oil-in-water emulsions were prepared as described by Gomes et al. (2008). The FPHs samples (C, EST and UT) were used as antioxidants in concentrations of 3 g FPH per 100 g. The salad dressing was also produced with 0.5 g butylhydroxytoluene (BHT) per 100 g, and also a control formulation without antioxidants (FC) was obtained. First, tomato extract (9 g/100 g), skimmed milk powder (8 g/100 g), sugar (5.7 g/100 g), vinegar (3 g/100 g), salt (2 g/100 g), garlic powder (0.5 g/100 g), sweet paprika (0.5 g/100 g), mustard powder (0.2 g/100 g), monosodium glutamate (0.5 g/100 g), antimicrobial potassium sorbate (0.1 g/100 g) and FPHs or BHT (described above), were solubilized in water (32 g/100 g). Next, sunflower oil (40 g/100 g) was added slowly under agitation and then the salad dressing was homogenized in Ultra-turrax at 15,000 rpm for 1 min in an ice bath. The salad dressings were stored at 4ºC and the next day (day 1 of storage) the analyses of proximate composition (same methodology described in 2.2), optical microscopy, and rheology were performed. Color, texture, and oxidative stability analyses were performed at 0, 15, and 30 days of storage.

The morphology of the emulsions was evaluated using an optical microscope (Nikon Eclipse E200). The samples were diluted in the proportion of 1:5 (v:v) with distilled water and a drop of this dilution was placed on a glass slide and carefully covered

with a coverslip. The images were captured using a 100× objective lens with a Moticam 2.0 MP digital camera.

The rheological behavior of the salad dressings was evaluated in duplicate at 25 °C using a rheometer (Brookfield DV-III Ultra) with spindle velocity from zero to 100 rpm. The results obtained by the shear stress and viscosity curves were adjusted to the models: Power Law (Equation (7)), Herschel-Bulkley (Equation (8)), Casson (Equation (9)), and Bingham (Equation (10)), where "τ" is the shear stress ($N.m^{-2}$), "η" is the apparent viscosity (mPa.s), "γ" (s^{-1}) is the shear rate, "K" ($Pa.s^{-1}$) is the consistency index, "n" is the dimensionless flow behavior index. Rheological model parameters were determined using non-linear regression using Statistica 7.0 software (Statsoft, USA).

$$\tau = \eta \times \dot{\gamma}^{n} \quad (7)$$

$$\tau = \tau_0 + K \times \dot{\gamma}^{n} \quad (8)$$

$$\tau^{\frac{1}{2}} = K_0 + K \times \dot{\gamma}^{\frac{1}{2}} \quad (9)$$

$$\tau = \tau_0 + \eta \times \dot{\gamma} \quad (10)$$

Fresh salad dressing texture measurements were carried out in a texture analyzer (TA.XT Express, Stable Micro Systems) with a 10 kg load cell, with a back extrusion cell (compression probe of 35 mm diameter). The methodology was adapted from Rojas et al. (2019), in which the samples were subjected to 50% depth compression with a speed of 1 $mm.s^{-1}$ in cylindrical containers with 50 mm diameter and 75 mm height (50 mL of the sample). The firmness parameter was obtained through the maximum force, the consistency parameter was calculated through the area under the curve until the maximum force, the cohesion parameter was obtained by the maximum negative force and, the adhesion parameter was obtained by the area under the curve until the maximum negative force (Liu et al., 2007). Samples of each treatment were analyzed in triplicate.

Colorimetric analysis was performed with a Delta Color colorimeter (Delta Vista 450G) in triplicate from each salad dressing formulation. The color parameters L*, a*, b*, and Chroma (C) were obtained using the colorimeter.

The salad dressing oils (stored for 0, 15, and 30 days) were separated by the Bligh & Dyer method to evaluate their oxidative stability. After separation, the oils were filtered in 0.45 µm syringe filters and diluted 1,000 × with isooctane solvent. The procedure was performed in duplicate, and its oxidation state was evaluated using a UV-Vis spectrophotometer (Ocean Optics model USB-650-UV-VIS).

To evaluate oxidation, the absorbance of the solutions at 232, 266, 270, and 274 nm (10 mm quartz cuvette) were evaluated, respectively, and the extinction coefficients were determined according to Equations 11, 12, and 13, respectively (P. Santos et al., 2020).

$$K_{270} = \frac{A_{270}}{cl} \quad (11)$$

$$K_{232} = \frac{A_{232}}{cl} \quad (12)$$

$$\Delta K_{232} = A_{270} - \frac{A_{266} + A_{274}}{2} \quad (13)$$

K_{270} and K_{232} are the extinction coefficients at 270 and 232 nm, respectively; A_{270}, A_{232}, A_{266}, and A_{274} are the absorbance at 270, 232, 266, and 274 nm, respectively; *c* is the oil concentration (g.100 mL^{-1}); *l* is the optical pathway (1 cm). The extinction coefficients obtained were analyzed by hierarchical cluster analysis (HCA) (Santos et al., 2018) using the software MATLAB R2021a (Mathworks Inc., Natick, MA).

2.7 *Statistical analysis*

Results were evaluated using analysis of variance (ANOVA), and averages were compared using the Tukey test at a 5% significance level ($p < 0.05$) using the Statistica 7.0 software (Statsoft, USA). *A. cepa* results were analyzed by analysis of variance (ANOVA) and the mean values were compared by the Scott-Knott test with a significance of 0.05, using the software BioEstat®.

3 RESULTS AND DISCUSSION

3.1 *Characterization of FPHs*

The yield, degree of hydrolysis (DH), free amino acids, and antioxidant activity (DPPH, FRAP, and ABTS) of fish protein hydrolysates obtained with different pretreatments are described in Table 1.

Table 1. Yield, degree of hydrolysis (DH), free amino acids, antioxidant capacity (DPPH, FRAP, and ABTS), and AChE activity inhibition of FPHs undergoing pretreatment before hydrolysis (Control - without pretreatment (C), sterilization (EST), and Ultra-turrax (UT)).

Sample	Yield (%)	DH (%)	Free amino acid ($\mu mol_{Gly\ E}/g_{sample}$)	Antioxidant capacity ($\mu mol_{TE}/g_{sample}$)			AChE activity inhibition (%)		
				DPPH	FRAP	ABTS	15 $mg.mL^{-1}$	45 $mg.mL^{-1}$	60 $mg.mL^{-1}$
C	81.01^{a}±3.07	37.90^{a}±0.34	1.057^{a}±0.008	53.77^{a}±0.78	16.57^{a}±0.26	410.49^{a}±2.99	7.46±1.50	20.07±1.68	30.09±2.52
EST	81.68^{a}±2.87	37.66^{a}±0.05	1.073^{b}±0.001	52.89^{a}±2.35	15.45^{b}±0.25	348.37^{b}±3.66	9.54±1.70	20.99±2.18	27.39±1.23
UT	77.19^{a}±3.30	40.55^{b}±0.25	1.013^{c}±0.004	45.46^{b}±1.12	15.80^{ab}±0.45	318.26^{c}±0.33	12.14±3.90	29.58±1.12	45.87±3.20

^{a,b} columns with different letters indicate the significant difference with a significance level of 5% by the Tukey test.

The hydrolysis yield values were 81.01, 81.68, and 77.19% for hydrolysates submitted to C, EST, and UT samples, respectively, demonstrating that enzymatic hydrolysis was conducted properly. DH represents the percentage of cleavage of peptide bonds (Kristinsson & Rasco, 2000) and the values found ranged from 37.66 to 40.55%, which was close to those obtained by Yarnpakdee et al. (2014) (30 and 40%) and, higher than Robert et al. (2015) (22.1%) and Foh et al. (2010) (25.43%). This difference may be due to the parts of the fish, enzymes, and their concentrations used in hydrolysis.

In the analysis of free amino acids, it can be verified that the free amino acid content was influenced by the different pretreatments ($p < 0.05$). The FPH in which the substrate was submitted to the pretreatment of sterilization (EST) presented a higher value of free amino acids, possibly due to the modifications in protein structure and denaturation resulting from the thermal treatment (X. Li et al., 2021; Rivero-Pino et al., 2020). On the other hand, among all FPH samples, the one in which substrate was obtained by homogenization (UT) presented the lowest free amino acids result.

3.1.1 *Antioxidant capacity of FPHs*

Through the antioxidant analyses presented in Table 1, it was possible to verify significant differences ($p < 0.05$) between the samples, demonstrating that substrates' pretreatment can influence the bioactive properties of the FPH.

In all the evaluated antioxidant methods (DPPH, ABTS, and FRAP), the UT sample presented the lowest values among the samples. This may have occurred as a consequence of the protein aggregation having initially decreased the exposed sites of alcalase recognition. The enzyme interacts with insoluble protein particles to a greater extent in the initial phase of the hydrolysis process, then the polypeptide chains that are weakly bound to the surface are hydrolyzed, and ultimately the more compacted proteins in the nucleus are cleaved more slowly (Benjakul & Morrissey, 1997; Klompong et al., 2007). Therefore, as hydrolysis may have occurred with greater access limitations of the enzyme in the initial phase, peptide radicals contained in FPH with antioxidant properties were not released easily, remaining inactive within the sequence of precursor protein molecules (Chalamaiah et al., 2012).

In the DPPH analysis, the C and EST samples did not differ significantly ($p > 0.05$), however, in the FRAP and ABTS analyses, there was a significant difference ($p < 0.05$), with higher antioxidant properties determined for the control sample (16.57 –

410.49 $\mu mol_{TE}/g_{sample}$, FRAP, and ABTS respectively) when compared to the heat treatment (EST) (15.45 – 348.37 $\mu mol_{TE}/g_{sample}$). Korczek et al. (2020) obtained a similar result and attributed this lower antioxidant activity to changes in secondary and tertiary protein structures after heating.

Despite this, all FPHs presented antioxidant capacity in the range of 15.45 to 410.49 $\mu mol_{TE}/g_{sample}$, values higher than those found in the literature for FPHs. Guo et al. (2019) for instance, reported antioxidant activity of 174.68 $\mu mol_{TE}/g_{fish}$ for FPH from Armoured Catfish using the ABTS method. Korczek et al. (2020) obtained FRAP results equal to 2.30 $\mu mol_{TE}/mg_{fish}$ for fish (Mackerel, *Scomber scombrus*) treated by frying before hydrolysis and 1.73 $\mu mol_{TE}/mg_{fish}$ for fish that has been hydrolyzed in its raw state.

3.1.2 *AChE activity inhibition of FPH*

Because AChE is one of the main enzymes of Alzheimer's disease, its inhibition leads to increments of communication between the activities in the cholinergic pathway and nerve endings, resulting in lesser symptoms of Alzheimer's disease (Yener et al., 2020). AChE inhibition by FPHs ranged between 7.46 and 45.87% (Table 1). The AChE inhibition increased for all samples according to the concentration analyzed (15, 45 e 60 $mg.mL^{-1}$). The results were similar to those found in a in previous work, Moreira et al. (2022) obtained inhibitions from 10.51 to 40.45% at concentrations of 20, 30, and 50 $mg.mL^{-1}$ of FPHs obtained from Nile Tilapia residues under different hydrolysis conditions. Naik et al. (2021) obtained inhibitions of 27.48 and 25.43% but with lower concentrations (1 $mg.mL^{-1}$) for FPHs fractions obtained from a blend of Mesopelagic fish (*Maurolicus muelleri* and *Meganyctiphanes norvegica*).

3.1.3 *Functional and chemical properties of FPHs*

Figure 1 presents the results of the solubility (A), emulsifying activity index (EAI) (B), emulsifying stability index (ESI) (C), and FTIR (D) analysis.

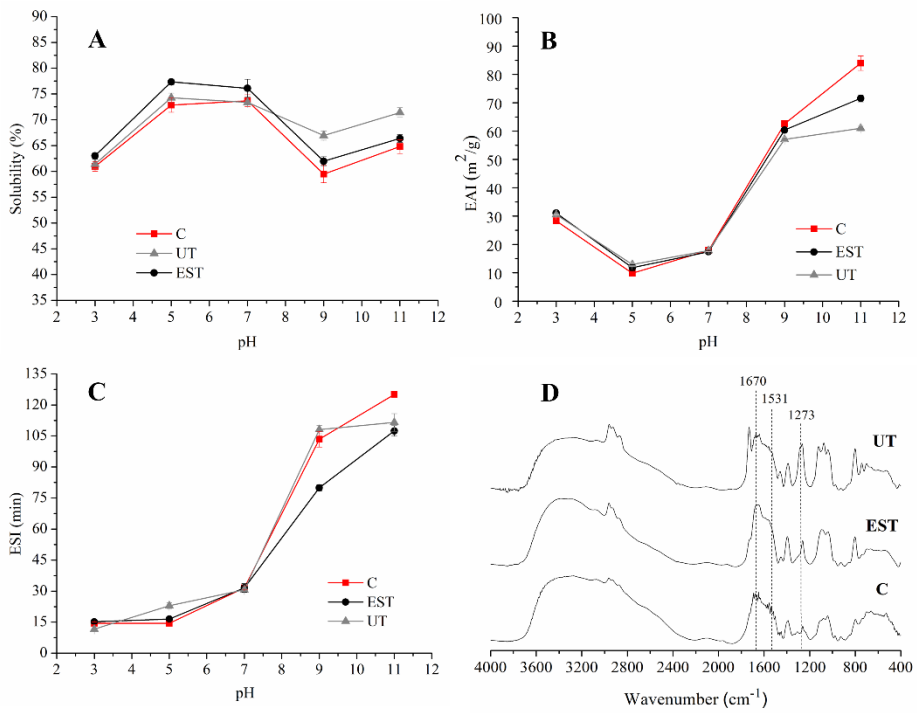

Figure 1. Solubility (A), emulsifying activity index (EAI) (B), emulsifying stability index (ESI) (C), and FTIR spectra (D) of FPHs undergoing pretreatment before hydrolysis (Control - without pretreatment (C), sterilization (EST) and Ultra-turrax (UT)).

The solubility of the FPHs was evaluated in the pH range from 3 to 11, as can be observed in Figure 1 (A). Solubility is an indicator of protein functionality widely used to evaluate the denaturation or aggregation of proteins. This property is highly pH-dependent and is linked to the performance of proteins when applied to foods, mainly in emulsions, foams, and gels (Chalamaiah et al., 2010; Zhang et al., 2019). The solubility remained stable in the range of pH 5 to 7. The lowest solubility for all sampled was observed at the pHs 3 and 9, possibly by the difference in isoelectric points of the peptides that were influenced by a load of acid and basic lateral groups. At pH 11, an intermediate solubility was found for all samples, however, the UT sample had the highest solubility among the samples.

This difference may have occurred due to different modifications according to the structure of the proteins in the substrate (Thoresen et al., 2020). The high solubility obtained may have occurred by peptide hydrolysis (related to the highest DH found for

the UT sample), resulting in smaller sizes and greater hydrophilic properties, thus suggesting that the application in food formulations can contribute to the appearance and softness in the mouth (Thiansilakul et al., 2007).

An increase in the emulsifying capacity and stability in basic pH can be observed in Figures 1 - B and C. In addition, EAI results corroborated the solubility values, since EAI was higher in the pH range where the solubility was lower. This is possibly related to the increased hydrophobicity of proteins in pH 9 and 11. Due to negative charges, peptides can aggregate or perform self-assembly interactions creating a protective membrane in the oil droplets during homogenization that prevents the coalescence of the droplets (Gbogouri et al., 2004; Yesiltas et al., 2021). On the other hand, the higher solubility found from pH 5 to 7 may have resulted in an increased exposition of the peptides' hydrophilic sites, decreasing the hydrophobic-hydrophilic balance between the interaction sites at the oil-water interface and, consequently, decreasing the emulsifying property. With this, there may be undissolved peptides that accumulate at the bottom of the oil drop and can cause deformation and decrease the surface tension of the emulsion (Yesiltas et al., 2021).

In the FTIR spectra (Fig. 1 - D) the chemical structure of FPHs peptide-binding groups were observed in the characteristic bands: amide I (1670 cm^{-1}), amide II (1531 cm^{-1}), and amide III (1261 cm^{-1}) (Andrade et al., 2019). Amide I peak at 1700-1600 cm^{-1} usually results from C=O stretching vibration, C – N stretching, and N – H bending vibrations (Stani et al., 2020). Amide I bands are widely associated with the secondary structure of proteins, the 1670 cm^{-1} peak observed in FPH may be related to β-turn or β-sheet structures, which occur in 1675-1662 cm^{-1} (Vaskoska et al., 2021). Amide II (1600-1500 cm^{-1}), observed at 1531 cm^{-1}, may have been caused by the combination of the C – N elongation vibrations of the peptide with vibrations of curvature N – H. Amide III (1310-1175 cm^{-1}) band identified at 1261 cm^{-1} may be related to the elongation C - N, bending vibrations N – H, elongation C – C and flexion C – H (Stani et al., 2020).

3.1.4 *Cytotoxic and genotoxic analysis in Allium cepa L.*

Table 2 presents the results of cytotoxicity and genotoxicity *in vivo* analyses of the *Allium cepa* L. bulbs submitted to contact with FPHs.

Table 2. Mitotic indices (%) observed in root meristems of *A. cepa* exposed to FPH at 24 and 48 h exposure times.

Positive control		MI (%)/SD	
Co: MMS ($4x10^{-4}$ $mol.L^{-1}$)		4.78±0.94	
		MP (%)/SD	
FPH	Co (0 h)	24 h	48 h
EST	23.7[a]±0.97	14.5[a]±1.82	16.9[a]±1.92
C	19.6[a]±1.23	17.4[a]±1.74	15.3[a]±1.99
UT	20.4[a]±1.34	10.8[a]±1.69	13.5[a]±1.52

control; MI: mitotic index; TR: treatment; h: hour. MMS: methyl methanesulfonate (positive control). The same letters refer to similar results means between the exposure times considered (Co-0h, 24h, and 48h) at the same treatment by the Scott-Knott test at 0.05. SD: Standard deviations reported for each analysis time evaluated for each concentration. *Mitotic index equal to the mitotic index observed for the MMS control.

It can be verified that there were no differences between the mitotic indices obtained for each exposure time within the same treatment. Therefore, none of the FPH treatments evaluated caused cytotoxicity to the meristematic cells of *A. cepa* root meristems. The results obtained through the bioassay with *A. cepa* have a satisfactory correlation with the results observed in genetic tests performed in other bioassays, such as those with mammals and in cell culture (Herrero et al., 2012). This indicates that the FPH produced in this work can be safely applied as additives in food.

3.2 *Application of FPHs in salad dressing*

Figure 2 shows the emulsion microstructure and the appearance of the salad dressings. The emulsion characteristics of BHT and C samples were similar and the FC sample presented smaller drops when compared to BHT and C samples. For the EST sample, a smaller number of large drops can be observed, and the UT sample presented a greater amount of large drops. This result may be associated with the solubility and emulsifying activity. As the hydrolysate UT showed greater solubility, in the emulsion the proportion of amphiphilic proteins decreased, containing most hydrophilic proteins, thus the protective membrane was formed in smaller amounts resulting in larger and less stable oil drops.

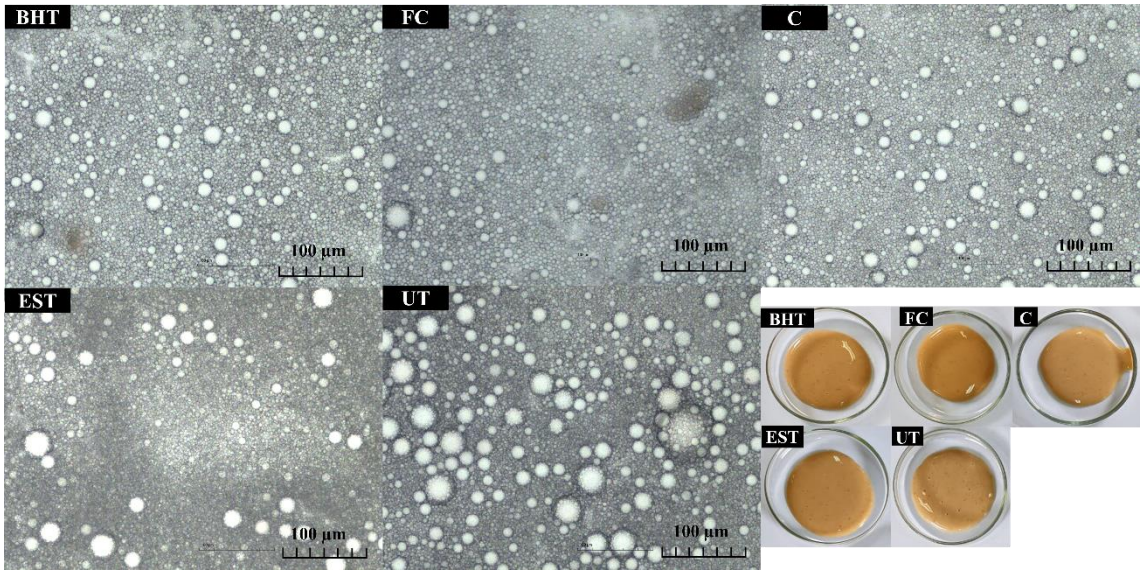

Figure 2. Optical microscopy (10×) and images of salad dressing emulsions added from BHT antioxidant, control formulation (FC, without antioxidants or FPHs), and, FPH samples (C, EST, and UT).

Table 3. Proximate composition of Nile Tilapia muscle (*Oreochromis niloticus*) and French salad dressing formulations.

Formulation	Proximate composition (%)			
	Moisture	Ash	Protein	Lipids
Fish muscle	62.15 ± 2.35	0.87 ± 0.09	35.25 ± 2.29	1.19 ± 0.74
FC	32.44^{ab} ± 0.09	2.99^{a} ± 0.10	4.21^{a} ± 0.15	44.39^{a} ± 0.92
BHT	34.27^{ab} ± 0.31	3.044^{ab} ± 0.14	4.19^{a} ± 0.62	45.30^{a} ± 3.37
C	33.12^{ab} ± 0.35	3.12^{ab} ± 0.02	8.65^{bc} ± 0.09	46.19^{a} ± 1.36
EST	31.48^{a} ± 2.12	4.22^{c} ± 0.02	9.19^{c} ± 0.09	45.38^{a} ± 2.10
UT	34.78^{b} ± 1.26	3.41^{b} ± 0.29	7.78^{b} ± 0.15	45.85^{a} ± 3.14

^{a,b} means of the different treatments (FC, BHT, C, EST, and UT) followed by different letters show the significant difference as a function of the treatment ($p < 0.05$) by the Tukey test.

From the analysis of the salad dressing proximate composition (Table 3), it was possible to verify an increase in the proportion of protein existing in the added formulations of FPH, with 8.65, 9.19, and 7.18% for C, EST, and UT, respectively, differently from the formulations without the FPHs addition, who presented 4.21 and 4.19% for FC and BHT, respectively, a result close to that found by Gomes et al. (2008) for French salad dressing in the sample without the addition of the protein (3.78%). The other ingredients were similar for all formulations and, to that found by de Melo et al.

(2015) in French salad dressing with small differences related to the sources and proportions of ingredients used in the formulation.

3.2.1 *Rheological behavior*

Shear stress and viscosity curves (Figure 3) for salad dressing formulations were adjusted to the mathematical models (Power Law, Herschel-Bulkley, Casson, and Binghan) and are presented in Table 4.

All the mathematical models evaluated demonstrated the ability to adjust the experimental data, however for all samples the best fit was determined for the Herschel-Bulkley model. All the samples presented pseudoplastic behavior, in addition, all samples containing antioxidants (BHT and FPH) had a higher consistency index (K) and shear stress (τ_0) when compared to the control sample for all the mathematical models evaluated. This demonstrates the viscous nature of the samples, and the shear stress is an important factor for salad dressing because it relates to a greater retention capacity of the sauce on the surface of the salad (Bortnowska et al., 2014; Liu et al., 2007).

Concerning the Herschel-Bulkley model parameters, the behavior index (n) showed an increase in the function of FPH addition to the salad dressing formulations. The highest value was found for UT, followed by C and EST.

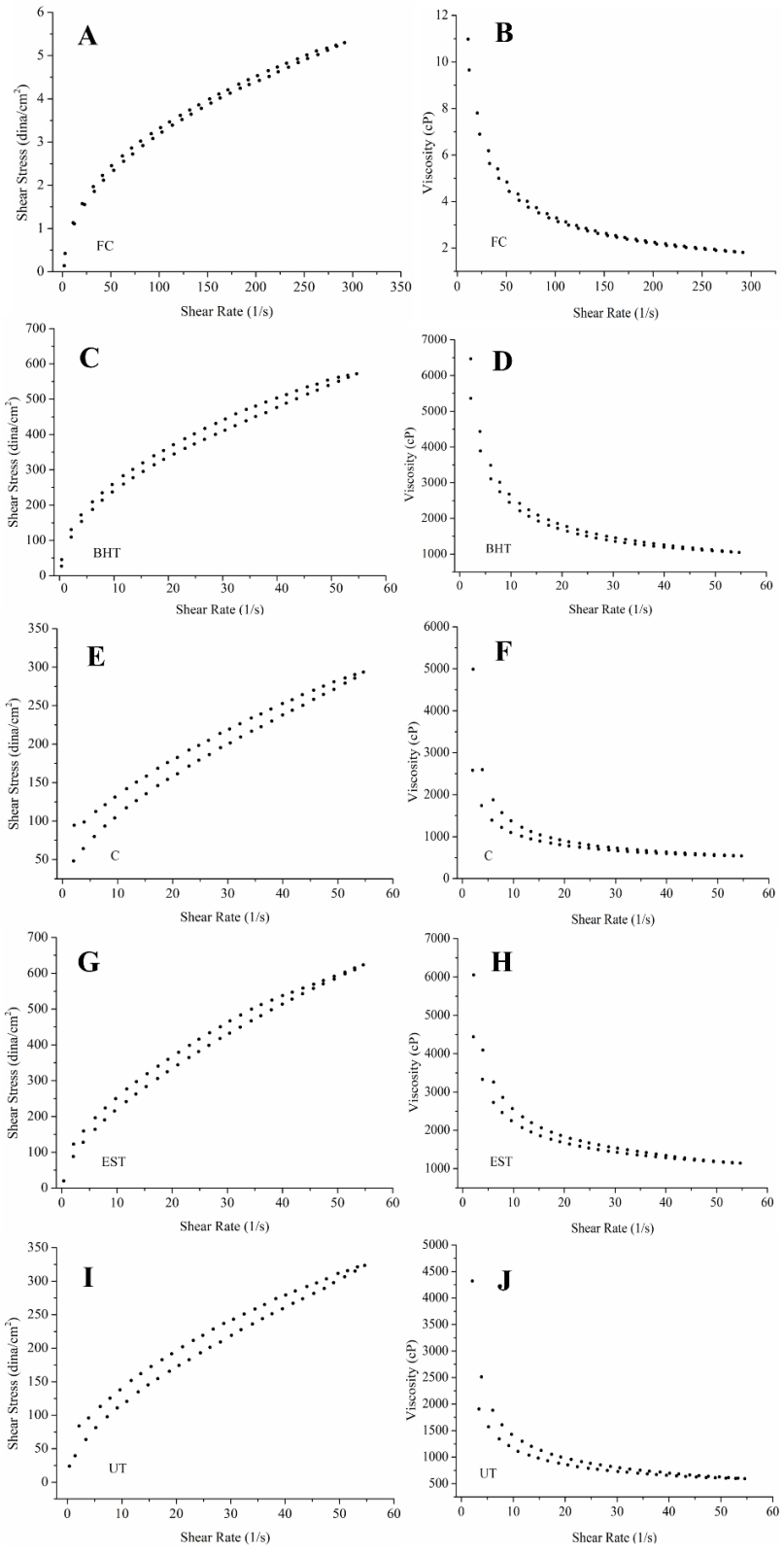

Figure 3. Shear stress and viscosity curves to salad dressing formulations. Shear stress results: (A) FC; (C) BHT; (E) C; (G) EST and, (I) UT. Viscosity results: (B) FC; (D) BHT; (F) C; (H) EST and, (J) UT.

The samples presented shear thinning behavior ($0 < n < 1$), in emulsions, this behavior represents that the structure was irreversibly broken due to the shear rate,

resulting in the redistribution of the oil drops that form the emulsion. The parameter K > 0 represents consistency and indicates that the FPH and BHT provided the natural viscosity of the fluid (Kumar et al., 2021; Saramito, 2009). On the other hand, the values of the τ_0 parameters observed in the samples indicate a high suspension capacity, an important property for FPH to be used as stabilizers in food, especially in sauces and mayonnaises (Hosseini-Parvar et al., 2010).

Among the samples containing FPH, the EST formulation presented lower τ_0 and n and higher K, this may indicate that the structure was not broken properly, thus affecting its suspension capacity. These factors, together with the high consistency of the sample, can demonstrate that the EST formulation presents a trend of phase separation.

Table 4. Parameters determined for the rheological mathematical models applied to salad dressing formulations data: control formulation (FC), added with BHT, and FPH samples (C, EST, and UT).

Samples	Power Law			Herschel-Bulkley				Casson			Bingham		
	K (mPa.s)	n	R^2	K (mPa.s)	τ_0 (N/m^2)	n	R^2	η (mPa.s)	τ_0 (N/m^2)	R^2	η (mPa.s)	τ_0 (N/m^2)	R^2
FC	22.8	0.57	91.6	60.3	0.05	0.40	99.8	0.90	0.06	93.9	1.44	0.15	71.2
BHT	7166	0.53	95.3	8916	1.23	0.47	99.2	513.2	6.38	95.4	842.5	15.2	81.2
C	3533	0.53	92.6	2633	1.95	0.58	97.4	247.7	3.32	95.1	422.3	7.53	84.1
EST	5675	0.61	94.6	6708	0.53	0.56	99.1	639	4.88	96.0	962	13.5	82.8
UT	3967	0.52	93.4	2870	1.60	0.59	98.1	291.4	3.24	95.9	481.2	7.64	88.1

η- apparent viscosity; n-behavior index; K- consistency index, τ_0 - shear stress and R^2- coefficient of determination.

3.2.2 *Color and texture parameters*

Color parameters (L*, a, b and C) and texture parameters (firmness, cohesiveness, adhesion work, and consistency) are presented in Table 5.

In general, samples presented a small variation concerning color during storage. Samples showed high luminosity (L*), the tendency to red (+a), yellow (+b), and relatively low color intensity (C), which confirm the characteristic orange color of French salad dressing.

It can be observed that the most important reduction in luminosity occurred in the UT sample (67.75 on the initial day of analysis and 60.01 on the 30^{th} day). Chung et al. (2014) found variations in luminosity by adding whey protein microparticles to salad dressing. Also, Melo et al. (2015) observed a decrease in this parameter after storage of 28 days of French salad dressing and relates the luminosity to the efficiency of the compound in the formation of regular fat crystals in the emulsion.

In the texture analysis, the formulations also presented a little variation during storage. The formulations BHT and FC showed statistical similarity ($p > 0.05$) between themselves but differed ($p < 0.05$) from the formulations containing FPHs, which were similar to each other in all parameters. Formulations containing FPHs showed firmness, cohesiveness, adhesion work, and consistency values close to those obtained by Rojas et al. (2019) in mayonnaise samples, by the characteristics of this product, may indicate a possible action of the FPHs as emulsifiers on the salad dressing.

Table 5. Color and texture parameters of salad dressing: control formulation (FC), added with BHT, and FPH samples (C, EST, and UT).

Sample	Day	Color parameters				Texture parameters			
		L*	a	b	C	Firmness (N)	Cohesiveness (N)	Adhesion work (N.s)	Consistency (N.s)
FC	0	$71.43^{ab,A}$±0.70	$9.79^{ab,A}$±0.57	$31.17^{a,A}$±0.39	$32.68^{a,A}$±0.45	$0.25^{a,A}$±0.02	$-0.10^{a,A}$±0.00	$-1.58^{a,A}$±0.10	$3.64^{a,A}$±0.08
	15	$69.95^{a,B}$±0.49	$10.88^{a,B}$±0.16	$33.67^{a,B}$±0.50	$35.38^{a,B}$±0.39	$0.19^{a,B}$±0.00	$-0.09^{a,A}$±0.00	$-0.98^{a,B}$±0.12	$2.98^{a,B}$±0.01
	30	$71.20^{a,A}$±0.64	$10.20^{a,AB}$±0.81	$33.84^{a,B}$±0.47	$35.36^{a,B}$±0.36	$0.25^{a,A}$±0.01	$-0.09^{a,A}$±0.00	$-1.12^{a,B}$±0.05	$2.98^{a,B}$±0.13
BHT	0	$71.58^{b,A}$ ±0.47	$10.23^{ab,A}$±0.38	$31.23^{a,A}$±0.49	$32.86^{a,A}$±0.49	$0.18^{a,A}$±0.00	$-0.10^{a,A}$±0.00	$-1.39^{a,A}$±0.06	$3.14^{a,A}$±0.07
	15	$70.22^{a,AB}$ ±0.60	$10.73^{a,A}$±0.28	$32.56^{b,B}$±0.33	$34.29^{b,B}$±0.35	$0.22^{a,B}$±0.00	$-0.11^{a,A}$±0.00	$-1.49^{a,A}$±0.20	$3.52^{a,B}$±0.05
	30	$69.11^{a,B}$±1.50	$10.64^{a,A}$±0.47	$34.41^{a,C}$±0.64	$36.02^{a,C}$±0.66	$0.23^{a,C}$±0.00	$-0.10^{a,A}$±0.00	$-1.30^{a,A}$±0.08	$3.45^{a,B}$±0.04
C	0	$65.22^{c,AB}$±3.47	$9.29^{b,A}$±1.023	$33.15^{b,A}$±1.15	$34.44^{b,A}$±1.13	$1.33^{b,A}$±0.05	$-0.78^{b,A}$±0.05	$-15.25^{bc,A}$±1.11	$19.85^{bc,A}$±0.94
	15	$67.11^{b,A}$±0.54	$10.84^{a,A}$±0.33	$34.07^{a,A}$±0.20	$35.77^{a,A}$±0.15	$1.31^{b,A}$±0.08	$-0.92^{b,A}$±0.11	$-15.30^{b,A}$±0.23	$19.32^{b,A}$±1.28
	30	$62.85^{b,B}$±2.53	$10.03^{a,A}$±1.45	$32.80^{a,A}$±1.45	$34.30^{a,A}$±2.10	$1..41^{b,A}$±0.10	$-0.85^{b,A}$±0.10	$-14.70^{b,A}$±0.42	$19.74^{b,A}$±1.37
EST	0	$68.74^{abc,A}$±3.02	$10.83^{a,A}$±0.86	$33.31^{b,A}$±0.71	$35.05^{b,A}$±0.74	$1.50^{c,A}$±0.09	$-0.80^{b,A}$±0.12	$-16.72^{b,A}$±0.97	$21.80^{b,A}$±0.84
	15	$65.64^{c,AB}$±1.45	$10.93^{a,A}$±0.19	$33.87^{a,A}$±0.21	$35.59^{a,A}$±0.20	$1.39^{b,AB}$±0.09	$-0.82^{b,A}$±0.05	$-14.66^{b,AB}$±0.35	$19.55^{b,A}$±2.05
	30	$62.28^{bc,B}$±1.99	$10.33^{a,A}$±1.02	$33.25^{a,A}$±2.25	$34.82^{a,A}$±2.40	$1.26^{b,B}$±0.09	$-0.83^{b,A}$±0.05	$-14.37^{bc,B}$±1.03	$18.30^{b,A}$±1.22
UT	0	$67.75^{ac,A}$±1.75	$10.53^{a,A}$±0.55	$33.05^{b,AB}$±0.38	$34.72^{b,AB}$±0.49	$1.23^{b,A}$±0.05	$-0.68^{b,A}$±0.02	$-13.51^{c,A}$±0.44	$18.54^{c,A}$±1.40
	15	$67.06^{b,A}$±0.67	$10.74^{a,A}$±0.74	$33.78^{a,A}$±0.63	$35.46^{a,A}$±0.75	$1.42^{b,B}$±0.09	$-0.86^{b,B}$±0.07	$-15.26^{b,B}$±0.40	$19.42^{b,A}$±1.81
	30	$60.01^{c,B}$±0.62	$9.75^{a,A}$±1.88	$32.19^{a,B}$±1.11	$33.67^{a,B}$±1.60	$1.26^{b,AB}$±0.04	$-0.78^{b,AB}$±0.03	$-13.16^{c,A}$±0.06	$18.29^{b,A}$±1.11

Mean ± standard deviation (n = 3); ^{a,b} means of the different treatments (FC, BHT, C, EST, and UT) from the same day of analysis followed by different letters (for each parameter and different formulations) show the significant difference as a function of the treatment ($p < 0.05$) by the Tukey test; mean $^{A, B}$ of the same formulation followed by different letters (for each parameter and different days) show significance difference ($p < 0.05$) by Tukey's test.

3.2.3 *Oxidative stability*

The oxidative stability of salad dressing was evaluated using the extinction coefficients (K_{270}, K_{232}, and ΔK) as shown in Table 6 and Figure 4 by *hierarchical cluster analysis* (HCA).

Table 6. Extinction coefficients (K_{270}, K_{232} and ΔK) of salad dressing added from BHT, control formulation (FC) and, FPH samples (C, EST and UT)

Sample	Days	K_{270}	K_{232}	ΔK
FC	0	$4.39^{ac,A} \pm 0.39$	$6.50^{a,A} \pm 0.41$	$4.04^{ac,A} \pm 0.35$
	15	$6.11^{a,AB} \pm 0.42$	$8.14^{ab,AB} \pm 0.03$	$5.61^{a,AB} \pm 0.40$
	30	$6.65^{a,B} \pm 0.70$	$10.12^{b,B} \pm 0.93$	$6.11^{a,B} \pm 0.64$
BHT	0	$5.37^{b,A} \pm 0.09$	$8.30^{c,A} \pm 0.09$	$4.93^{b,A} \pm 0.08$
	15	$6.43^{a,B} \pm 0.11$	$9.46^{c,B} \pm 0.46$	$5.90^{a,B} \pm 0.10$
	30	$6.80^{a,B} \pm 0.04$	$9.76^{ab,B} \pm 0.10$	$6.23^{a,B} \pm 0.04$
C	0	$5.14^{ab,A} \pm 0.05$	$6.95^{a,A} \pm 0.12$	$4.73^{ab,A} \pm 0.05$
	15	$6.24^{a,B} \pm 0.16$	$9.06^{bc,B} \pm 0.17$	$5.73^{a,B} \pm 0.14$
	30	$6.751^{a,C} \pm 0.007$	$9.23^{ab,B} \pm 0.18$	$6.197^{a,C} \pm 0.008$
EST	0	$5.26^{ab,A} \pm 0.23$	$6.55^{a,A} \pm 0.05$	$4.83^{ab,A} \pm 0.20$
	15	$5.83^{a,A} \pm 0.16$	$6.97^{a,AB} \pm 0.04$	$5.356^{a,A} \pm 0.008$
	30	$6.31^{a,A} \pm 0.54$	$7.79^{ac,B} \pm 0.50$	$5.80^{a,A} \pm 0.50$
UT	0	$4.02^{c,A} \pm 0.27$	$5.20^{b,A} \pm 0.14$	$3.70^{c,A} \pm 0.25$
	15	$5.91^{a,B} \pm 0.39$	$7.11^{a,B} \pm 0.49$	$5.43^{a,B} \pm 0.36$
	30	$5.57^{a,AB} \pm 0.47$	$6.98^{c,B} \pm 0.48$	$5.12^{a,AB} \pm 0.43$

Mean ± standard deviation (n = 3); $^{a, b}$ means of the different treatments (FC, BHT, C, EST, and UT) from the same day of analysis followed by different letters (for each parameter and different formulations) show the significant difference as a function of the treatment ($p < 0.05$) by the Tukey test; mean $^{A, B}$ of the same formulation followed by different letters (for each parameter and different days) show significance difference ($p < 0.05$) by Tukey's test.

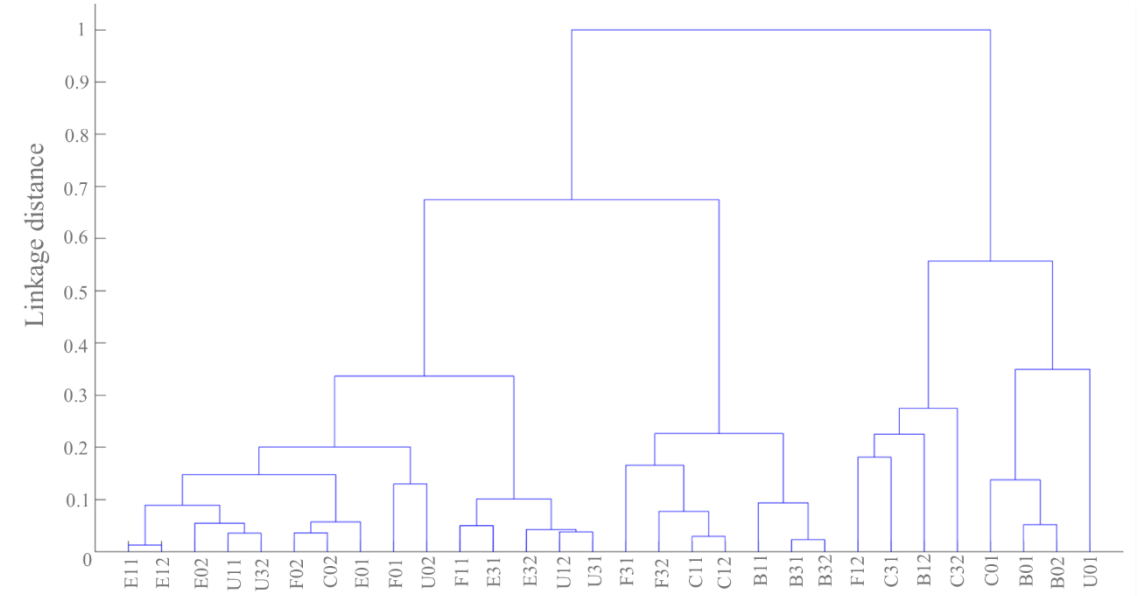

Figure 4. Hierarchical Cluster Analysis (HCA) of the extinction coefficients (K_{270}, K_{232}, and ΔK) data of salad dressing added from BHT (B), control formulation (F), and FPH samples: Control (C), EST (E), and UT (U).
*Samples codification: the letter represents the treatment followed by the storage day 0, 15, and 30 days (0, 1, and 3, respectively) and the replica of the experiment (1 or 2). For instance, B01 = BHT sample at 0 days of storage with its 1st replica.

The oxidation of polyunsaturated fatty acids can be verified at 232 and 270 nm by an increase in absorption in the ultraviolet region due to the formation of conjugated dienes and trienes (Santos et al., 2020). There was an increase in the extinction coefficient according to the storage time for all formulations, demonstrating the lipid oxidation in the salad dressing. It can be noted in Figure 3 that after 30 days of storage the samples without any antioxidant added (F31, F32) and added with FPH produced without any pretreatment (C31 and C32) are grouped, showing similarity. On the other hand, samples added with the FPH pretreated by sterilization, after 30 days of storage (E31, E32) were grouped with the control formulation analyzed after 15 days (F11), the Ultra-turrax treated samples that were analyzed after 15 days of storage (U12) and after 30 days (U31). This is an indication that the oxidation state of the oil present in the salad dressing prepared without antioxidants (FC) was reached by the samples added with Ultra-turrax pretreated FPH after 15 days of storage, and that the oxidation state of this sample was still similar after 30 days. Furthermore, the salad dressing prepared with FPH pretreated by sterilization only reached this oxidation state after 30 days of storage, showing an increased capacity of preservation among the FPHs tested. This result may be associated with larger agglomerated oil droplets due to the higher solubility of UT having decreased the proportion of amphiphilic proteins and thus decreased the number of protective

membranes in emulsions. Thus, by the lower contact of the oil with water molecules, the formulation containing UT may have oxidized less. On the other hand, BHT, FC, and C samples were shown to be more susceptible to lipid oxidation.

Despite this, in general, results demonstrated that FPH presented a high antioxidant capacity and can assist in the oxidative stability of emulsified foods acting in the elimination of radicals generated in lipid oxidation.

4 CONCLUSION

The pretreatments of the substrate by sterilization and homogenization were evaluated to obtain FPHs with antioxidant and AChE inhibition properties. These FPHs were also evaluated concerning modifications in functional properties and their behavior when applied to salad dressing. In hydrolysis, the FPHs (C, EST, and UT samples) rpesented satisfactory yield values (81.01, 81.68, and 77.19%) and DH (37.90, 37.66, and 40.55%). In addition, FPH showed antioxidant and AChE inhibition properties. Regarding functional properties, the highest solubility of FPHs occurred at pH 11, and therefore emulsifying and emulsion stability capacity was also observed at basic pH. In salad dressing, the FPH showed pseudoplastic behavior, in addition to increasing the protein content and maintaining the characteristics of color and texture of the food. FPHs also demonstrated efficacy in assisting in oxidative stability of the salad dressing, thus indicating possible action as radicals scavenger.

5 ACKNOWLEDGEMENTS

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6 DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information file.

7 CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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