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Research article

Optimization of fractionation with membranes of antioxidant enzymatic hydrolysate of Californian red worm (*Eisenia fetida*) protein

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ABSTRACT

Problem: Earthworm is a valuable source of biologically and pharmacologically active compounds, with applications in the treatment of various types of diseases; however, the main application they have been given is in the production of organic fertilizer. One of the alternatives for obtaining bioactive compounds is by means of enzymatic hydrolysis.
Aim: This study proposes the optimization of the fractionation of the antioxidant enzymatic hydrolysate from Californian red worm (*Eisenia fetida*) protein.
Methodology: For this purpose, the worms were separated and hydrolyzed using the enzyme Alcalase 2.4L for 4000s. The obtained hydrolysate was fractionated by means of a crossflow tangential ultrafiltration system, with a 3 kDa molecular weight cut-off ceramic membrane. A response surface design of the composite central factorial type was implemented to evaluate the effect of pH, transmembrane pressure, and flow factors on the response variables transmission, volume reduction factor (VRF) and permeate flow resistance. The transmissions focused on the antioxidant peptides, measured by three conventional methods such as TEAC, FRAP, ORAC, also known as TTEAC, TFRAP and TORAC, respectively. The evaluated resistances were the total resistance (Rtotal), fouling resistance (Rfouling), and gel resistance (Rgel).
Result: The results showed that the three factors evaluated affect all the response variables either in their linear or quadratic terms or by some interaction. For each response variable, a mathematical model was obtained, with statistical significance and a non-significant lack of adjustment. The models obtained were used for a multi-objective optimization process in which transfers were maximized, and resistances were minimized. The efficiency of the optimum ultrafiltration process was 25 %.
Conclusion: The neutral-alkaline pH is ideal for the ultrafiltration process of bioactive peptides, as it is where the highest transmissions of peptides with antioxidative capacity are found. Under optimal conditions, the 3 kDa membrane permeate was found to exhibit higher antioxidant capacity than the retentate and feed. Based on this, the fraction of less than 3 kDa emerges as a potential multifunctional ingredient, thanks to its antioxidant properties.

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1. Introduction

Agro-industrial development is essential to ensure food availability for the population in the modern world. Inevitably, the vast amount of agro-industrial products generated are accompanied by equally large volumes of wastes and by-products, either during harvest, such as leaves, stems and roots, or during post-harvest, such as deteriorated fruits, products with non-commercial sizes, or processing residues [1]. All these wastes generate a high environmental impact due to their high content of organic matter [2], which, in turn, makes them an opportunity to obtain value-added products. One of the alternatives with the most significant potential in this regard is lumbriculture, which takes advantage of vegetable waste and animal excreta for the production of agricultural inputs and worm meat [3]. The cultivation of the Californian red worm (*Eisenia fetida*) has certain advantages thanks to its rapid growth, high reproduction rate and ease of handling [4]. Although the protein obtained from worm meat is of very high quality, in developing countries its cultivation has focused mainly on producing organic fertilizer [2]. On the other hand, earthworms have been used in various ancient cultures as a source of therapeutic compounds [5], while in recent years, knowledge on the compounds responsible for these properties has been deepened [6]. Along with a growing interest in bioactive compounds from natural sources, a therapeutic capacity in worm compounds has been found, opening a new horizon in the use of these organisms, given that they have been attributed antioxidant capacity, among other properties [7]. Antioxidants are molecules capable of neutralizing free radicals, thereby reducing oxidative damage, which has been related to the prevention of various chronic diseases and cellular aging [7]. Free radicals can cause damage to biologically important molecules, such as DNA, proteins, and phospholipids, which in turn can contribute to the development of various diseases, including atherosclerosis, arthritis, hypertension, diabetes, and cancer [8].

On the other hand, membrane separation technology represents a highly efficient and non-destructive physicochemical technique, in which the compounds in the permeate maintain the biological activity they contained in the feed stream [9,10]. This technology is characterized by being cost-effective and environmentally friendly [11]. For its application in industrial scenarios, the standardization of operating conditions is required, which guarantees minimum fouling and maximum permeate transmissions and flows, in addition to avoiding deterioration of the membrane and protecting its integrity [12]. In this sense, ceramic membranes surpass polymeric membranes, because they have greater chemical, thermal, and mechanical resistance, although they generally have a higher cost [13]. Various studies have been conducted on the purification and extraction of proteins and peptides possessing diverse biological activities. These include the utilization of porcine collagen hydrolysates for their anti-oxidative and anti-aging properties [14], vegetable protein hydrolysates like douchi with antioxidant properties [15], and the employment of food industry by-products to obtain ultrafiltered fractions of hydrolysates with antioxidant and antimicrobial capacities [16]. However, these studies generally do not assess the optimization of ultrafiltration process conditions. Moreover, to date, no studies have been reported on the fractionation of red Californian earthworm hydrolysates. In the present study, the optimization of the fractionation of enzymatic hydrolysates with antioxidant capacity of Californian red worm (*Eisenia fetida*) protein in a crossflow tangential ultrafiltration system is proposed, using a 3 kDa molecular weight cut-off ceramic membrane.

2. Materials and methods

2.1. Reagents and chemicals

Reagents, chemicals, and solvents used for preparation, hydrolysis, and characterization were supplied by Sigma-Aldrich and Merck (Darmstadt, Germany). Alcalase® 2.4 L (a commercial protease obtained from the fermentation of Bacillus licheniformis, a non-specific serine endopeptidase) was supplied by Novozymes (Bagsværd, Denmark). A ZrO2 ceramic membrane on an Al2O3 support (Tami Inc., France) was used for fractionation via the tangential ultrafiltration system (Standex Electronics, Inc., USA). The reagents 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromo-2-carboxylic acid (Trolox), 3-(2-pyridyl), 2, 2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) and Fluorescein-12-UTP were obtained from Sigma-Aldrich (Oakville, Ontario, Canada) and 2,4,6-Tri-2-pyridyl-*s*-triazine (TPTZ) was supplied by Merck (Darmstadt, Germany).

2.2. Raw material handling

The red Californian earthworm (*Eisenia fetida*) was sourced from "Lombrices de Tenjo" (Cundinamarca, Colombia) and was transported in a feeding substrate until the commencement of the process. Initially, the worms were manually separated from the substrate in which they were found, and subsequently subjected to a wash with potable water to remove any residue from said substrate. A purge process was then performed for 4 h using a sodium bicarbonate ($NaHCO_3$) solution at 4 %. After purging, an additional wash was carried out with potable water to remove any bicarbonate residue. The worms were then sacrificed by immersing them in a 7 % saline (NaCl) solution for a period of 30 min. Finally, the resulting paste was subjected to another wash with potable water and then frozen to be used in subsequent tests [17,18].

2.3. Physicochemical characterization

The samples of Californian red worm (CRW) were analyzed according to the official AOAC, (2000) [19] analysis methods: the moisture content was determined following the methodology established in the AOAC 930.1 standard [19], by drying the sample at 105 °C for 8 h. Protein was quantified by the Kjeldahl method, AOAC 954.010 [19] with a DK 12 heating digester model equipment (Velp Scientifica, USA). The ethereal extract content was carried out according to the AOAC 920.39 standard [19], using a starfish

equipment (Radleys, United Kingdom) soxhlet extractor, with petroleum benzine as solvent. To determine the total ash content, it was analyzed according to the AOAC 942.05 standard [19] by calcining the samples at 550 °C in a model D8 muffle (Terrígeno, Colombia).

2.4. Enzymatic hydrolysis of Californian red worm

The hydrolysis was carried out in a Bioflo & Celligen 310 bioreactor of the New Brunswick brand (Eppendorf, Germany) with a working volume of 6 L. The CRW was added at a concentration of 20 g of protein per liter and the pH was adjusted to 8.5 with 2 N NaOH. Subsequently, the enzyme Alcalase® 2.4 L was added in an enzyme/substrate ratio (E/S) of 18 % at 45 °C, maintaining an agitation of 240 rpm. The hydrolysis time was 67 min. Alcalase 2.4L was selected for the hydrolysis of Californian red worm protein based on its superior efficacy, as demonstrated in previous comparative studies of various enzymes. It has been identified as achieving the highest degree of hydrolysis in the shortest amount of time [20]. Subsequently, the enzyme was inactivated by heating the hydrolysate at 85 °C for 10 min. The hydrolysate was cooled and centrifuged at 8500 rpm for 5 min at 4 °C in a U-320R refrigerated centrifuge (Boeco, Germany); finally, the supernatant was collected and stored at −18 °C.

The reaction was monitored over time by means of the degree of hydrolysis (DH; %), which is defined as the ratio between the number of hydrolyzed peptide bonds (h) and the number of total peptide bonds in the native protein per unit weight (ht), which is calculated by adding the total moles of the amino acids contained in the substrate [21]. The method used for the determination of DH was the proton titration or pH-static method, using equation (1).

$$GH = \frac{\mathbf{BN_B}}{\mathbf{M_P}} \frac{\mathbf{1}}{\alpha} \frac{\mathbf{1}}{\mathbf{h_t}} * 100 \quad (1)$$

Where B corresponds to the volume of sodium hydroxide consumed in L, MP is the mass of protein loaded in the reactor in kg, NB the normality of sodium hydroxide (N), and α the degree of dissociation of the amine groups released in the hydrolysis, calculated as a function of the temperature (Kelvin) and pK of the reaction, according to equations (2) and (3), respectively [22].

$$\alpha = \frac{\mathbf{10^{pH-pK}}}{\mathbf{1 + 10^{pH-pK}}} \quad (2)$$

$$pk = 7.8 + \frac{\mathbf{298\text{-}T}}{\mathbf{298 * T}} * 2400 \quad (3)$$

2.5. Tangential filtration system

The fractionation of the hydrolysate was carried out by means of a tangential ultrafiltration system such as the one presented in Fig. 1. The system consists of a 10-liter capacity tank, a 140-gph food-grade stainless-steel Procon positive displacement rotary vane pump series 3 with NPT 3/8″ connection (Standex Electronics, Inc., USA), two pressure gauges at the inlet and outlet of the membrane, and a flow meter for the flow of retentate (Georg Fischer Signet LLC, USA). For this, 7-channel membranes of 0.013 m^2 filtration surface area and 250 mm in length were used (Tami Inc., France), formed by a filter layer of ZrO_2 on an Al_2O_3 support with molecular weight cut-off of 3 kDa.

2.6. Membrane activation and cleaning

The completely new 3 kDa membrane was conditioned by a hydration process recirculating distilled water with a flow rate of 400 L/h, 50 °C and transmembrane pressure (TMP; Pas) of 1.5 bar for a time of 1.5 h, in a procedure called activation. After this, a cleaning treatment was applied by recirculating a 20 g/L sodium hydroxide solution at 80 °C for a total time of 1 h [23], initially with the valve fully open (TMP = 0) for 15 min and subsequently using a TMP of 4.5 bar for the remaining 45 min [24,25]. Finally, the membrane was

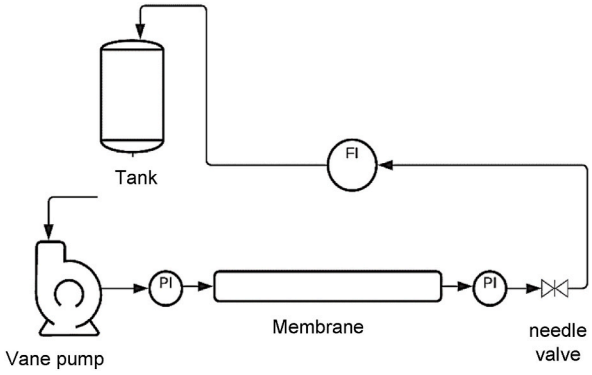

Fig. 1. Diagram of the crossflow filtration system.

rinsed using deionized water to neutral pH, to remove sodium hydroxide residues in the ultrafiltration system [26]. At the end of each hydrolysate filtration test, the same cleaning process was applied to the membrane (20 g/L NaOH, 80 °C, 1 h).

2.7. Antioxidant activities

The biological activities of the hydrolysate and the fractions obtained from the Californian red worm were evaluated by three tests: TEAC (Trolox Equivalent Antioxidant Capacity), FRAP (Ferric ion Reducing Antioxidant Power), and ORAC (Oxygen Radical Absorbance Capacity).

2.7.1. Measurement of TEAC (Trolox Equivalent Antioxidant Capacity)

The method described by Re et al. [27] was followed. 20 μL of the Trolox sample or standard was mixed with 180 μL of an ABTS* solution (7 mM ABTS and 2.45 mM potassium persulfate) and incubated at 30 °C for 30 min. Absorbance readings were then taken at 730 nm using a Varioskam Lux spectrophotometer (Thermo Fisher Scientific, USA). The calibration curve was constructed using Trolox concentrations between 0 and 500 μM, and the results were expressed in micromoles of Trolox equivalents per gram of hydrolyzed protein (μmolET/g).

2.7.2. Measurement of FRAP (Ferric ion reducing antioxidant power)

It was carried out according to the methodology of Benzie & Straint[28]. 175 μL of the FRAP reagent (TPTZ, FeCl and sodium acetate buffer) was mixed with 25 μL of the Trolox sample or standard, and incubated at 37 °C for 30 min. The absorbance at 595 nm was then measured using the Varioskam Lux spectrophotometer. The calibration curve was performed using Trolox concentrations between 0 and 500 μM, and the results were expressed in micromoles of Trolox equivalents per gram of hydrolyzed protein (μmolET/g).

2.7.3. Measurement of ORAC (Oxygen Radical Absorbance Capacity)

The method of Ou et al. [29] was implemented. All solutions, including AAPH (2,2'-Azobis-(2-amidinopropane), 2HCl, 2,2'-Azobis-(2-methylpropionamidine)), fluorescein and earthworm hydrolysate, were diluted in 75 mM phosphate buffer (pH 7.4) to obtain a final reaction mixture of 200 μL. 50 μL of the hydrolysate solutions and 50 μL of fluorescein (78 nmol/L final concentration) were added to a black 96-well plate. The mixture was preincubated for 15 min at 37 °C before AAPH (25 μL; 40 mmol/L final concentration) was added. Fluorescence was recorded every minute for 120 min, at a temperature of 37 °C, using a Varioskam Lux spectrophotometer. Fluorescence measurements were performed using excitation and emission filters of 485 and 520 nm, respectively. ORAC assay results were expressed as units of antioxidant capacity in terms of micromoles of Trolox equivalent per gram of hydrolyzed protein (μmolTE/g).

2.8. Transmission of antioxidant peptides

The transmission of antioxidant peptides corresponds to the ratio between the concentration of peptides in the permeate with respect to the feed solution. This is calculated using equation (4). Peptide transmission was determined based on TEAC (TTEAC), FRAP (TFRAP), and ORAC (TORAC) antioxidant activities.

$$T\,(\%) = \frac{C_p}{C_f} * 100 \tag{4}$$

Where C_p corresponds to the concentration of antioxidant peptides in the permeate, taken as the antioxidant activity of the permeate, and C_f is the concentration of antioxidant peptides in the retentate at the end of filtration, taken as the antioxidant activity of the retentate.

2.9. Volume reduction factor (FRV)

The volume reduction factor refers to the ratio between the initial feed volume (Vo; mL) and the retentate volume (Vr; mL) at the end of the ultrafiltration process. This was calculated according to equation (5), with Vp being the total volume of permeate obtained.

$$FRV = \frac{V0}{Vr} = \frac{V0}{V0\text{-}Vp} \tag{5}$$

2.10. Fouling resistances

The evaluation of the fouling resistance was carried out applying Darcy's law (equation (6)), where J represents the outflow of the permeate, TMP (Pas) is the arithmetic mean between the pressures applied to both sides of the membrane, called transmembrane pressure, μ is the viscosity of the fluid, and ∑R is the sum of the resistances against the permeation of the fluid [30,31].

$$J = \frac{TMP}{\mu \sum R} \tag{6}$$

The sum of the resistances ($\sum$R) includes both the intrinsic resistance of the membrane (Rm; Kpa/(Lm^{2}h)) and the resistance derived from the gel polarization layer, and the fouling resistance (Rf). The Rm is calculated by equation (7), for which it is required to measure the flow of distilled water through a new (virgin) membrane. In this condition, there is no blocking in the membrane (Rf = 0), and the permeate flow of distilled water is represented as Jwi.

$$J_{wi} = \frac{TMP}{\mu R_m} \tag{7}$$

As the filtration process proceeds and membrane fouling occurs, the resistance associated with blocking and cake formation must be considered in the calculation. Therefore, equation (7) should be modified according to equation (8), where Jww corresponds to the permeate flux with the working fluid at a given filtration time.

$$J_{ww} = \frac{TMP}{\mu R_f} \tag{8}$$

In each filtration test, the initial and final resistance of the membrane was determined in order to establish the efficiency of chemical cleaning. This process was carried out by recirculating distilled water at a rate of 400 L/h, taking permeate flux data at different transmembrane pressure (TMP) values, and the slope of the graph of these two variables represents the corresponding resistance [32].

2.11. Optimization of operating conditions in tangential filtration

To optimize the filtration process, 15 experimental runs were carried out by means of a composite central factorial response surface experimental design (Table 1), with three factors and two levels: TMP (2 and 5 bar), pH (6 and 10) and flow rate (400 and 500 L/h). Their effect on the response variables (VRF), total resistance (Rf), gel resistance (Rg), TTEAC, TFRAP and TORAC was evaluated. Design analysis was done by analysis of variance (ANOVA), using Design Expert software (Stat-Ease Inc, USA). The mathematical models obtained were optimized after verifying compliance with the assumptions of independence, homogeneity, and lack of adjustment, to experimentally verify the predicted values in said optimization.

3. Results and discusión

3.1. Proximal composition of the Californian red worm (Eisenia fetida.)

Table 2 shows the values of the proximal composition of the paste derived from the Californian red worm (*Eisenia fetida*). It can be observed that the paste has a high protein content compared to other macro compounds, which are mainly albumin, globulin, and glutelin. This protein concentration is within the normal range reported in the literature, which indicates that the earthworm has protein values greater than 60 % [33,34]. Regarding ash content, the values found were slightly higher than those reported by Garczyńska for the earthworm (*Dendrobaena veneta Rosa*); however, the fat content was significantly lower compared to theirs (12.21 %) [34]. The high percentage of proteins of high biological value makes this substrate a viable alternative for biotechnological applications such as enzymatic hydrolysis [33,35].

Kinetic analysis of the enzymatic hydrolysis reaction of Alcalase® 2.4 L protease with Californian red worm protein paste resulted in a final degree of hydrolysis (DH) of 16.4 %, as can be seen in Fig. 2. Approximately 40 min into the experiment (or after 2500 s), the enzymatic hydrolysis curve begins to exhibit an asymptotic behavior, indicating diminishing changes in the rate of hydrolysis as time advances. Consequently, the maximum hydrolysis time was established at 4000 s. This deceleration in enzymatic hydrolysis can

Table 1
Composite central response Surface Design.

Run	Factor			Responses variables						
	A: TMP	B: pH	C: FLow	FRV	RT	Rf	Rg	TTEAC	TFRAP	TORAC
	Bar		L/h		Kpa/(Lm2h)	Kpa/(Lm2h)	Kpa/(Lm2h)	%	%	%
1	2.00	6.00	400.00	93	51.28	32.74	21.42	246.40	84.99	120.20
2	3.25	10.22	441.50	355	19.76	16.12	8.81	117.36	82.53	186.17
3	4.50	6.00	483.00	170	48.78	41.57	1.83	144.57	64.04	303.22
4	5.02	7.75	441.50	325	32.26	23.94	4.25	125.67	38.18	284.43
5	3.25	7.75	441.50	215	31.95	18.08	9.07	181.60	55.97	100.51
6	3.25	5.28	441.50	140	66.67	40.86	12.67	168.77	97.44	154.20
7	3.25	7.75	382.81	260	21.69	17.25	0.05	288.90	74.87	96.32
8	4.50	9.50	400.00	440	20.00	9.81	5.14	157.29	87.91	249.53
9	2.00	9.50	483.00	125	19.80	6.97	7.78	127.04	60.26	217.39
10	3.25	7.75	441.50	220	34.36	14.71	14.72	188.90	61.09	145.39
11	1.48	7.75	441.50	90	30.80	16.06	11.91	141.82	60.29	125.00
12	3.25	7.75	441.50	245	33.22	15.94	12.86	181.28	55.75	144.17
13	3.25	7.75	500.19	265	26.53	13.69	9.07	170.48	56.80	327.02
14	3.25	7.75	441.50	250	30.21	15.97	9.84	182.92	52.14	141.94
15	3.25	7.75	441.50	240	28.21	16.18	9.84	167.19	55.94	142.45

Table 2
Bromatological composition of red Californian earthworm paste.

Component	Californian red worm
Moisture (%)	81.41 ± 0.07
Protein (%)[a]	70.16 ± 0.12
Fat (%)	1.62 ± 0.08
Ash (%)	6.02 ± 0.05
Albumins (%)	65.25 ± 0.28
Globulins (%)	20.12 ± 0.12
Prolamins (%)	2.85 ± 0.07
Glutelins (%)	11.87 ± 0.09

[a] Dry base (g/g).

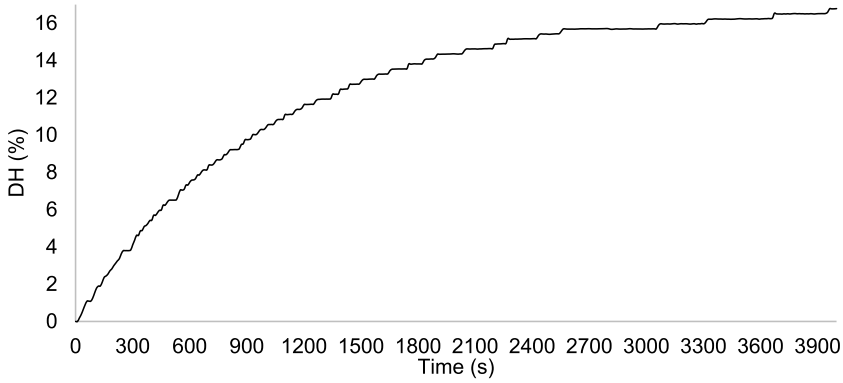

Fig. 2. Kinetics of the degree of hydrolysis to Red Californian Earthworm (*Eisenia Foetida*).

primarily be attributed to three critical factors: (1) a gradual decrease in the availability of peptide bonds that are susceptible to proteolytic enzymes, which naturally occurs as the substrate becomes increasingly hydrolyzed; (b) the accumulation of hydrolysis products that may act as inhibitors, thereby reducing the enzyme's efficacy; and (c) the thermal denaturation of the enzyme, a process where the enzyme's structure is altered due to prolonged exposure to the operational temperature, leading to a loss of catalytic activity [36]. This DH value suggests that the reaction product has promising potential as a hydrolysate with components of biological interest, as values of DH above 10 % have been shown to tend to generate hydrolysates with bioactive properties [37]. Such hydrolysates are potentially valuable in various applications, ranging from nutraceuticals to functional food ingredients, owing to their capacity to exert beneficial health effects. These effects may include antioxidant, antimicrobial, and anti-inflammatory activities, among others.

3.2. Effect of factors on response variables

The analysis of variance (ANOVA) of the experimental design is presented in Table 3, which includes the statistical significance (P value) of the adjusted model, the factors, their interactions and their quadratic terms, the adjusted coefficient of determination of the model (R^2) and the lack of adjustment of each of the response variables. It can be observed that the coefficient of determination is

Table 3
Analysis of variance (ANOVA) for the response variables of the experiments desing

Source	p-value						
	FRV	RF	RT	RG	TTEAC	TFRAP	TORAC
Model	<0.0001	<0.0001	<0.0001	0.0004	<0.0001	0.0003	<0.0001
A-TMP	<0.0001	0.0021	–	0.0012	0.0137	0.0239	<0.0001
B-pH	<0.0001	<0.0001	<0.0001	0.1896	0.0003	0.2048	0.0704
C-Flow	–	0.8589	0.5090	0.0015	<0.0001	0.0016	<0.0001
AB	0.0008	–	–	0.0004	–	–	0.0040
AC	–	0.0023	–	–	–	–	–
BC	–	–	–	–	–	0.0440	–
A^2	–	0.0354	–	–	0.0004	–	0.0004
B^2	–	<0.0001	<0.0001	–	0.0020	<0.0001	0.00156
C^2	–	–	0.0013	0.0016	0.0001	0.0411	0.0002
R^2 adjust	0.9448	0.9827	0.9814	0.9011	0.9687	0.9303	0.9810
Lack of Fit	0.1094	0.1285	0.7203	0.2625	0.1951	0.0693	0.9934

above 90 % for all the response variables. The lack of adjustment does not show statistical significance for any of the response variables, indicating that the data fit correctly to the proposed mathematical models obtained.

From the ANOVA, the mathematical models were determined for each of the response variables (equations (9)–(15)); these only include the terms that have a statistically significant effect ($p < 0.05$). It can be seen that the VRF exhibited a first-degree linear model, while the other response variables had second-degree models. Based on the mathematical models, the response surfaces were constructed (Fig. 3a–d and 4a-c), which describe the behavior of each response variable as a function of the factors.

$$FVR = 228.867 + 90.5425 * A + 75.757 * B + 59.5 * AB \quad (9)$$

$$RT = 31.5741 - 15.8241 * B + 0.516954 * C + 6.14643 * B^2 - 3.40665 * C^2 \quad (10)$$

$$Rf = 15.9762 + 2.84974 * A - 8.74656 * B + 0.117399 * C + 5.6351 * AC + 1.64481 * A^2 + 5.8899 * B^2 \quad (11)$$

$$Sqrt(Rg) = 3.24 - 0.7204 * A - 0.2201 * B + 0.9884 * C + 1.68 * AB + 0.7041 * C^2 \quad (12)$$

$$TTEAC = 179.53 - 11.80 * A - 22.42 * B - 37.44 * C - 21.82 * A^2 - 17.16 * B^2 + 26.15 * C^2 \quad (13)$$

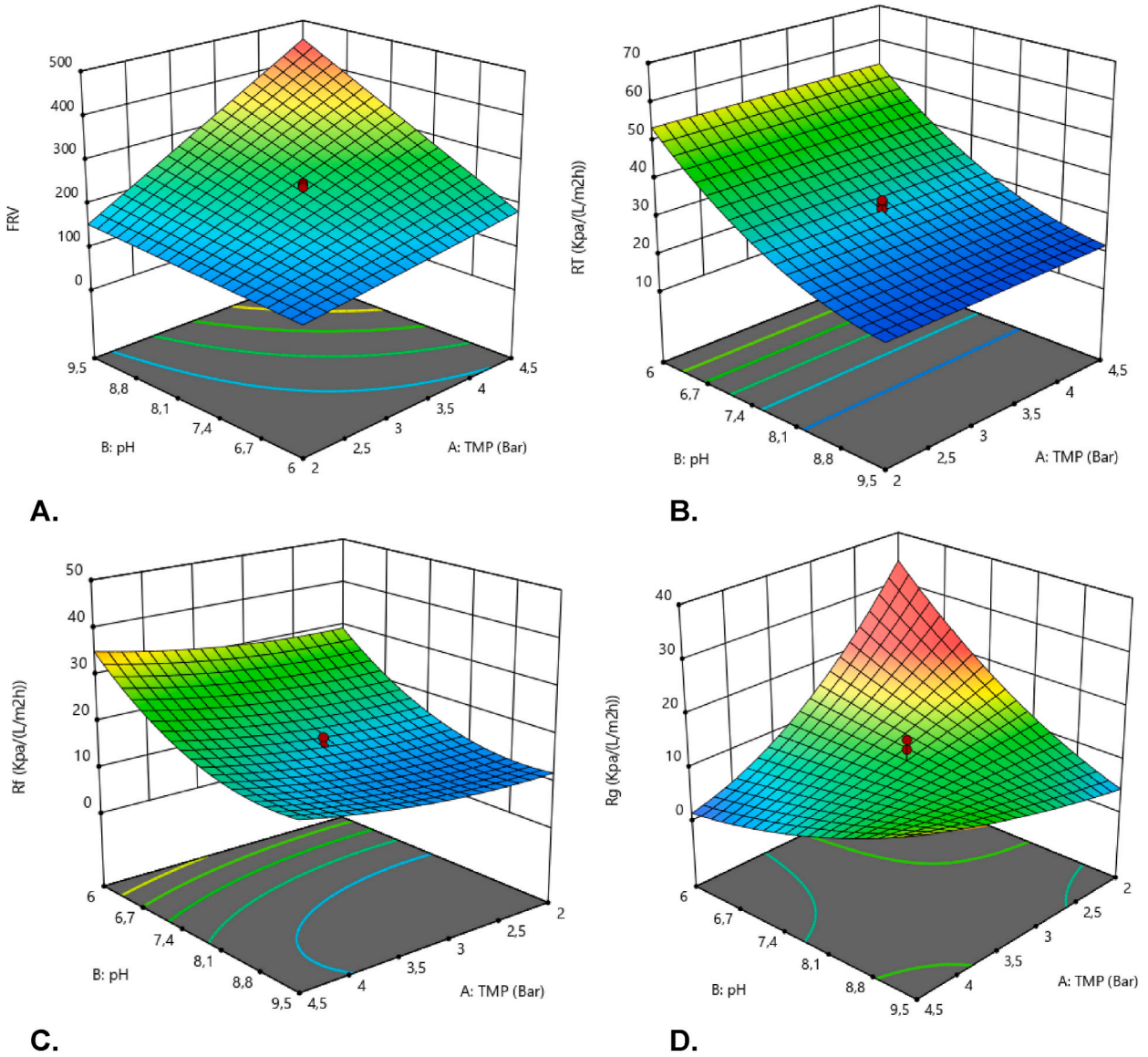

Fig. 3. Response surface graphic of filtration resistance of Red Californian earthworm hydrolysate. (*Eisenia Foetida*). **A.** Volume Reduction Factor (FRV) **B.** Total resistance. **C.** Fouling resistance. **D.** Gel resistance.

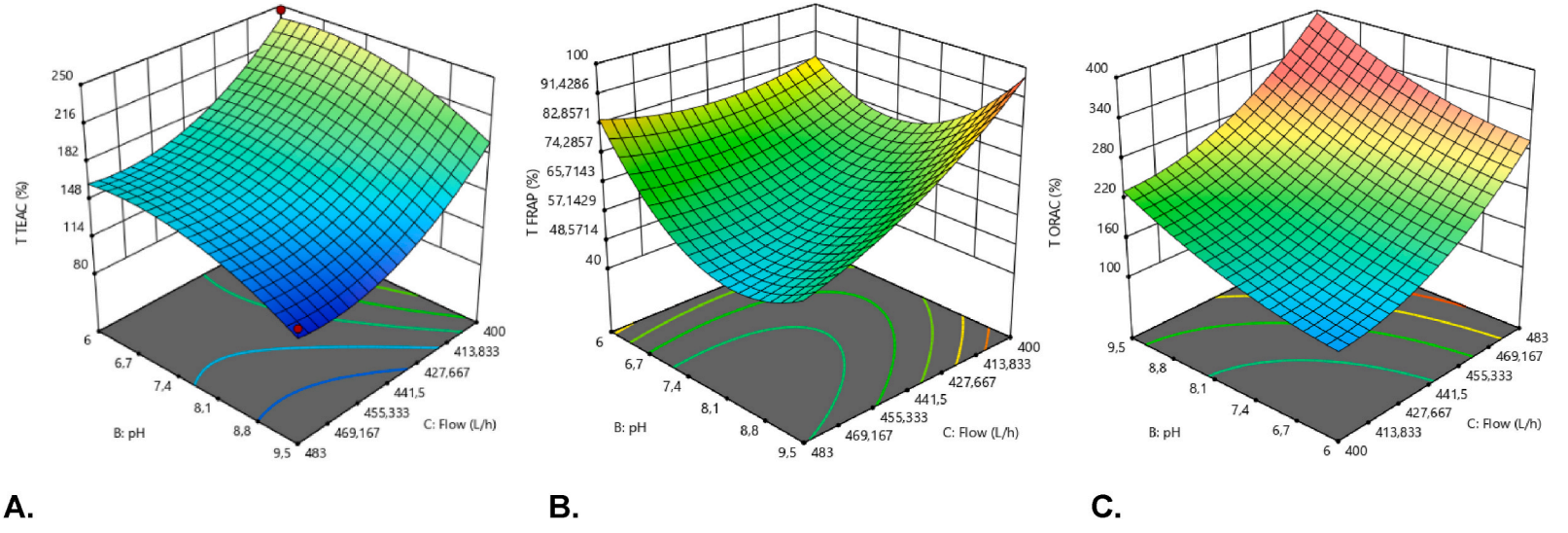

Fig. 4. Response surface plot for the transmission of antioxidant peptides of Red Californian earthworm hydrolysate permeate. (*Eisenia Foetida*). **A.** Trolox Equivalent Antioxidant Capacity (TTEAC) **B.** Ferric Reduction Antioxidant Power (TFRAP). **C.** Oxygen Radical Absorbance Capacity (TORAC).

$$TFRAP = 54.19 - 7.82 * A - 2.74 * B - 9.27 * C - 9.49 * BC + 16.99 * B^2 + 4.92 * C^2 \quad (14)$$

$$TORAC = 135.37 + 55.08 * A + 11.09 * B + 81.56 * C + 43.84 * AB + 34.07 * A^2 + 16.80 * B^2 + 37.55 * C^2 \quad (15)$$

Increases in pH favor the conditions of the process since they increase the VRF and reduce filtration resistances, decreasing the clogging of the membrane, as well as the clogging of the membrane pores. This may be because, at high pH, electrostatic repulsion between proteins and the membrane helps reduce scale deposition in the pores, increasing permeate flux [38]; Although the membrane does not initially possess an electrostatic charge, since it is not a charged membrane, the adsorption of H^+ and OH^- ions on the pore walls to acid or basic pH, can modify its surface electrical charge. Consequently, this alteration in charge affects the membrane's interaction with the desired compounds, either facilitating attraction or causing repulsion [39]. At low pH values (pH = 6), the highest resistances occur (Fig. 3b–d), mainly because the proteins have their isoelectric point in acidic pHs, usually around 3 to 6 [40]. At this point, the protein load approaches zero, which increases precipitation and aggregation, increasing resistance to elution through membranes [41]; additionally, the repulsion by the membrane is reduced, which promotes fouling and plugging of the pores [42]. Even small amounts of aggregates in whey proteins have been reported to cause a strong reduction in filtration efficiency [43]. Similar trends were found in the ultrafiltration of quinoa proteins (*Chenopodium quinoa Willd*), where the highest permeate fluxes were found at pH 9.5 [41]. Likewise, Almécija et al. (2007) found that pH values close to 10 increase the permeate flux in the filtration of whey proteins, producing a significant reduction in pH values close to 4 [44].

On the other hand, transmembrane pressure (TMP) increases the volume reduction factor in a directly proportional manner, producing a greater volume of permeate, thanks to a greater driving force [45]. However, high TMP values favor the increase in fouling resistance (Fig. 3c) because high pressures promote the entry of particles into the pores of the membrane, thus increasing fouling [46]. These trends are consistent with what was reported by Qi et al. (2022), who observed that at higher transmembrane pressure, the irreversible fouling of the membrane increased, possibly due to the formation of a layer on the surface or the blocking of the pores; however, the permeate flux was also increased, thus increasing filtration performance [47].

Regarding the transmission of antioxidant peptides, it can be observed that at low pH, the transmission of antioxidant peptides equivalent in Trolox (TTEAC) is increased, as is the iron-reducing antioxidant power (TFRAP). In the case of TORAC, at a higher pH value, the highest peptide transmissions are obtained. This may be fundamentally due to the different mechanisms of action of each of the methodologies, since in the antioxidant activity against the cationic ABTS radical (TEAC), the peptides act as hydrogen or electron donors in the free radical reactions, with these being favored in neutral pH as can be seen in the results obtained (Fig. 4a) [16]. On the other hand, the flow rate had a statistically significant effect on the transmissions of peptides with antioxidant activity, favoring transmission at low flow rates, mainly because the selectivity of the membrane is increased by improving the concentration of compounds of interest in the permeate and increasing the yield of the process of obtaining antioxidant peptides [45].

3.3. Optimization of factors based on response variables

The mathematical models presented in equations (8)–(13) were subjected to optimization based on the response variables, where the fouling resistances (total resistance, gel resistance, and fouling resistance) were minimized and the transmissions of antioxidant peptides (TTEAC, TFRAP, and TORAC) and the VRF were maximized. The results showed that the optimal filtration conditions are TMP = 4.5, pH = 9.31 and fluid flow = 400 L/h with a desirability of 86 %, which indicate that the set of answers are near to its desired values. Under these conditions, the predicted values of the response variables that appear in Table 4 were obtained, which were verified experimentally. The efficiency of the optimum ultrafiltration process was 25 % in a time of 2 h. The experimental values evaluated do not present a high relative error compared to the values predicted by the mathematical models optimized in the software, and only the transmission of antioxidant peptides through the FRAP mechanism exhibits an error of 39 %; however, the experimental value is greater than the predicted one, with this being positive since the sample would present a higher activity than theoretically expected from the optimization. Additionally, these antioxidant activities are superior to those reported by Sierra and Zapata (2021) in the optimization of the conditions of the enzymatic hydrolysis of protein extracted from red tilapia scales (*Oreochromis* Spp.), indicating that the permeate (fraction <3 kDa) of Californian red worm hydrolysate shows potential for supplementation in different matrices as an antioxidant alternative [48,49].

Fig. 5 shows the antioxidant activities of the Californian red worm hydrolysate and the fractions obtained by means of the tangential ultrafiltration system. This shows that the lower molecular weight fraction has the highest values of antioxidant activity in the TEAC and ORAC tests and does not present statistically significant differences for FRAP activity with respect to the complete

Table 4
Experimental verification of the predicted values of the response variables.

Variable	Predicted	Experimental	Error relativo (%)
TTEAC (%)	116.99	116.99 ± 0.16	0.127
TFRAP (%)	74.05	100.6 ± 0.730	39.42
TORAC (%)	122.78	128.24 ± 0.130	4.1
FRV	440	457 ± 4.210	3.86
Total Resistance	18.43	18.251 ± 0.48	0.97
Fouling Resistance	11.60	11.2 ± 0.131	3.45
Gel Resistance	4.5318	4.582 ± 0.002	1.12

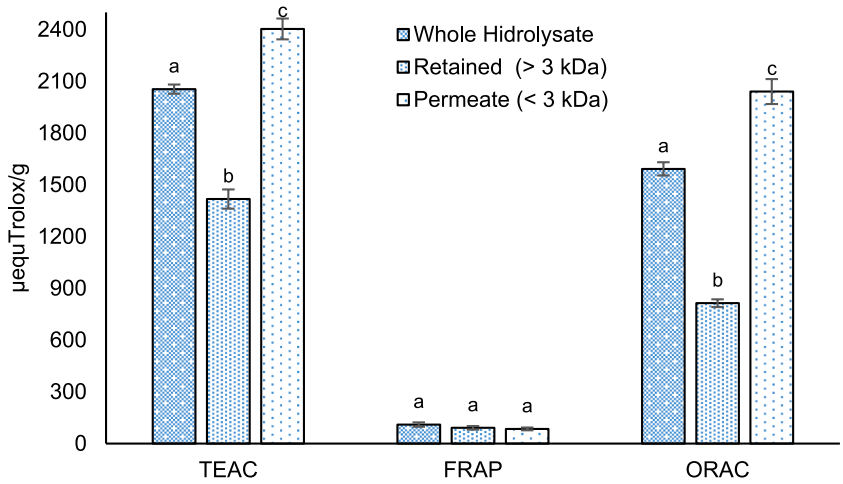

Fig. 5. Antioxidant activity values of Red Californian earthworm hydrolysate (*Eisenia Foetida*) and fraction less than 3 kDa and greater than 3 kDa. Two-way analysis of variance followed by Student's t-test, significance level of p-value <0.05. Different letters within the same parameter indicate significant differences.

hydrolysate and the fraction greater than 3 kDa. This is associated with the release of medium and small peptides, which have greater exposure to hydrophobic and reactive groups, with greater antioxidant power [50]. Given the composition of the Californian red worm, proteins emerge as the predominant macromolecules (see Table 2). The biological activities identified are primarily linked to these proteins and the peptides derived from them. Moreover, the methodologies employed in protein extraction, enzymatic hydrolysis, and tangential ultrafiltration indicate that these bioactivities are likely attributable to peptides derived from proteins [18,33,49].

Similar results were reported by Zhang et al. (2017), who found that the fraction less than 3 kDa of carp (*Hypophthalmichthys nobilis*) protein hydrolysates had significantly higher bioactivities compared to the complete hydrolysates and the other two peptide fractions (3–5 kDa and >5 kDa); additionally, the largest fraction was the one with the lowest antioxidant activities, as in the Californian red worm hydrolysate [51]. Likewise, Pezeshk et al. (2019) obtained peptide fractions from the viscera of yellowfin tuna (*Thunnus albacores*) by ultrafiltration with membranes and evaluated the antioxidant and antibacterial activity of the fractions, finding that peptides less than 3 kDa present the best antioxidant capacities expressed in terms of DPPH and TEAC at different concentrations [16].

4. Conclusions

The fractionation of Californian red worm hydrolysate through tangential ultrafiltration using 3 kDa membranes has demonstrated a significant impact on the isolation of peptides with antioxidant potential, particularly within the fraction possessing a molecular weight below 3 kDa. Our findings highlight the crucial role of neutral to alkaline pH conditions in enhancing the ultrafiltration process for extracting bioactive peptides. Notably, optimal permeate yields and the highest transmission rates of antioxidant peptides were achieved under these conditions. Conversely, lower pH values were observed to exacerbate protein precipitation and aggregation within the membrane, resulting in increased resistance to elution.

The isolated fraction below 3 kDa, owing to its demonstrated antioxidant properties, emerges as a promising ingredient for the development of novel functional food products. These findings not only underscore the significance of pH modulation in optimizing ultrafiltration processes but also pave the way for harnessing the bioactive potential of Californian red worm hydrolysates in the formulation of value-added food products with enhanced nutritional and health benefits. Moving forward, further characterization of the isolated peptides and exploration of their synergistic effects, as well as investigation into their application in various food matrices, will be key areas of focus for advancing this field.

Data availability statement

Publicly available datasets were analyzed in this study.

CRediT authorship contribution statement

Yhoan S. Gaviria: Writing – original draft, Writing – review & editing, Methodology, Formal analysis, Conceptualization. **Jose E. Zapata:** Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing 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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