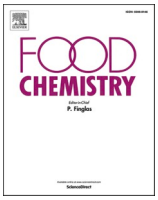
Food Chemistry 487 (2025) 144733

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Antioxidant bioactivity of sunflower protein hydrolysates in Caco-2 cells and *in silico* structural properties

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ARTICLE INFO

Keywords:
Sunflower peptides
Antioxidant bioactivity
Caco-2 cells
Prediction analysis tools
Hydrophobic peptides
Functional foods
Structure peptide

ABSTRACT

Sunflower protein hydrolysate (SPH), with 95 % reduced phenolic content, was studied for its protective effects against oxidative stress in intestinal cells (Caco-2). Produced *via* alcalase hydrolysis, SPH's molecular weight, amino acid composition, and hydrophobicity were characterized. The antioxidant activity of SPH, tested by ABTS, was maintained in Caco-2 cells under oxidative stress, modulating glutathione and catalase enzymes. LC/MS/MS identified 196 peptides, which were cross-referenced with a bioactive database and found to contain several di- and tripeptides with antioxidant activity. Higher hydrophobicity and molecular weight correlated with predicted antioxidant and anti-inflammatory activity scores, provided by tools that use machine learning methods. The study shows that SPH exhibits antioxidant properties in enterocyte cell models, even with reduced phenolic content, suggesting its potential use in functional foods.

1. Introduction

Food-derived peptides have been widely studied due to their ability to promote antioxidant effects against diseases associated with oxidative stress (OS) (Zou et al., 2016). The human intestine, a tissue that has primary functions in the absorption of nutrients, is susceptible to OS, which can potentiate the onset of several gastrointestinal disorders, such as inflammatory bowel diseases (Qiao et al., 2022). Therefore, the ingestion of antioxidant peptides may be an alternative for the treatment or prevention of diseases associated with OS.

Bioactive peptides can be obtained from enzymatic hydrolysis, fermentation, gastrointestinal digestion, and other methods. Their biological effect depends on cell interaction, where they perform their biological roles. Among these functions, bioactive peptides can attenuate/eliminate free radicals or even activate and inhibit specific signaling pathways, acting as antioxidant, anti-inflammatory, antimicrobial, depressant, and other agents (Duffuler et al., 2022; Manzoor et al., 2022; Zaky et al., 2022).

Sunflower meal, a by-product of oil extraction, is a protein-rich source, yielding over 60 % protein post-processing. Given sunflower's global prominence as a major oilseed crop and the large-scale production of its meal, this readily available and inexpensive protein source holds significant potential for applications in the food industry (Hadidi et al., 2024; Kaur & Ghoshal, 2022). Sunflower protein isolates can be used as an ingredient to increase the nutritional and functional value of foods, thus contributing to the alternative and sustainable green protein market. However, the application of this ingredient in the production of new functional foods is limited, partly due to a lack of knowledge about the physiological and structural properties of proteins and peptides (de Oliveira Filho & Egea, 2021).

Furthermore, the large amount of chlorogenic acid in sunflowers, while beneficial in many contexts, may be unfavorable for technological

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https://doi.org/10.1016/j.foodchem.2025.144733
Received 15 February 2025; Received in revised form 29 April 2025; Accepted 11 May 2025
Available online 12 May 2025

purposes. The interaction of peptides with phenolic compounds may affect peptide stability in several ways. For example, phenolic oxidation may generate reactive quinones that can bind covalently to peptide residues, potentially reducing their functional integrity; promote peptide aggregation, leading to precipitation and reduced solubility, which affects their usability in food formulations; and inhibit or enhance proteolytic enzyme activity, which can either slow or accelerate peptide degradation, influencing their stability in storage and digestion (Kaur & Ghoshal, 2022).

The antioxidant activity of sunflower peptide hydrolysates has been primarily observed in conjugation with phenolic compounds and through *in vitro* chemical assays (Bisinotto et al., 2023; Dabbour, He, Mintah, & Ma, 2019; Dabbour, He, Mintah, & Ma, 2019; do Prado et al., 2021; Habinshuti et al., 2007). While these assays serve as valuable screening tools, their results may not always accurately reflect biological system responses. Moreover, the existing literature does not report on the effects of sunflower peptides on antioxidant cell enzymes, nor does it elucidate the relationship between peptide structure and antioxidant activity.

In this study, we physicochemical characterized sunflower protein alcalase hydrolysates with low phenolic content in terms of size, amino acid composition and hydrophobicity profile. The antioxidant activity of sunflower protein hydrolysates was evaluated with *in vitro* chemical assays, as well as in a cellular model of enterocytes (Caco-2), simulating a gastrointestinal oxidative environment. The sequences of the hydrolyzed peptides were identified, and their structure and bioactivity were correlated with prediction bioinformatic tools and cross-referenced with bioactive data-base. Finally, the frequency of the most abundant amino acids was analyzed in terms of their location in the peptide structure.

2. Material and methods

2.1. Chemical reagents, characterization, and phenolic compounds

- *Sample and chemicals:* Defatted sunflower meal (*Helianthus annuus* L.) was produced and donated by Sunbloom protein Company *(München, Germany)*. Enzymes, standards, and chemicals were obtained from Sigma Aldrich (St. Louis, USA). Pepsin (P7000), Pancreatin (p1750), o-phthaldialdehyde (OPA- P1378), DL-dithiothreitol (DTT- S43819), α-Lactalbumin (L6385), Insulin (I2643), Vitamin B12 (V2876), 3,4-Dihydroxy-*L*-phenylalanine (D9628), DL-2-Aminobutyric acid (AAAB - 162,663), phenyl isothiocyanate (PITC - P1034), 2,2-Diphenyl-1-picrylhydrazyl (DPPH - D9132), 2,2′-Azino-bis(3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt (ABTS -A1888), (±)-6-Hydroxy- 2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox - 238,813), 2,2-Azobis (2-methylpropionamidine), dihydrochloride (APPH - 440,914), Fluorescein (46955). Amino acid standards (P/N20088, Standard H) and L-Serine (36323) were obtained from Pierce Biotechnology (Massachusetts, USA). *In vitro* analysis with cell culture were used Thiazolyl Blue Tetrazolium Bromide or MTT (M5655), L-Glutathione reduced, or GSH (G6529), 2′,7′-dichlorofluorescin diacetate or DCF-DA (D6883), and bovine serum albumin (A4503) obtaining from Sigma Aldrich (St. Louis, USA). Deionized water and other reagents were used in the analytic grade, and the assays were performed in triplicate.

-*Phenolic Compounds Extraction:* Phenolic compounds were extracted by sequential extraction with 70 % ethanol according to Salgado et al. (2011). Briefly, sunflower flour at a 1:15 (*w/v*) ratio, pH 5.0, was stirred for 1 h at room temperature, then centrifuged at 11000 ×*g* for 20 min at room temperature. The phenolic compounds remained in the soluble portion.

-*Protein Solubilization:* The precipitate (fibers and proteins) was dispersed in distilled water (5 mL) and the pH was adjusted to 9.0 (NaOH 1 mol/L), stirred for 1 h, and centrifuged at 11000 ×*g* for 20 min at room temperature. Supernatants were collected.

-*Protein Isolate:* Supernatants were subjected to isoelectric precipitation (pH 4.5/HCl 1 mol/L), rested overnight, and centrifuged at 11000 ×*g* for 20 min at 4 °C. This procedure was repeated twice to optimize phenolic extraction. Decanted sunflower protein isolate was centrifuged at 11000 ×*g* for 20 min at 4 °C and lyophilized.

-*Total Phenolics Quantification:* Phenolic compounds were determined by reaction with Folin-Ciocalteu according to Singleton and Rossi (1965), and the result was expressed in mg gallic acid equivalent (GAE)/g sample.

-*Protein Determination:* Moisture content and total protein (Kjeldahl method, Nx 5.75) of samples were performed as described by AOAC methods (Latimer, 2012).

-*Amino Acid Profile:* The amino acid (AA) profile of the samples was determined by RP-HPLC, coupled to a UV detector (Shimadzu Corporation, Tokyo, Japan), equipped with a Luna/Phenomenex C18 column (250 mm × 4.6 mm × 5 μm; Phenomenex Inc., Torrance, USA). Mobile phases A and B consisted of sodium acetate, acetonitrile, ultra-pure water, and disodium EDTA. Identification and quantification were performed using the external standard (Pierce / PN 20088) and α-aminobutyric acid (AAAB) as the internal standard (Aldrich, Milwaukee, USA), using a wavelength of 254 nm, as described by White et al., 1986.

2.2. Preparation of sunflower hydrolysates with Alcalase

Sunflower protein isolate (SFPI) was hydrolyzed according to Dabbour et al. (2018). Briefly, the sample was diluted to 5 % in water and sonicated (37 kHz – Unique ultrasonic cleaner / 8000) at 50 °C, pH 9.0 for 20 min. Hydrolysis was carried out by the addition of Alcalase® (0.3 U/g protein) at pH 9.0 for 90 min at 50 °C; the reaction was stopped by reducing the pH to 5. The hydrolysates were fractionated and filtered using an ultrafiltration system (Labscale TFF 29751 model - Millipore) and cellulose membrane (< 5 kDa). All the fractions obtained (filtered (< 5 kDa) and retained (> 5 kDa)) were lyophilized. The Alcalase concentration was corrected by its enzymatic activity measured by de Castro and Sato (2013) with azocasein as substrate. The degree of hydrolysis (DH) was determined according to Anvar et al. (2024) using the pH-stat method and the following equation:

$$DH\ (\%) = \frac{BNb}{\alpha Mphtotal}\ x\ 100.$$

Where: DH = degree of hydrolysis; B = volume of consumed base (mL); Nb = normality of the base; α = average degree of dissociation; Mp = mass of protein; htotal = total peptide bond content (mmol/g).

2.3. Molecular exclusion chromatography

Samples were analyzed by size exclusion chromatography (SE-FPLC) using a Fast Protein Liquid Chromatograph (Akta Pure Chromatograph, GE Healthcare, Chicago, Illinois, USA) with gel filtration columns connected in series (models Superdex 200 and Superdex 30). Samples (5 mg/mL) were diluted and sonicated for 20 min. Both the sample and standard (1 mg/mL) were diluted in 25 mM sodium phosphate buffer (pH 7.4 with 150 mM NaCl) and filtered through a hydrophilic polytetrafluoroethylene (PTFE; 0.45 μm) membrane. Samples were injected (0.5 mL/min), and readings were taken at 280 nm UV detection. The standards were α-Lactalbumin (14,178 Da), Insulin (5807.6 Da), Vitamin B12 (1355.4 Da), L-β-4-Dihydroxyphenylalanine (197.2 Da), and L-Norleucine (131.17 Da). A wavelength of 280 nm was employed (Sisconeto Bisinotto et al., 2021; Vander Heyden et al., 2002).

2.4. Electrophoresis

Protein samples were analyzed in sodium dodecyl sulfate-polyacrylamide gel (12 %) (SDS-PAGE) under reducing conditions. Samples at 2 mg/mL were homogenized in a sample buffer (0.0625 M tris–HCl, pH 6.8, 2 % (*w/v*) SDS, 5 % (w/v) β-mercaptoethanol, 10 % (*v/v*) glycerol, 0.002 % (w/v) bromophenol blue) and subsequently boiled for 5 min and centrifuged at 10,000 *g*. Protein content was measured by Lowry's method, using bovine serum albumin as the standard (Peterson,

1979). Equal amounts (20 μg) of total protein were electrophoresed in a 10 % (w/v) SDS–polyacrylamide gel. The gel was run at a constant 120 V for 1.5 h, and the proteins were fixed and stained with 0.006 % (w/v) Coomassie Blue R and 6 % (v/v) acetic acid. Molecular weights of the polypeptides were estimated using low molecular weight standards (Sigma) with molecular weights ranging from 6500 to 66,000 Da.

2.5. Hydrophobic profiles

Reverse phase high-performance liquid chromatography (RPHPLC) was performed on an Agilent liquid chromatograph with a semi-preparative and analytical quaternary pump system and a diode array detector (DAD) (Agilent, Waldbronn, Germany) on a Microsorb – MV™ C18 column (4.6 mm × 250 mm; 5 μm particle size) (Rainin, Woburn, MA, USA), following Caetano-Silva et al. (2017). The composition of solvent A was 0.04 % TFA in ultrapure water, and solvent B was 0.03 % TFA in acetonitrile. The gradient elution conditions were 100 % A from 0 to 40 min, 70 % B and 30 % A from 40 to 45 min, and 100 % A from 45 to 60 min. Detection was at 214 nm, and the sample injection volume was 50 μL (3 mg protein/mL for the samples).

2.6. In vitro antioxidant chemical assays

Oxygen Radical Absorbance Capacity (ORAC): The ORAC assay was performed as described by Corrochano et al. (2019). Samples and the standard curve were diluted in 75 mM potassium phosphate buffer (pH 7.4). In a microplate, 20 μL of sample or standard, 120 μL of fluorescein solution (0.17 μM), and 60 μL of AAPH solution (40 mM) were added and monitored for 2 h at 90-s intervals at 485 nm excitation and 520 nm emission wavelength in a microplate reader (Varioskan Lux, Thermofisher, Singapore). A Trolox standard curve (12.5–400 μM) was used to express the results as μmol Trolox equivalent/g sample.

−2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay: The DPPH assay was performed as described by Santos et al. (2017), with minor modifications. The radical 2,2-diphenyl-1-picrylhydrazyl was diluted in methanol at 80 μM and stored in a dark bottle. The solution was used to dilute samples or Trolox standard (1.5 to 100 μM), stirred, and incubated at room temperature for 30 min. Then, a volume of 200 μL was dispensed into a 96-well microplate and read at an absorbance of 517 nm. The scavenging activity of the tested samples was extrapolated into the DPPH inhibitory percentage using the equation: AA (%) = ((Abs White - Abs Sample) / Abs White) × 100.

−2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS): The ABTS assay was performed as described by Dryáková et al. (2010). A solution of potassium persulfate at 2.45 mM and 7 mM $ABTS^{+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid radical) was prepared and left to stand in the dark for 12 h. Its absorbance was adjusted to 0.7 ± 0.02 at 734 nm using a UV–Vis spectrophotometer before analysis. Then, a 10 μL aliquot of the sample was placed in a microplate, followed by the addition of 190 μL of ABTS+ solution (Abs 0.7 ± 0.02). A Trolox standard curve (50–1000 μM) in 75 mM phosphate buffer (pH 7.4) was used. After 6 min, the absorbance was read at 730 nm on the microplate. The results were expressed as μmol Trolox equivalent/g sample in triplicate or by the percentage of antioxidant activity (AAT), calculated and expressed according to the equation: AAT (%) = ((Abs White - Abs Sample) / Abs White) × 100.

2.7. Caco-2 cell culture

Human adenocarcinoma cells derived from human intestinal epithelium (Caco-2) were obtained from the Cells Bank in Rio de Janeiro (code 0059) and donated by Dr. Juliana Alves Macedo's laboratory. The Caco-2 cell line was cultured according to the protocol of Hubatsch et al. (2007). These cells were seeded in 25 cm^2 flasks and cultured in DMEM containing 10 % fetal bovine serum, 1 % penicillin/streptomycin, 8.4 mM HEPES, 1 % sodium pyruvate, 1 % non-essential amino acids, and 1 % L-glutamine under 5 % CO_2 at 37 °C. The medium was changed every 2 days. The exponentially growing cells were trypsinized and seeded in 24-well plates (with a cell density of 10 × 10^4 cells/well) or 96-well plates (2 × 10^4 cells/well), depending on the type of treatment. To assess the antioxidant effects of sunflower hydrolysate, undifferentiated Caco-2 cells were used as a model of intestinal progenitor cells rather than mature enterocytes. These cells exhibit higher metabolic activity and greater sensitivity to oxidative stress, allowing measurable effects to be observed with lower stimulus concentrations.

2.7.1. Cell treatment design

Caco-2 cells were pretreated for 1 h with sunflower hydrolysate, followed by 3 h with sunflower hydrolysates and the presence or absence of hydrogen peroxide (H_2O_2) at 1 mM. The total treatment time of 4 h was chosen based on the physiological time of *in vitro* digestion. For this evaluation, the following concentration curve of peptide hydrolysates was adopted: 0.6, 0.3, and 0.15 mg/mL. The control group did not receive any treatment.

2.7.2. Assessment of cell viability by MTT

The effect of metabolic activity on cell internalization of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT; M5655, Sigma-Aldrich, St. Louis, MO, USA) was investigated. The dissolution of the crystals, resulting in the conversion of MTT into insoluble purple crystalline formazan, was measured by reading the absorbance in a microplate reader (Varioskan Lux, Thermo Fisher, Singapore) at wavelengths of 560 and 650 nm, respectively (Leite et al., 2009).

2.7.3. Assays for evaluation of antioxidant activity in cells

-Evaluation of reactive oxygen species by DCF: The formation of DCFH from the action of intracellular esterases was assessed after the entry of 2′,7'-Dichlorofluorescein (DCF-DA; D6883, Sigma Aldrich, St. Louis, USA), by passive diffusion (Wan et al., 2015). The cells were seeded in black 96-well plates (density 2 × 10^4 cells/well) and treated for 3 h. In the final 30 min of treatment, DCF-DA was incubated at a concentration of 20 μM per well. After 3 h, the wells were washed with PBS and incubated with 1 mM hydrogen peroxide to stimulate the oxidation of DCFH to dichlorofluorescein (DCF). Fluorescence was then read in kinetic time, with readings every 5 min for a total of 1 h at 37 °C (Varioskan Lux, Thermo Fisher, Singapore) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The results were expressed as an arbitrary unit of fluorescence per microgram of protein (UF/mg of protein).

-Nitric oxide production (NO): Nitric oxide production was evaluated using the Griess assay (Soliman & Mazzio, 1998) and adapted as Chen & Kitts, 2008. After treating the cells, 100 μL of the supernatant was collected and incubated with the same volume of Griess reagent for 10 min at 25 °C. The absorbance was then read at a wavelength of 550 nm using a microplate reader (Varioskan Lux, Thermo Fisher, Singapore). A standard curve was prepared with sodium nitrite (1 mM $NaNO_2$, N101 5.01.AG, Synth, São Paulo, Brazil) at the following concentrations: 40, 20, 10, 5, 2.5, and 1.25 μM/mL. Data were expressed as nM nitrite/mg protein/h.

-Determination of glutathione content (GSH): Reduced glutathione content was determined using a fluorogenic assay as previously described (Browne & Armstrong, 1998 and Galland et al., 2024). Briefly, samples were homogenized in sodium phosphate buffer (0.1 M, pH 8.0) containing 5 mM EDTA. Proteins were precipitated with 1.7 % metaphosphoric acid and centrifuged at 1000 ×*g* for 10 min at 4 °C. The supernatant was incubated with o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was created using standard GSH solutions (G-6529, Sigma Aldrich, St. Louis, USA) (0–500 μM). Readings were corrected for the blank, and the result was expressed as μmol GSH/mg protein

-Soluble protein content (Lowry assay): Soluble protein content in cell

culture was quantified using the Lowry method (Peterson, 1979) and revised by Waterborg, 2009. Bovine albumin (1 mg/mL; A4503, Sigma Aldrich, St. Louis, USA) was used as the standard at concentrations of 2 to 40 μg/mL. The protein amount was expressed in μg/μL.

- *Superoxide dismutase and Catalase activity:* SOD and CAT activity in the treated Caco-2 cells was evaluated using the Cayman Chemical ELISA kit (706,002; 707,002). The methodology followed the manufacturer's instructions.

2.8. Identification of sequences by LC/MS/MS (Nano-coupled liquid chromatography QExactive mass)

For the test, SPHf and digested samples were analyzed. First, the samples were resuspended in LC/MS water with 0.1 % formic acid, and their protein content was quantified using the Qubit Protein Assay method. Chromatography was then performed on a PicoChip source (Model 1PCH-550; 75 μm ReproSil Pur C18 3 μm silica matrix; New Objective, USA) at a continuous flow rate of 0.300 μL/min. One microgram of sample was injected into an Acclaim PepMap 100 trap (75 μm ID, C18 3 μm; Thermo Fisher Scientific). Sample separation was conducted in a gradient of 2–40 % mobile phase B for 120 min, followed by 10 min in 80 % mobile phase B and a 10 min column reequilibration in 2 % mobile phase B. The mobile phases of the chromatography were: mobile phase A, water/0.1 % formic acid, and phase B, acetonitrile/0.1 % formic acid. Mass spectra were acquired using a QExactive mass spectrometer (Thermo Fisher Scientific) in the DDA method (data-dependent acquisition, Full MS/MS) with the selection of the 10 best counts. The precursor ion search was performed from 300 to 1750 *m/z* at a resolution of 70,000. An isolation window of 2 m/z was selected, with collision energies NCE 15 and 30, followed by MS/MS acquisition at 17,500 resolution. The automatic gain control (AGC) target was set to 1 and 4, with a maximum injection time of 100 ms. Loads 1 and greater than 5 were excluded, and a dynamic exclusion time of 30 s was used. Samples were analyzed in duplicate. The spectra were analyzed with PatternLab for Proteomics (Carvalho et al., 2016) using the *Helianthus annuus* database from the UNIPROT database (https://www.uniprot.org). Peptide searches were conducted using the NOVOR software (Ma, 2015) (available at https://novor.cloud/), with predefined definitions and the *Helianthus annuus* database as the reference.

2.9. In silico bioactive analysis

The sequencing results were checked against antioxidant bioactivity using the 'Profiles of proteins potential biological activity' tool in the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/en/biopep-uwm-2/). Predictive tools were used to evaluate the probability of peptides being: (a) bioactive, using the PeptideRanker server (http://distilldeep.ucd.ie/PeptideRanker/) (Mooney et al., 2012); (b) antioxidant, using the AnOxPePred webserver (https://services.healthtech.dtu.dk/service.php?AnOxPePred-1.0), which predicts free radical scavenging (FRS) and metal chelating properties of peptides (CHEL) (Olsen et al., 2020); and (c) anti-inflammatory, using the PreAIP software (http://kurata14.bio.kyutech.ac.jp/PreAIP/) (Khatun et al., 2019). The molecular size, isoelectric pH, number, and sequence of amino acids were analyzed using the Peptide Property Calculator software (https://pepcalc.com/). The percentage of hydrophobic, acidic, basic, and neutral amino acids were analyzed using the Peptide 2.0 software (https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php). To visualize the frequency of each amino acid in each position, a heat map graph was constructed based on iceLogo, a free, open-source Java application for analyzing and visualizing consensus patterns in aligned peptide sequences (Colaert et al., 2009). The online software (https://iomics.ugent.be/icelogoserver/) was used.

2.10. Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL). An ANOVA was conducted, followed by Duncan's post-test to assess differences between groups. The mean ± standard error was used to represent the data from each *in vitro* experiment, which was repeated at least five times. Statistical significance was determined at $p < 0.05$.

3. Results and discussion

3.1. Production of sunflower protein isolates with low phenolic content

Before sunflower protein hydrolysis, we isolated the protein and reduced the phenolic content, which was mainly composed of chlorogenic acid (Friolli et al., 2023). Table 1 shows that the protein content increased from 48 % in defatted sunflower meal (DS) to 92 % in the sunflower protein isolate fraction (SFPi). The phenolic compound content in SFPi was reduced by 95 % compared to the original flour. The reduction of phenolic compounds in the sunflower sample was important for several reasons: firstly, covalent and non-covalent interactions between polyphenols and peptides may lead to conformational and functional modifications, impacting the bioaccessibility and bioavailability of peptides (Jiang et al., 2019). Secondly, while phenolic compounds and food peptides can both exhibit antioxidant properties, their interaction can result in either a synergistic effect or, conversely, a reduction in antioxidant activity. This reduction might occur by phenolic masking active sites or altering peptide conformation, thus limiting their ability to scavenge free radicals or chelate metal ions (Muntaha et al., 2025). Therefore, removing phenolic compounds in this study aimed to enhance peptide absorption and utilization in the body, as well as to preserve intrinsic bioactive functions.

The use of Alcalase enzyme to form bioactive sunflower peptides has been one of the most commonly used methods due to the industrial technological advantages of this enzyme (de Oliveira Filho & Egea, 2021). According to the literature, Alcalase shows a higher degree of hydrolysis and is often more effective than other proteases, such as pepsin and trypsin, in generating bioactive peptides (Tacias-Pascacio et al., 2020). Alcalase has been found to produce a higher degree of hydrolysis than trypsin and pepsin in whey proteins and collagen from jellyfish (Barzideh et al., 2014; Pein et al., 2018). Alcalase hydrolysates from silk sericin and insect larvae proteins showed stronger scavenging activity against free radicals than those obtained with trypsin and other enzymes. Compared to pepsin and trypsin, Alcalase produced higher DPPH radical-scavenging activity and lipid peroxidation inhibition (Fan et al., 2010; Yu et al., 2017). Thus, for this study, we chose Alcalase enzyme to produce potential bioactive peptides. Using this enzyme, we achieved a degree of hydrolysis (DH) of 30.3 ± 2.6 %. These results align with previously published data on sunflower protein hydrolysis using Alcalase at similar concentrations, which reported DH values ranging from 20 % to 30 % (do Prado et al., 2021; Ren et al., 2017; Ugolini et al., 2015).

Table 1
Protein content and quantification of total phenolic compounds of the samples.

Sample	Protein content (%)	Phenolic content (mg AG /100 g*)	Total phenolic elimination (%)
DS	48 ± 1.1	488 ± 4.7	–
SFPi	92 ± 0.1*	26 ± 2.4*	95

DS = Defatted sunflower meal; SFPi = Sunflower protein isolates. * mg of Gallic acid per 100 g of sample. Values are means of three (3) measurements. Each value is the mean (± standard error) of triplicates. Statistical analysis was performed by Student's *t*-test, with a significance level of $p < 0.05$.

3.2. Sample characterization regarding amino acid profile, hydrophobicity and peptide molecular size

According to the FAO reference standard (FAO, 2007), SFPi presented all the essential amino acids in desirable proportions, except lysine (Suppl. material: Table S1), reflecting the behavior of a pseudocereal although it is an oilseed (Carrão-Panizzi & Mandarino, 1994). In general, sunflower protein can be considered a good quality protein, and the low lysine level can be corrected by adding other protein sources to the diet that have significant values for the limited amino acid (Alexandrino et al., 2017; Gilbert et al., 2011; Salgado et al., 2011; Salgado et al., 2012). The majority of the amino acids in SFPi were classified as hydrophobic and acidic, which may directly impact the bioactivity of the formed peptides. After hydrolysis and ultrafiltration, two fractions were generated: the filtered sunflower protein hydrolysate (SPHf, < 5 kDa) and retained sunflower protein hydrolysates (SPHr, > 5 kDa). Because peptides with high hydrophobicity and low molecular size have been linked to antioxidant bioactivity, these two parameters were evaluated in isolated sunflower protein and hydrolysate fractions, and the results are shown in Fig. 1.

Specifically, Figs. 1 A, B, and C show the peptide hydrophobicity of SFPi, SPHr, and SPHf, respectively, based on their retention time during RP-HPLC on a hydrophobic stationary phase. Peptides with more hydrophobic amino acids stay longer in the column and elute later. The chromatograms were divided into three zones, (I) Low hydrophobicity zone (0 to 20 min, 0–25 % elution gradient) (II) medium hydrophobicity zone (20–40 min, 25–50 % elution gradient); and (III) high hydrophobicity zone (> 40 min., 50 % elution gradient). Notably, the chromatographic peaks for all three samples were predominantly observed in the medium hydrophobicity zone (II). This suggests that the majority of peptides in these samples possess moderate hydrophobicity. Peptides with this characteristic often strike a beneficial balance between high hydrophobicity—which can enhance bioavailability and antioxidant activity but compromise digestive stability—and low hydrophobicity, which improves stability but may reduce bioactivity (Xie et al., 2015). Consequently, moderate hydrophobicity is frequently considered optimal for maximizing antioxidant potential by improving solubility and facilitating interactions in both lipid and aqueous environments.

The hydrolysate fractions (SPHr and SPHf) presented peptide peaks between zones I and II, representing lower hydrophobicity than the isolated protein (SFPi). The hydrophobicity profile of the peptide may be influenced by the degree of hydrolysis (DH). Previous studies on sunflower protein reported an increase in hydrophobicity at lower DH values (0–18 %), followed by a decrease at higher DH levels (18–24 %). This trend was attributed to protein-protein interactions and the confinement of hydrophobic regions within aggregates (Dabbour, He, Mintah, Xiang, & Ma, 2019). Notably, these findings align with our observations at a higher DH (>27 %).

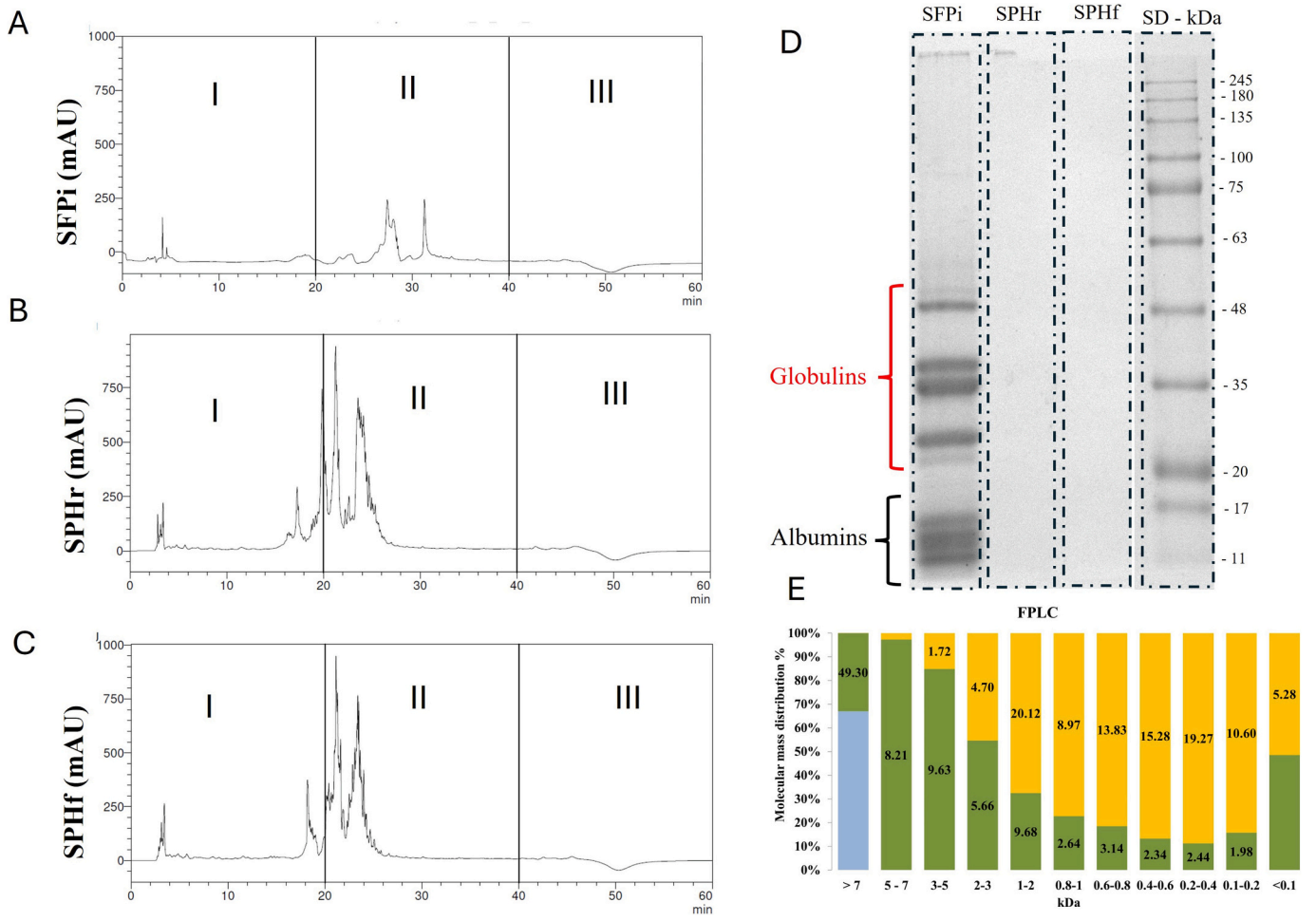

Fig. 1. Hydrophobic profile and molecular size distribution of the sunflower protein isolate and hydrolyzed fractions. (A, B and C) show the chromatograms of hydrophobic profile which are divided into three zones, related to retention time and mobile phase concentration, where: 0–25 % Mobile Phase B corresponds to low hydrophobicity (I), 25–50 %, of the samples, medium hydrophobicity (II), 50–100 % and high hydrophobicity (III). (D) show the SDS-PAGE gel (SD = molecular mass standard); (E) molecular weight (MW) distribution profile. SFPi = sunflower protein isolates; SPHr = sunflower hydrolysates retented and SPHf = sunflower protein filtered.

The molecular size of proteins and peptides was observed by electrophoresis and molecular size exclusion chromatography (SEC). In the electrophoretic profiles, the SFPi sample shows bands in the range of 10 to 50 kDa, which may correspond to globulins (range of 20–60 kDa) and albumins (range of 11–20 kDa), classically present in sunflower protein (González-Pérez et al., 2002; González-Pérez & Vereijken, 2007). As expected, no bands were observed in the hydrolyzed fractions (Fig. 1D), showing the predominance of low molecular weight peptides, which migrated rapidly through the electrophoresis and were lost in the gel.

SEC results are shown in Fig. 1E. All proteins in SFPi were above 7 kDa (blue column), which corroborates with electrophoresis analysis with integral proteins. The SPHr fraction presented a wide distribution in different molecular weights (green columns) but still showed a large proportion in the range above 7 kDa. This data indicates that alcalase hydrolysis was not complete, and some proteins remained intact after the hydrolysis process, being retained in the retained fraction. On the other hand, the SPHf fraction showed all peptides smaller than 3 kDa (yellow columns), predominantly below 600 Da. A study on African yam bean seed protein hydrolysate fractions showed that peptides with molecular weights <1 kDa exhibited superior antioxidant activity compared to larger peptides (Ajibola et al., 2011). Similarly, sacha inchi protein hydrolysate fractions with low molecular weight (<1 kDa) demonstrated strong antioxidant properties, including DPPH and ABTS radical scavenging (Suwanangul et al., 2022).

3.3. Chemical antioxidant bioactivity

In the literature, several studies have evaluated the antioxidant activity of sunflower protein hydrolysates using chemical models (ABTS, DPPH, and ORAC). However, few of these studies have analyzed samples with reduced phenolic content in protein hydrolysates, which may contribute to antioxidant action (Bisinotto et al., 2023; Dabbour, He, Mintah, & Ma, 2019; Dabbour, He, Mintah, & Ma, 2019; Dabbour, He, Mintah, Xiang, & Ma, 2019; do Prado et al., 2021; Habinshuti et al., 2007).

In this study, sunflower hydrolysate fractions (SPHf and SPHr), with low phenolic content, exhibited better antioxidant bioactivity than isolates (SFPi) in the ABTS assay (Fig. 2A), demonstrating that peptides *per se* present radical scavenging activity (Fig. 2). However, this effect was not observed in the DPPH and ORAC assays (Fig. 2B and C). Several factors may have influenced the antioxidant test results using chemical models, including chemical composition, polarity, and solubility in water or organic solvents. The ABTS radical is soluble in both water and organic solvents, making it suitable for evaluating the antioxidant capacity of both hydrophilic and lipophilic compounds. Although the DPPH assay, like ABTS, is based on electron donation, it may not be ideal for hydrophilic antioxidants due to its solubility limitations. The ORAC assay, which follows a hydrogen atom transfer (HAT) mechanism, measures the inhibition of peroxyl radical oxidation—a naturally occurring radical—making it biologically relevant. However, it is a more complex assay and less effective for lipophilic antioxidants due to its aqueous-based reaction system (Munteanu & Apetrei, 2021).

Previous studies evaluating the antioxidant activity of sunflower hydrolysates using ABTS, DPPH, and ORAC assays found a significant increase compared to non-digested protein (Bisinotto et al., 2023; Dabbour et al., 2018). Therefore, the reduction of phenolic compounds in our peptide hydrolysates may have contributed to lower sensitivity in *in vitro* tests. A potential synergistic effect between peptides and phenolics in antioxidant activity is possible, though this interaction was not directly assessed in our study (Munteanu & Apetrei, 2021).

Despite chemical antioxidant methods providing guidance for antioxidant capacity, they do not necessarily indicate the ability to suppress oxidation in biological systems (Moharram & Youssef, 2014; Niki, 2010). To obtain information about the real antioxidant activity and interaction with cell machinery, we continued the study with Caco-2 cells, evaluating the antioxidant action of SPHf, with low molecular weight peptides (< 5 kDa).

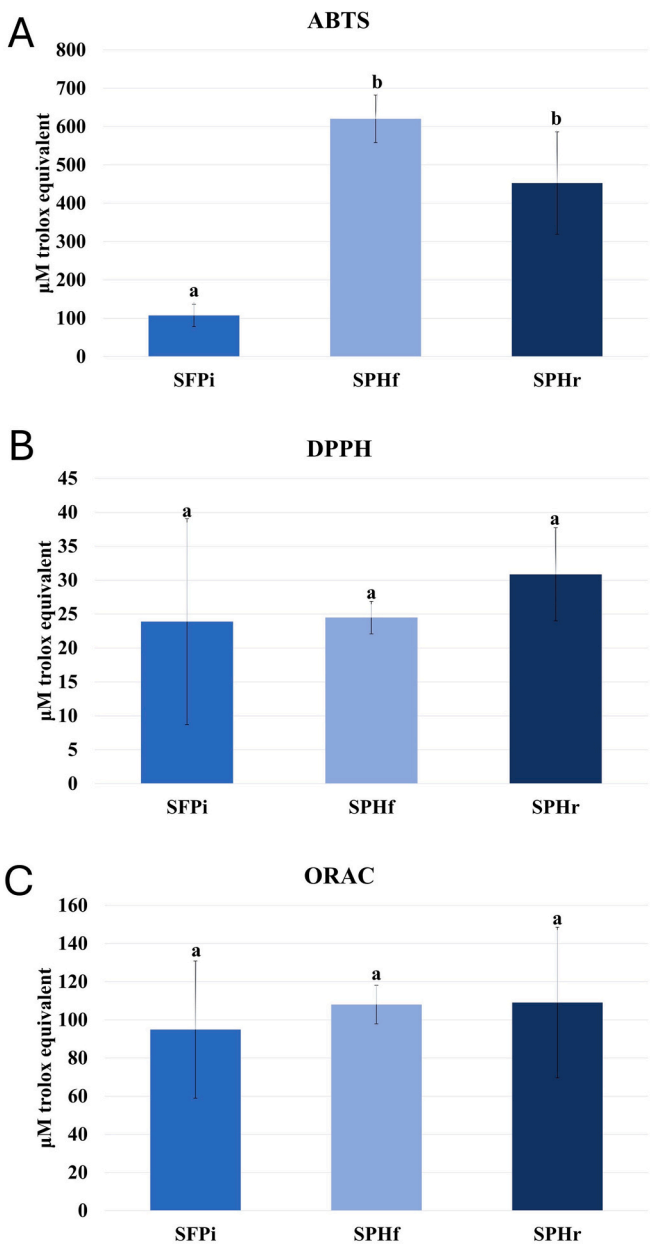

Fig. 2. Antioxidant chemical activity of the sunflower protein hydrolysates. (A) ABTS; (B) DPPH and, (C) ORAC. Samples were analyzed in 0.6 mg/mL. Each value is the meaning of the standard error of at least 5 experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey test, with $p < 0.05$. SFPi = sunflower protein isolates and SHPf = sunflower protein filtered.

3.4. Evaluation of peptide antioxidant activity on Caco-2 cells

The antioxidant capacity of sunflower peptides (SPH) with low phenolic content and reduced molecular size (< 5 kDa) was evaluated in Caco-2 cells following oxidative stress induction with hydrogen peroxide (H_2O_2). The experiment aimed to induce oxidative stress without significantly compromising cell viability, as low to moderate oxidative stress can activate signaling pathways, trigger adaptive responses, and cause cellular damage without immediate cell death.

A 3-h exposure to 1 mM H_2O_2 did not induce cell death (Fig. 3A) but did generate oxidative stress, as indicated by increased reactive oxygen species (ROS) production, measured using the DCF assay (Fig. 3B).

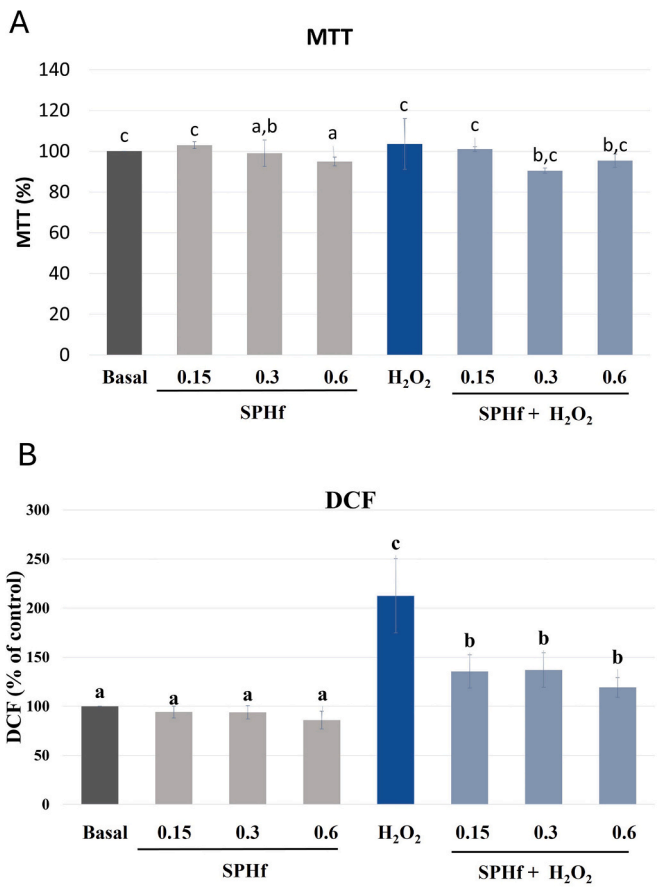

Fig. 3. SPHf prevents ROS production in Caco-2 cells stimulated with H_2O_2. (A) The viability of the cells was tested with different concentrations of SPHf with and without H_2O_2 for 4 h. (B) Cells were pre-treated with different concentrations of SPHf (1 h) and stimulated with 1 mM H_2O_2 for 3 h. Stimulation with H_2O_2 increased the production of reactive oxygen species (ROS) by fluorescence increase of DCF, which was attenuated with SPHf treatments. Treatments were compared by ANOVA followed by the Duncan test. Different lowercase letters indicate statistical difference ($p < 0.05$).

Regarding SPH exposure, the highest concentrations (0.3 and 0.6 mg/mL) caused a slight reduction in cell viability (Fig. 3A). In the absence of oxidative stress, SPH alone did not significantly affect ROS levels compared to basal production. However, pre-exposure to SPH at various concentrations before oxidative stress reduced ROS production. Notably, SPH mitigated H_2O_2-induced ROS generation in a non-dose-dependent manner (Fig. 3B). The lack of protection against oxidation at higher concentrations likely resulted from reduced cell viability caused by sunflower protein hydrolysates at elevated concentrations. This reduced number of viable cells would inherently limit the overall oxidative response and subsequent DCF oxidation, potentially masking any antioxidant effects. Therefore, subsequent experiments focused solely on the lowest concentration (0.15 mg/mL). This effective concentration is higher than the 0.05 mg/mL reported by Tonolo et al. (2024) for antioxidant and anti-inflammatory activity of sunflower seed-derived bioactive peptides in Caco-2 cells (Tonolo et al., 2024). In contrast, the concentration of sunflower hydrolysates used in Caco-2 cell treatments was not reported in other studies (Megías et al., 2009).

It is pertinent to note that the observed effects of sunflower hydrolysate and its antioxidant activity were evident in undifferentiated Caco-2 cells, which are known to be more susceptible to oxidative stress than their differentiated counterparts. Differentiated Caco-2 cells, with their enhanced antioxidant defenses and lower basal ROS levels, may necessitate higher hydrolysate concentrations to elicit comparable antioxidant effects. These findings suggest that sunflower peptides retain radical-scavenging properties in biological systems, likely through interactions with cellular antioxidant mechanisms.

To further investigate the antioxidant peptide effect on cells, the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) content was evaluated. These enzymes represent the first line of cellular antioxidant defense, especially in relation to the superoxide anion radical (O_2^-). SPH was able to modulate these enzymes, reversing the increase in GSH content and CAT activity induced by the H_2O_2 stimulus (Fig. 4A and C). Although not statistically significant, the same behavior was observed with SOD activity (Fig. 4B). No change was observed in the secretion of nitric oxide by Caco-2 cells (Fig. 4D).

Bioactive peptides modulate antioxidant enzymes through multiple mechanisms. They can act as hydrogen donors to scavenge free radicals, inhibit pro-oxidant enzymes such as NADPH oxidase (Wu et al., 2024), directly modulate antioxidant enzymes (Liu et al., 2023), and induce the expression of antioxidant-related genes by activating transcription factors like Nrf2 (nuclear factor erythroid 2-related factor 2), leading to the upregulation of antioxidant enzymes. Notably, chronic exposure (24 h) to specific sunflower-derived peptide sequences (DVAMPVPK, VETGVIKPG, TTHTNPPPEAE, LTHPQHQQQGPSTG, and PADVTPEEKPEV) has been shown to activate the Keap1/Nrf2 pathway, promoting the upregulation of antioxidant response element-regulated enzymes (Tonolo et al., 2024).

In our experiment, however, we observed a decrease rather than an increase in antioxidant enzyme levels following sunflower protein hydrolysate (SPH) exposure. Unlike previous studies, our treatment involved acute oxidative stress induced by peroxide (3 h), which may not have been sufficient to elicit significant changes in antioxidant enzyme expression. It is possible that the acute oxidative stimulus initially activates antioxidant enzymes as a cellular defense mechanism, a response that may be mitigated by pre-exposure to sunflower hydrolysates due to their electron-donating properties.

In this study, we highlight that sunflower protein hydrolysates can act as an antioxidant agent by themselves, with minimal phenolic interference. Antioxidant peptides were able to modulate important antioxidant enzymes in Caco-2 metabolism, probably acting as hydrogen donors and interacting with intracellular oxidant agents. This result suggests that the application of sunflower hydrolysate in food supplements could beneficially affect gastric-intestinal health, acting as protective molecules against oxidative damage and preventing gastric-intestinal disorders.

3.5. *Peptide identification sequence and alcalase hydrolysis pattern*

Peptide sequences of the SPHf fraction were identified to better understand the structure-function relationship of antioxidant activity (Suppl. material: Table S2). Spectral analysis was performed with PatternLab using the *Helianthus annuus* database from Uniprot, and 196 peptide sequences exclusive to sunflower were identified. These peptides originated from 10 different proteins, mostly represented by 11S globulin (73 %), followed by glutelin (10 %). In fact, globulins are predominantly found in seeds and constitute most of the sunflower proteins (González-Pérez & Vereijken, 2007).

Regarding the alcalase hydrolysis pattern, several homologous peptides were observed, which vary in length, with a few amino acids upstream or downstream of the protein sequence (Suppl. material: Table S2). This result indicates that the alcalase enzyme recognized very close regions for cleavage. Furthermore, alcalase was able to form peptides not only from the surface regions of the protein but also from internal regions of its structure. For example, the labeling of some peptides formed from the globulin structure is shown in Suppl. material Table S3. Alcalase hydrolysis was able to fragment most parts of the globulin protein, including internal regions. This is consistent with an endopeptidase, which promotes a wider range of cleavage sites and, therefore, is widely applied to produce bioactive peptides (Tacias-Pascacio et al., 2020).

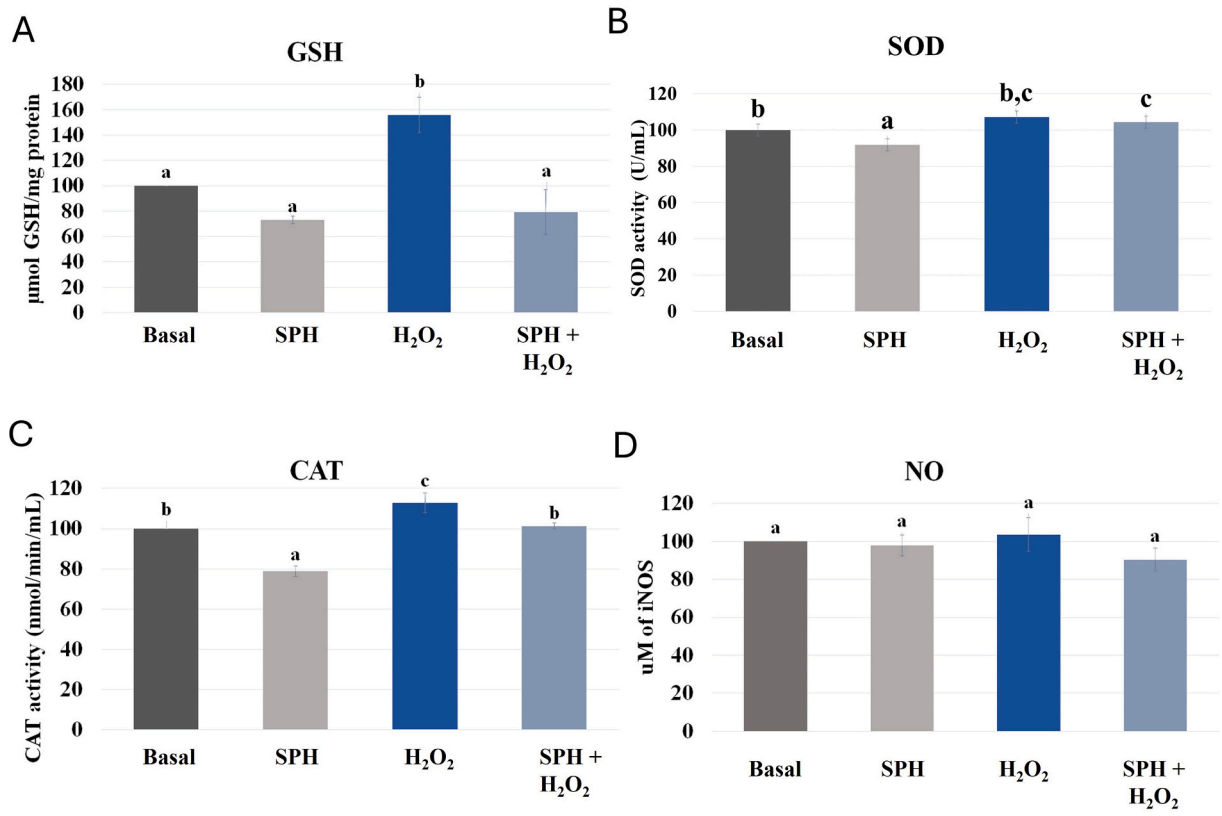

Fig. 4. SPHf modulates GSH and CAT levels after oxidative stimulus. (A) GSH content; (B) SOD activity, (C) CAT activity; (D) NO secretion after treatment with 3 h of SPHf and H_2O_2. Treatments were compared by ANOVA followed by the Duncan test. Different lowercase letters indicate statistical difference ($p < 0.05$).

3.6. *In silico bioactive analysis of peptide sequences*

To evaluate the overall structural peptide profile generated by the Alcalase enzyme and its association with bioactivity, we utilized *in silico* analysis tools. These tools enable the simultaneous analysis of numerous peptide sequences and help identify the most promising sequences with potential bioactivity. The sequenced peptides identified were analyzed by their molecular weight, hydrophobicity, and the probability of being bioactive, antioxidant, and anti-inflammatory (Suppl. material: Table S2). The predictive analysis was conducted using software such as PeptideRanker, Free Radical Scavenging Activity (FRS), Metal Chelating Properties (CHEL), and Anti-Inflammatory Activity Predictor (Pre-AIP) that help predict the bioactivity of peptides based on sequence features, physicochemical properties, and machine learning models (Khatun et al., 2019; Mooney et al., 2012; Olsen et al., 2020). The machine learning-based algorithm trained on experimentally validated bioactive peptides. All these computational tools assigns a probability score, where higher scores indicate greater bioactivity potential. For Peptide Ranker and FRS tools were considered the output score above 0.5 and for Pre-AIP scores above 0.468 to provide higher confidence in prediction results (Khatun et al., 2019; Mooney et al., 2012; Olsen et al., 2020).

Over half of the identified peptides (55.7 %) had a molecular size smaller than 1 kDa (approximately 10 amino acids) and hydrophobicity greater than 50 %, which is consistent with our previous experimental results (Fig. 1). In terms of predictability analysis, 33 % of our samples had PeptideRanker scores over 0.5 (predicting bioactivity), 9 % had FRS scores above 0.5 (predicting free radical scavenging activity), and 28.3 % had Pre-AIP scores above 0.468 (predicting anti-inflammatory activity) (Table S2).

To better explore the predictability results, correlations were made between the different parameters, and Pearson values and correlation graphs are shown in Fig. 5. The most significant correlation shows that molecular weight was positively correlated with PeptideRanker, FRS, and Pre-AIP, meaning that larger peptide sequences are more likely to be bioactive and exhibit radical scavenging and anti-inflammatory activities. Hydrophobicity was highly positively correlated with PeptideRanker, FRS, and CHEL, indicating that the greater the hydrophobicity, the higher the probability of having a bioactive effect, free radical scavenging activity, and metal chelating properties. Furthermore, PeptideRanker was positively correlated with FRS, CHEL, and Pre-AIP, which is expected, as antioxidant and anti-inflammatory capacities should reflect the overall ability to be bioactive.

Literature data show that low molecular weight and higher hydrophobicity are highly associated with antioxidant activity. The small size of the peptides allows better cellular diffusion and resistance to digestive processes (Zaky et al., 2022). In our results, most of the peptides are smaller than 1 kDa, which was positively associated with bioactivity. The chemical structure of the peptides includes hydrophobic amino acids with aromatic rings, such as Tyr and Phe, which allow the donation of protons to radicals, forming a peptide with a resonance-stabilized radical (Tonolo et al., 2024; Zaky et al., 2022). Additionally, hydrophobic peptides protect against the formation of oxidized polyunsaturated fatty acids and tend to cross cell plasma membranes more effectively (Zou et al., 2016).

Peptide sequences identified were cross-checked with the BIOPEP-UWM database (Table 2). This database compiles food peptide sequences and their related bioactivity in previous *in vivo* or *in vitro* studies (Minkiewicz et al., 2022). No sequence identified in our sample was 100 % identical to those already reported. This result was expected, as there is limited data in the literature relating functionality to the sequence structure of plant-derived peptides. However, smaller sequences that could be integrated within the structure of the identified peptides were analyzed, and several di- and tripeptides were found to be previously associated with antioxidant activity (red fragments, Table 2). These short antioxidant fragments were extracted from plant sources with similar protein structures to sunflower, such as soybean (Table 2 –

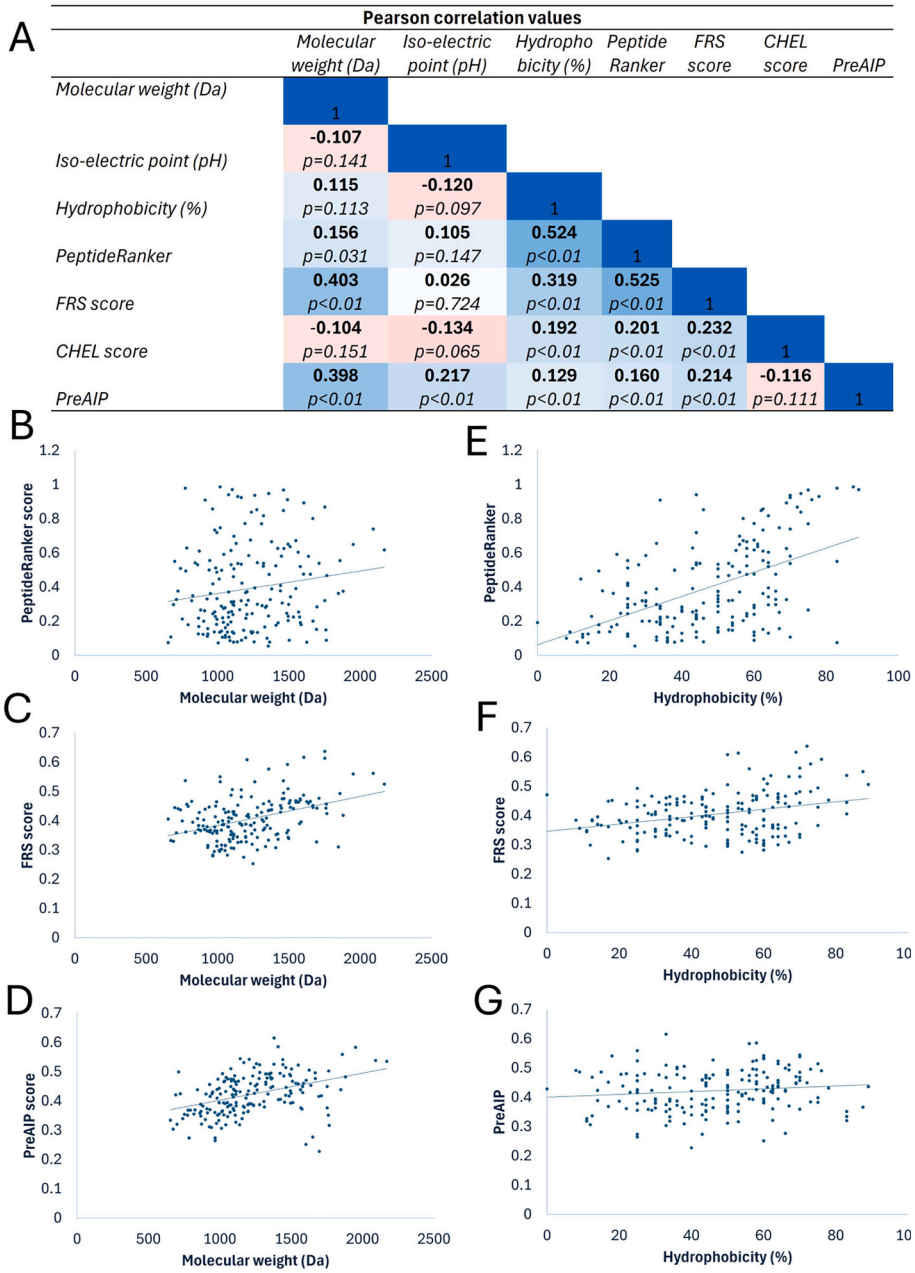

Fig. 5. Pearson Correlations of molecular weight, hydrophobicity and bioactive prediction scores. (A) Pearson scores and statistical differences with "p" values. Pearson correlation graph between (B) Molecular weight and peptide ranker; (C) Molecular weight and free radical scavenging activity (FRS); (D) Molecular weight and probability to act as anti-inflammatory (PreAIP); (E) Peptide Ranker and Hydrophobicity; F: FRS and Hydrophobicity; G: PreAIP and Hydrophobicity.

"Origin of biopeptide extracted"). It is important to mention that small molecular peptides (di- and tripeptides) were not identified in our analysis, as they were below the ionization.

The 30 peptides with the best *in silico* scores were selected for amino acid sequence analysis (Table 3). The frequency of each amino acid in the center of the peptide structure, as well as in the amino (N-terminal) and carboxyl (C-terminal) regions, are shown in the IceLogo and heatmap in Fig. 6. A high incidence of the amino acids proline (Pro, P) and tryptophan (Trp, W) in the center of the sunflower bioactive peptide structures can be observed. These hydrophobic and aromatic amino acids, respectively, are commonly associated with antioxidant properties due to their ability to scavenge the hydroxyl (OH) radical and/or inhibit lipid peroxidation (Zaky et al., 2022). Trp mainly acts through single electron transfer (Esfandi et al., 2019). The presence of proline residues in the peptide structure contributes to an open structure, which has been linked to better resistance to digestive enzymes and improved absorption in the body (Ichikawa et al., 2010; Sabeena Farvin et al., 2010).

The presence of proton-donating amino acids and their specific locations within the peptide sequence are important for their bioactivity (Esfandi et al., 2019). Both the N- and C-terminal regions were rich in phenylalanine, a hydrophobic and aromatic amino acid with strong radical-scavenging activity. The negatively charged amino acid aspartic acid (Asp), which was highly present in the N-terminal region of the identified peptides, may contribute to radical quenching activity due to its ability to transfer electrons (Zou et al., 2016).

There are limited studies on the secondary structure of peptides. However, since most bioactive peptides are short molecules, their folding capacity is likely limited. Unlike proteins, which have amino acids hidden in their structure, the majority of amino acid residues in

Table 2
Identification of sunflower peptides with potential shorter sequences with antioxidant activity and antioxidant predictability.

Origin of biopetide extracted	Peptide sequence Identified	Reference	Predicted free radical scavenger (FRS) score	Predicted chelation (CHEL) score
Synthetic peptides of soybean protein	AEKG**HL**QPN	https://doi.org/10.1021/jf950833m	0.46477	0.25367
	SFKFPILE**HL**		0.38542	0.24877
	AIQS**PHW**TIN	https://doi.org/10.1021/jf021191n	**0.50619**	0.22474
	IQS**PHW**TIN		0.48768	0.23813
	LL**PYY**PNTPE	https://doi.org/10.1021/jf950833m	**0.60884**	0.20892
	TG**RHQ**QSQRPGWE		0.33960	0.22997
	TG**RHQ**QTQRPSWE		0.36449	0.22708
	GERLPFDED**RHQ**		0.33966	0.25427
	RLPFDED**RHQ**	https://doi.org/10.1021/jf021191n	0.40462	0.24493
	RLPFDED**RHQ**KVE		0.44578	0.22719
	TD**RHQ**KIH		0.38660	0.25712
	GGRRGGGEGNQD**RHQ**		0.47151	0.25095
Hydrolysetes of Sardinelle by-products proteins (*Sardinella aurita*)		https://doi.org/10.1016/j.foodchem.2009.05.021		
Hydrolysates of potato protein	GLLL**PYY**PNTPELVY	https://doi.org/10.1021/jf101556n	**0.61410**	0.20455
Synthetic peptides of soybean protein		https://doi.org/10.1021/jf950833m		
	AERG**EL**RPN		0.38660	0.25712
	GNSVFDN**EL**RE		0.34326	0.22190
Synthetic casein protein	GNSVFDN**EL**REG	https://doi.org/10.1016/s0955-2863(99)00083-2	0.31211	0.21325
	HNDGNT**EL**		0.38281	0.25362
	HNDGNT**EL**VVV		0.34314	0.20508
	NNGQD**EL**VII		0.30653	0.19021
	SADRG**EL**RPN		0.35424	0.21353
	TRDN**VY**AGF		0.38825	0.22466
Hydrolysates of potato protein	DN**VY**AGF	https://doi.org/10.1021/jf101556n	0.45668	0.21403
	LTRDN**VY**AGF		0.39129	0.22000
	ENIDNPSH**ADF**VNPQ		0.44330	0.28437
	H**ADF**VNPQ		0.38009	0.28488
	IDNPSH**ADF**VNPQ		0.47559	0.26434
	NIDNPSH**ADF**VNPQ		0.46417	0.25458
Synthetic peptide from Okara protein	PSH**ADF**VNPQ	https://doi.org/10.3136/fstr.8.357	0.35244	0.26510
Synthetic of mung bean meal protein	LLPSYVNTPI**LAF**	https://doi.org/10.3390/molecules26061515	0.44343	0.20053
	RIQPGGLLLPSYVNTPI**LAF**		**0.52577**	0.21034
Hydrolysates peptide from soybean protein	NIDDPSNAD**LY**NPQ	https://doi.org/10.1007/s00217-009-1093-1	0.39043	0.25190
Hydrolysates of potato protein	RDN**VY**AGF	https://doi.org/10.1021/jf101556n	0.44729	0.22303
Synthetic peptide from Okara protein	VVL**AY**EPVWAIGTGK	https://doi.org/10.3136/fstr.8.357	0.48772	0.15001
Hydrolysates fraction of marine bivalve peptides (*Mactra veneriformis*)		https://doi.org/10.1016/j.foodchem.2014.06.113		
	RAGEQGS**RW**VSF		0.43841	0.17751
	TNRAP**LK**SPL		0.30750	0.26199
	NRAP**LK**SPL		0.31359	0.25321
Hydrolyzed of egg protein	GVDF**IR**H	https://doi.org/10.1016/j.foodchem.2010.04.083	0.35857	0.21452
	IIRPPQ		0.44182	0.27872
	IIRPPQAR		0.36838	0.28227
	QI**IR**PPQAR		0.41122	0.25741
Synthetic from β-Lactoglobulin	N**NEN**QLDEY	https://doi.org/10.1007/s13594-015-0226-5	0.34481	0.23910
Synthetic peptides of soybean protein	RGFQD**RHQ**KIR	https://doi.org/10.1021/jf021191n	0.45211	0.23101
Synthetic peptide from hemp seed (*Cannabis sativa L.*)	K**LPL**LQ	https://doi.org/10.1016/j.jff.2013.11.005	0.35917	0.29142
	QVVRPP**IR**IQ		0.41680	0.22782
Hydrolyzed of egg protein	RGLQVVRPP**IR**IQ	https://doi.org/10.1016/j.foodchem.2010.04.083	0.44215	0.18801
	VERGLQVVRPP**IR**IQ		0.44334	0.16774
	VVRPP**IR**IQ		0.43123	0.23234
	DI**PW**PF		**0.53734**	0.28542
	DMPFDI**PW**PFRPS		**0.61714**	0.26588
	EIPFDMPFDI**PW**PFRPS		**0.56245**	0.23478
Peptide fraction of buckwheat protein	FDI**PW**PFRPS	https://doi.org/10.1016/j.foodchem.2009.05.024	**0.63753**	0.26920
	FDMPFDI**PW**PFRPS		**0.59280**	0.26175
	MPFDI**PW**PFRPS		**0.55048**	0.26555
	PFDI**PW**PF		**0.57696**	0.26467
	PFDI**PW**PFRPS		0.41910	0.19481
Hydrolysates fraction of marine bivalve peptides (*Mactra veneriformis*)	AGEDKGR**LW**PF	https://doi.org/10.1016/j.foodchem.2014.06.113	**0.63753**	0.26920
Synthetic peptide from Okara protein	KDDDLK**AY**	https://doi.org/10.3136/fstr.8.357	0.27976	0.24703
Hydrolysated of tuna (*Katsuwonus pelamis*)		https://doi.org/10.2331/suisan.65.92		
Hydrolyzed of egg protein		https://doi.org/10.1016/j.foodchem.2010.04.083		
Synthetic peptide from Okara protein	SEDK**AD**FR	https://doi.org/10.3136/fstr.8.357	0.28384	0.22623

(*continued on next page*)

Table 2 (*continued*)

Origin of biopetide extracted	Peptide sequence Identified	Reference	Predicted free radical scavenger (FRS) score	Predicted chelation (CHEL) score
Synthetic casein peptide	ADRG**EL**RPN	https://doi.org/10.1016/s0955-2863(99)00083-2	0.36833	0.21247
Peptides from sea squirt (*Halocynthia roretzi*)	**WLP**SPFFPI	https://doi.org/10.1039/d2fo00729k rsc.li/food-function	**0.50706**	0.26398
Hydrolyzed of ovotransferrin	VVRPP**IR**IQ	https://doi.org/10.1016/j.foodchem.2010.04.083	0.43123	0.23234
Hydrolyzed of ovotransferrin	**IIR**PPQ	https://doi.org/10.1016/j.foodchem.2010.04.083	0.44182	0.27872

Source: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Tre; V = Val; W = Thr; Y = Tyr. In red are the di- and tri-peptides contained in the sequence that have previously been linked to antioxidant activity.

Table 3
Best 30th peptides that obtained the highest values of prediction scores in the *in silico* analysis.

	Peptide sequence Identified	Molecular weight (Da)	Hydrophobicity (%)	Peptide Ranker	FRS score	CHEL score	PreAIP
1	AIQSPHWTIN	1166.29	50	0.535	0.506	0.225	0.478
2	DIPWPF	773.87	83	0.980	0.537	0.285	0.352
3	DMPFDIPWPFRPS	1604.83	69	0.894	0.617	0.266	0.471
4	EIPFDMPFDIPWPFRPS	2091.39	70	0.740	0.562	0.235	0.539
5	FDIPWPFRPS	1261.43	70	0.938	0.534	0.264	0.458
6	FDMPFDIPWPFRPS	1752	72	0.870	0.638	0.269	0.45
7	FEIPFDMPF	1142.32	78	0.931	0.453	0.261	0.432
8	GDIFPVPQ	871.98	62.5	0.612	0.429	0.281	0.322
9	GDIFPVPQFF	1166.32	70	0.925	0.482	0.312	0.36
10	IFPVPQ	699.84	83	0.551	0.445	0.299	0.321
11	IQSPHWTIN	1095.21	44	0.532	0.488	0.238	0.474
12	KLPILSLMDL	1142.45	70	0.539	0.326	0.247	0.545
13	LLPYYPNTPE	1206.34	50	0.374	0.609	0.209	0.391
14	MPFDIPWPFRPS	1489.74	76	0.912	0.593	0.262	0.491
15	NLNSFKFPIL	1192.41	60	0.773	0.365	0.224	0.423
16	NSFKFPIL	965.14	62.5	0.859	0.358	0.233	0.361
17	NSFKFPILE	1094.26	55	0.616	0.364	0.228	0.414
18	PFDIPWPF	1018.16	87.5	0.987	0.550	0.266	0.367
19	PFDIPWPFRPS	1358.54	73	0.949	0.577	0.265	0.496
20	RGDIFPVPQFFAT	1494.69	61	0.652	0.510	0.292	0.435
21	RIQPGGLLLPSYVNTPIL	1951.31	56	0.651	0.560	0.227	0.584
22	RIQPGGLLLPSYVNTPILAF	2169.56	60	0.618	0.526	0.210	0.536
23	SFKFPILE	980.16	62.5	0.735	0.366	0.252	0.44
24	SFKFPILEHL	1230.45	60	0.676	0.385	0.249	0.486
25	SFKLPILQ	945.16	62.5	0.556	0.362	0.274	0.425
26	SRGDIFPVPQ	1115.24	50	0.507	0.431	0.277	0.434
27	SYPTLPGWIPSPF	1461.66	62	0.849	0.537	0.234	0.42
28	WLPSPFFPI	1103.31	89	0.972	0.507	0.264	0.437
29	YGPGGGGGGGRK	1019.07	67	0.749	0.534	0.189	0.494
30	YTNTPILFF	1115.28	56	0.699	0.422	0.231	0.457

Subscript numbers represent homologous peptides sequences. [a]PeptideRanker, probability to be bioactive (0 represent less active and 1 most active; threshold 0.5); [b] FRS predicts free radical scavenging activity (threshold 0.5); [c]CHEL predicts metal chelating properties; [d]PreAIP predicts anti-inflammatory activity of peptides (low scores <0.39 and high scores >0.47).

peptides are exposed and available to interact with the environment, thus contributing to their bioactive properties.

The *in silico* structural-bioactivity findings establish a crucial theoretical basis for the functionality of sunflower hydrolysate products, providing a framework for the development of high-value supplements and other functional products. Our current research, evaluating the total hydrolysate effect to mimic natural cellular exposure to the digestion peptide pool (comprising 196 identified peptides), suggests that lower molecular weight and higher hydrophobicity are key contributors to bioactivity, aligning with our computational predictions. Future validation through the synthesis of the most promising peptides will offer direct evidence of their individual antioxidant efficacy. Furthermore, *in vivo* studies using animal models and subsequent clinical trials are indispensable for a comprehensive understanding of these bioactive peptides' effects.

4. Conclusion

This study assesses the intrinsic antioxidant potential of sunflower peptides, even in the absence of phenolic compounds. A reduction in phenolic compound may increase the applicability of sunflower protein hydrolysates as an ingredient for functional food. The sunflower protein hydrolysate maintains its bioactive properties when interacting with cellular systems, enhancing intracellular antioxidant activity and reducing reactive oxygen species production. These bioactive effects are particularly significant for the potential application of sunflower hydrolysates in food supplements.

Structural analysis of the peptide sequences revealed an average length of 10 amino acids, with a hydrophobicity exceeding 50 %. Both hydrophobicity and molecular weight showed a positive correlation with computational bioactivity prediction scores, as well as with free radical scavenging and anti-inflammatory activity. The presence of hydrophobic amino acids, such as phenylalanine and tryptophan, at both

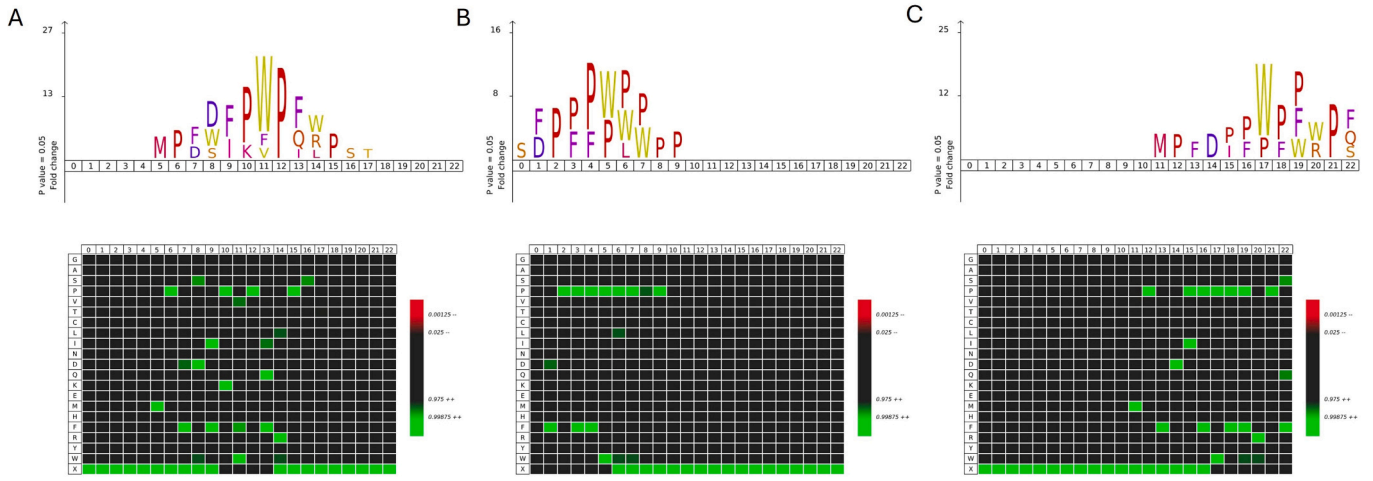

Fig. 6. IceLogo and Heat map graph of the 30th peptides with the best *in silico* score. (A) Central sctruture; (B) N-terminal alignment and (C) C-terminal alignment. Only significant amino acids ($P < 0.05$) are shown or colored in the heatmap. The difference in the frequency of an amino acid is expressed as the size of letters or color intensity. The *P* value of each amino acid at every position was calculated by testing the experimental frequency against the frequency of each amino acid in the reference set.

the internal and terminal positions of peptides may contribute to electron donation to radicals and the inhibition of lipid peroxidation in cells.

Future research should further explore the structure-function relationships of sunflower peptides. Advanced analytical approaches, including molecular docking and *in vivo* studies, are essential for validating these findings. Additionally, investigating potential synergistic effects with other natural antioxidants could enhance their practical applications in health and nutrition.

CRediT authorship contribution statement

Daniel S. Lopes: Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis. **Lilian G.V.C. Almeida:** Methodology, Investigation. **Agustina E. Nardo:** Software, Investigation, Data curation. **María Cristina Añón:** Writing – review & editing. **Lucilene D. dos Santos:** Methodology. **Bruno C. Rossini:** Methodology, Data curation. **Cristian M.B. Pinilla:** Methodology. **Maria T.B. Pacheco:** Writing – review & editing, Supervision, Investigation. **Fabiana Galland:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, 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.

Acknowledgment

This work was financially supported by FAPESP (grant 2019/01896-4 and 2018/12546-1). Daniel Saraiva acknowledges FAPESP for the fellowship (process 2022/01204-8).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.144733.

Data availability

Data will be made available on request.

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