Integrated ultrafiltration, nanofiltration, and reverse osmosis pilot process to produce bioactive protein/peptide fractions from sardine cooking effluent

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Abstract

Sardine cooking effluents contain a high level of organic matter, such as proteins and lipids, which allows them to be forward into a chain exploiting high added-value compounds attained from these effluents, increasing their economic value while reducing their environmental effect. Thus, the purpose of this work was to develop an innovative pilot-scale integrated membrane process, with or without enzymatic hydrolysis, to obtain fractions with high protein/peptide and low NaCl contents, as well as optimized bioactive properties. The research strategy followed involved the use of ultrafiltration (UF) and nanofiltration (NF) technologies of the pretreated sardine

cooking effluent followed by reverse osmosis (RO) at a pilot scale levels. Moreover, it allowed for the attainment of fractions rich in protein/peptides that might be used in the food, pharmaceutical, or cosmetic industries, particularly after RO, as they present a lower NaCl content.

The RO retentate (hydrolyzed sample) coupled with UF and NF resulted in the fractions with the best bioactive properties (higher antioxidant capacity and antimicrobial activity) of all the analyzed samples. Overall, the current work demonstrated the feasibility of exploiting liquid by-products as a source of functional components as well as reinforcing this strategy's potential relevance in future effective management strategies for this type of effluents.

Keywords: Canning industry by-products; Sardine cooking effluent valorisation; Bioactive compounds extraction; Membrane technology; Bioactive properties; Pilot-scale

1. Introduction

Globally, the processing of seafood results in high amounts of solid and liquid waste (AMEC Earth & Environmental Limited, 2003). In fact, operations such as trimming, eviscerating, peeling, and/or shelling may generate up to 70% of total incoming fish weight in by-products (Olsen et al., 2014).

Fish processing solid by-products are an important source of value-added compounds. Namely, fish skins are a potential source of collagen and gelatine, compounds used in the pharmaceutical, cosmetic, and food industries. Moreover, other discards (e.g. viscera, heads, tails, flesh residues and effluents) are rich in lipids and proteins, such as long-chain ω3 polyunsaturated fatty acids (PUFA), proteolytic

47 enzymes, and bioactive peptides, that can be used as fortifying food ingredients (Ferraro
48 et al., 2013; Etxabide et al., 2017; Ishak et al., 2018). Even though there is a significant
49 body of work focusing on the valorization of solid waste, we are aware of just a few that
50 have focused on industrial effluents. An example of a particularly interesting industrial
51 effluents are cooking effluents that result from canned fish production as they are rich
52 in soluble nutrients, particularly peptides and proteins (Hung et al., 2014), besides their
53 high content in PUFA.

54 The canning industry is the second largest source of fish-processing wastes and
55 by-products, generating more than 1.5 million tons of waste per year in the European
56 Union alone (Ferraro et al., 2010). Canning entails several steps, including raw material
57 washing, thawing, and cooking, all of which generate different effluents. Cooking is a
58 necessary operation that generates a large amount of effluent known as cooking
59 wastewater. It has been reported that a single fish canning plant produces
60 approximately 15 to 27 tons of cooking effluent per day. It comprises of ca. 4% water-
61 soluble protein, including sarcoplasmic proteins and other proteins such as collagen (Jao
62 and Ko, 2002; Hsu et al., 2009). Cooking waters do not produce the highest quantity of
63 effluents, but they pose an environmental concern due to their high organic pollutant
64 load (mainly proteins and lipids) (Cros et al., 2006). Because of their relatively high
65 concentration of proteins and potentially bioactive peptides, several strategies for
66 valorizing fractions of fisheries effluents have been proposed.

67 Membrane technology is suitable for the enrichment of valuable molecules from
68 fish processing effluents envisaging the production of marketable functional ingredients
69 because it is a sustainable cost-effective technology that does not require solvents or
70 absorbents. Based on the sieving effect as well as Donnan (electrostatics) repulsion for

71 ion fractionation, NF offers a great opportunity for fractionation of different solutes,
72 enabling mono-valent salts to partly cross the membrane and retaining molecules with
73 larger size. Considering the different molecular sizes of the peptides, membrane
74 processes such as UF, NF and RO may retain these molecules (Tonon et al., 2016).

75 Protein/peptide recovery from fish effluents using membrane technology has
76 received little attention so far with the few studies on the subject focusing primarily on
77 shrimp and sardine cooking effluents (Tonon et al., 2016; Ghalamara et al., 2020). The
78 filtration system, which consisted of an UF unit with ceramic membranes (Pall
79 Corporation, New York, USA) and average pore size of 10–20 kDa coupling enzymatic
80 hydrolysis processes, was used to obtain a protein hydrolysate from the waste produced
81 during shrimp cooking (Tonon et al., 2016). Ghalamara et al. (2020) recently studied the
82 sustainable production of fish by-products (codfish blood and sardine cooking effluents)
83 fractions enriched in small biopeptides using UF membrane at laboratory scale, with
84 enhanced biological properties compared to the raw materials.

85 There is still a lack of information regarding the bioactive properties of the
86 different proteins/peptides with different sizes recovered from fishery effluents at pilot
87 scale. To the best of our knowledge, the relationship between different sustainable
88 processes conducted at pilot scale for producing different protein/peptide fractions
89 from sardine cooking effluent and the detailed characterization of the resultant fractions
90 in terms of biochemical and bioactive properties has not been reported before. In this
91 sense, the objective of this study was to assess whether membrane technology,
92 combined or not with enzymatic hydrolysis, may originate added-value protein/peptide
93 fractions from sardine cooking effluent, at pilot scale, with enhanced bioactive
94 properties, when compared to the raw material. Particularly, the influence of the

different protein fractionation processes (processing the sardine cooking effluent by a tight-UF membrane combined or not with enzymatic hydrolysis) on the different fractions produced was evaluated, to obtaining fractions with maximized protein/peptide content and maximized bioactive properties (antioxidant, antimicrobial, antihypertensive, antifreeze activities). Due to the high content of salts in this sardine cooking effluent, further NF treatment was applied to these fractions to remove the mono-valent salts that remained in large quantities after the UF treatment, followed by RO to concentrate the NF retentate fractions. The fractions produced in this work were characterized in terms of their biochemical and bioactive properties.

The rest of the paper is organized as follows: Section 2 describes the materials used in this study, as well as the processing of sardine cooking effluent, analytical methods for biochemical characterization and bioactive properties of the obtained protein/peptide fractions. Section 3 discusses the biochemical characterization of sardine cooking effluent, protein/peptide fractions from sardine cooking effluent and the biological properties of relevant sardine cooking effluent fractions with the subsequent conclusions being summarized in section 4.

2. Materials and Methods

2.1. Materials

Sardine cooking effluent (*Sardina pilchardus*) was kindly supplied by Fábrica de Conservas A Poveira S.A., a company located in the North of Portugal (Póvoa de Varzim, Porto, Portugal). This effluent was collected directly from the cooker after one production day. To stabilize the sardine cooking effluent 1% (v/v) acorn shell extract (8%

w/v in water) was added immediately after collection (the raw material of this work) and then frozen at −20 °C for further analysis.

The materials used in this study are summarized in Table 1.

Table 1

List of materials used in this study.

Material	Company	Country
Cynara cardunculus extract (1.14 g/mL)	Ante Vegetal	Portugal
Potassium peroxodisulfate	Merck	Germany
Sodium dihydrogen phosphate dehydrate		
Zinc chloride		
Hydrogen peroxide 30%		
Nitric acid 65%		
PierceTM BCA protein assay kit	Thermo Scientific	USA
Glycerol	Thermo Scientific	Germany
Sodium chloride	Honeywell	Germany
Sulfuric acid 97%		
Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)	Sigma-Aldrich	USA
Flourescein sodium salt		
Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.9%)		
Sodium citrate dihydrate (trisodium salt, $C_6H_5Na_3O_7 \cdot 2H_2O$)		
Mercaptosuccinic acid (MSA, ≥99.0%)		
Standard proteins: thyroglobulin (669 kDa); aldolase (158 kDa); conalbumin (75 kDa); ovalbumin (43 kDa); carbonic anhydrase (29 kDa); ribonuclease A (13.7 kDa) and whey peptide (1.2 kDa)	Sigma-Aldrich GeneScript	USA
2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)	Sigma-Aldrich	Canada

2,2'-Azobis(2-methylpropionamidine) dihydrochloride	Sigma-Aldrich	China
Sodium hydroxide	LabChem	Portugal
Mueller hinton broth (MHB)	Biokar Diagnostics	France
Boric acid	PanReac AppliChem	Germany
Kjeldahl catalyst tablets	Thompson and Capper Ltd	United Kingdom

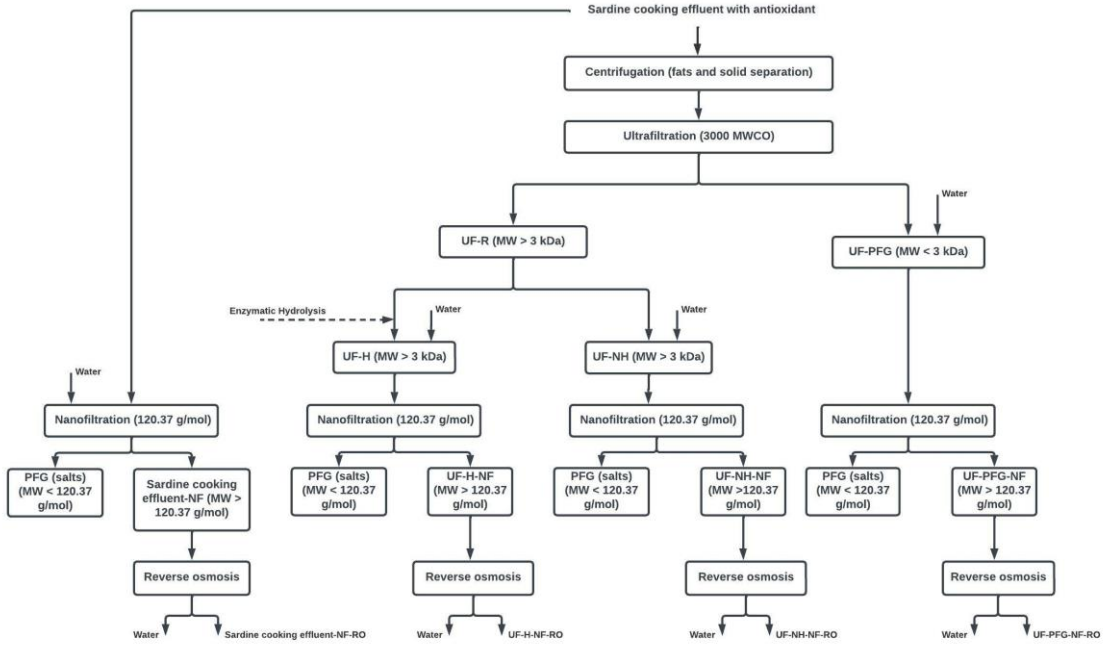
123

124 *2.2. Processing of sardine cooking effluent*

125 The scheme of the sardine cooking effluent (with acorn shell extract) process is
126 shown in Fig. 1. Before membrane processing, approximately 50 L of sardine cooking
127 effluent were centrifuged by means of a Westfalia separator type ADB (GEA, Oelde,
128 Germany) to remove lipids and solid components to facilitate the UF process. The
129 pretreated sardine cooking effluent by centrifugation was processed sequentially by UF
130 (combined or not with enzymatic hydrolysis), NF and RO. In order to separate the smaller
131 from the larger proteins, a spiral-wound UF polysulfone membrane (with 3 kDa pore size
132 membrane and an effective permeation area of 7 m^2) was used in a pilot batch plant
133 (Proquiga SA, Spain), at temperatures of 45-50 °C and a transmembrane pressure (TMP)
134 between 3 and 4 bar. Two fractions were obtained: 8 L of retentate (UF-R) enriched in
135 larger protein/peptides and 34 L of global final permeate (UF-PFG). The obtained
136 retentate fraction was divided into two equally separate parts, one of which was
137 hydrolyzed. Enzymatic hydrolysis using *Cynara cardunculus* extract (1.14 g/mL) was
138 carried out at 55 °C and a pH 5.2 (adjusted using 2 M HCl).

139 To reduce the amount of NaCl of the sardine cooking effluent and of all UF
140 fractions (the retentates UF-H and UF-NH and the permeate UF-PFG), 3 L of each were
141 mixed with deionized water up to a volume of 150 L and submitted to spiral-wound NF

142 experiments in a pilot batch NF plant (Setalact, Spain), using a 7 m^2 filtration area thin-
143 film composite (TFC)-PSF membrane with molecular weight cut-off (MWCO) of 120.37
144 g/mol at a TMP of 20 bar, with a final volume concentration factor (VCF) of 2.73.
145 Th obtained NF permeate fractions were discarded due to the high salt concentration
146 and the presence of just traces of nitrogen compounds.
147 Finally, to concentrate the NF retentate fractions obtained, the spiral-wound RO
148 plant (ORM, Portugal) concentrated each fraction to a VCF of 27.5 by using an AMFOR ®
149 ((INC, USA), 2540FF, TW30LE-254D)) membrane with an effective area of 2.6 m^2.



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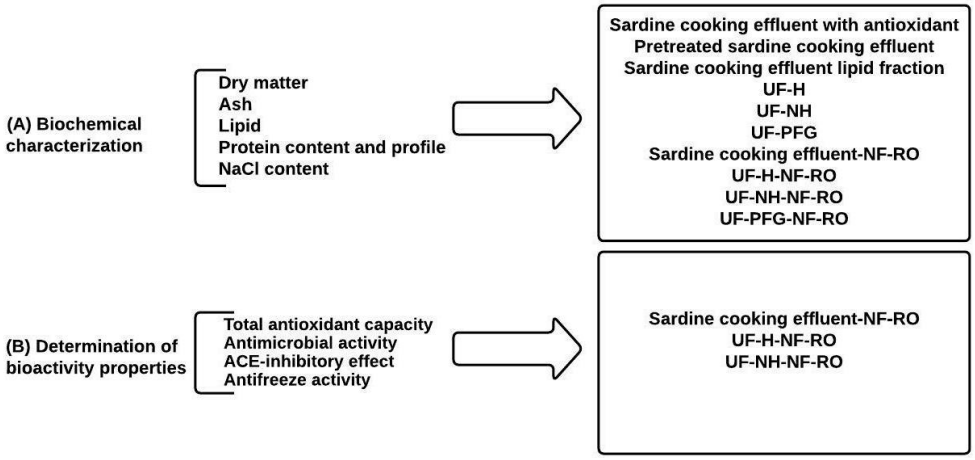

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Fig. 1. Flow diagram summarizing the process employed for the production of various protein/peptide fractions in this work. Observation: (A) samples characterized biochemically; (B) samples characterized in terms of their bioactivity properties.

The observed rejection of the protein/peptides for the UF membrane under study, at the end of the experiment (VCF of 5.25) was determined using equation 1:

$$R[\%] = 1 - \frac{c_{peptides,PFG}}{c_{peptides,FF}} \quad (1)$$

where $c_{peptidesPFG}$ and $c_{peptides,FF}$ are the peptides concentrations in the global final permeate and retentate at the end of the experiment, respectively. In equation 1, the peptides concentrations of each sample were determined by the product of their calibration factor and their chromatograms areas measured for each sample ($A_{peptides}$). Considering that the calibration factor of the peptides is constant for all samples, equation 1 was converted into equation 1':

$$R[\%] = 1 - \frac{A_{peptides,PFG}}{A_{peptides,FF}} \quad (1')$$

where $A_{peptides,PFG}$ and $A_{peptides,FF}$ are the areas (acquired by the size exclussion chromatography (SEC)) of the global permeate and retentate at the end of the experiment, respectively.

To evaluate the accuracy of the analytical results, partial mass balances to the peptides were determined using equation 2, which was converted into equation 2' for the SEC analysis (in the same way that equation 1 was converted into 1'):

$$c_{peptides,IF} \times m_{IF} = c_{peptides,FF} \times m_{FF} + c_{peptides,PFG} \times m_{PFG} \quad (2)$$

$$1 = \frac{A_{peptides,FF} \times m_{FF}}{A_{peptides,IF} \times m_{IF}} + \frac{A_{peptides,PFG} \times m_{PFG}}{A_{peptides,IF} \times m_{IF}} \quad (2')$$

where $A_{peptides,IF}$ is the chromatogram area achieved by SEC analysis and where the m_{IF}, m_{FF}, and m_{PFG} are, respectively, the total mass of the initial feed (pretreated sardine cooking effluent), retentate and global permeate after each experiment.

2.3. Analytical methods

In terms of biochemical characterization, the samples analyzed were the sardine cooking effluent (with acorn shell extract), pretreated sardine cooking effluent (the supernatant of centrifugation, the initial feed of UF), and all UF fractions (retentates, UF-H and UF-NH, and UF permeate UF-PFG). Additional samples analyzed were RO retentate fractions of NF retentate fractions: 1- from water-diluted sardine cooking effluent (sardine cooking effluent-NF-RO), 2- from water-diluted UF retentate hydrolyzed (UF-H-NF-RO), 3- from water-diluted UF retentate non-hydrolyzed (UF-NH-NF-RO) and 4- from water-diluted UF permeate (UF-PFG-NF-RO). In terms of bioactive properties, the samples analyzed were sardine cooking effluent-NF-RO, UF-H-NF-RO and UF-NH-NF-RO (see Fig. 1).

All samples were analyzed in triplicate, concerning dry matter (DM) content (EN 12880.2000), ash (AOAC 999.07) (William, 2000), NaCl (by inductively coupled plasma, ICP-OES), protein content (Kjeldahl, ISO R 973:1978) (William, 2000), protein molecular weight distribution by SEC, antioxidant capacity (by $ABTS^{+}$ and ORAC), antimicrobial potential (growth inhibition curves), antihypertensive (by ACE inhibitory fluorimetric assay), and antifreeze activity (by colorimetric assay). The raw material sardine cooking effluent (with acorn shell extract) and the lipid fraction separated by centrifugation from sardine cooking effluent were characterized in terms of lipid content (Soxhlet, AOAC 960.39) in triplicates (William, 2000).

197

198 *2.3.1. Determination of NaCl*

199 The Na profile of different samples was determined by inductively coupled
200 plasma–optical emission spectrometry–ICP-OES using Perkin Elmer, model Optima 8300
201 (Perkin-Elmer Corporation, USA) with a spectral line wavelength of 589.59 nm. To
202 accomplish this, each sample was digested with 65% HNO_3 using a Berghof microwave
203 digestion system (speed wave four with DAK - 100/4 flasks, Germany). Samples were
204 diluted with ultra-pure water before being measured. For Na, the standard solution from
205 Perkin Elmer (Perkin-Elmer Corporation, USA) was used, and the total amount of NaCl
206 was determined by multiplying the conversion factor of 2.50.

207

208 *2.3.2. Determination of protein and peptides profile by size exclusion chromatography*

209 The molecular weight distribution of different samples was determined using gel
210 filtration chromatography on a GE Healthcare Life Sciences AKTA pure 25 system
211 (Uppsala, Sweden), which included two gel filtration columns—the Superdex® 200
212 10/300 GL and Superdex Peptide 10/300 GL. The column was operated at flow rate of
213 0.5 mL/min with 0.05 M phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.2 g/L
214 NaN_3. The eluent's absorbance was measured at 280 nm. Standard proteins with known
215 molecular weights (thyroglobulin (669 kDa); aldolase (158 kDa); conalbumin (75 kDa);
216 ovalbumin (43 kDa); carbonic anhydrase (29 kDa); ribonuclease A (13.7 kDa) and whey
217 peptide (1.2 kDa)) were used to establish a molecular weight standard curve.

218

219 *2.3.3. Reversed phase high performance liquid chromatography (RP-HPLC)*

Due to the high salt content, for optimal chromatographic analysis, the samples were desalted by purifying the peptides. Peptides were purified by solid-phase extraction (SPE), using Sep-Pak Vac 3 cc (500 mg) C18 Cartridges (Waters, Dublin, Ireland). The sample was forced through the column using a vacuum flask and rubber stopper, with the vacuum pulling the solution through the SPE column. Eventually, the solution was collected in a test tube located inside the flask. Before use the column was conditioned by washing with 3 × 1 mL acetonitrile, 3 × 1 mL acetonitrile–water (1:1), and 5 × 1 mL water-0.1% trifluoroacetic acid. A 3 × 1 mL of each sample (Sardine cooking effluent-NF-RO, UF-H-NF-RO, and UF-NH-NF-RO) was transferred to a prewashed SPE column. After washing with acidified water (5 × 1 mL) the peptide was eluted from the cartridge with 1 mL of 70% acetonitrile in water in the presence of 0.1% TFA.

The purified peptides were analyzed by high-performance liquid chromatography (HPLC). The procedure was performed in a reverse phase column coupled with a guard column containing the same stationary phase (COSMOSIL 5C18-AR-II Packed Column – 4.6mm I.D.×250 mm). Separation of peptides was carried out with a mobile phase A – water-0.1% TFA – and a mobile phase B – acetonitrile-0.1% TFA. The operating conditions were: isocratic elution for 5 min at 100% phase A, then with a gradient until reaching 70% phase B at 35 min, at a continuous flow of 0.80 mL/min. The separation analysis was performed using a waters e2695 separation module system interfaced with a photodiode array UV/Vis detector (PDA 190–600 nm).

2.3.4. Determination of total antioxidant capacity

The antioxidant capacity of the different samples was determined according to the methods of 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation

244 ($ABTS^{+}$) (Re et al., 1999) and oxygen radical absorbance capacity (ORAC) (Contreras et
245 al., 2011) using a multi-detection plate reader (Synergy H1, Vermont, USA). Each
246 experiment's radical stock solution was freshly prepared. Lyophilized samples were
247 dissolved in deionized water to obtain a concentration of 10 mg/mL. The results were
248 represented as µmol of Trolox Equivalent (TE)/g sample.
249
250 *2.3.5. Determination of antimicrobial activity*
251 Samples were analyzed for antimicrobial activity against a Gram-negative
252 bacterium – *E. coli* (ATCC 25922), a Gram-positive bacterium – *S. aureu* (ATCC 25923),
253 and a yeast – *C. albicans* (CCGU 49242). Mueller hinton broth (MHB) was used to
254 prepare 4% (w/v) extract solutions, which were then inoculated (v/v) with an overnight
255 inoculum (ca. 10^{-5} CFU/mL). The mixtures were then incubated at 37 °C and the optical
256 density (OD) measured at 660 nm for 24 h (1 h intervals) using a microplate reader
257 (Fluostar, Optima; BMG Labtech, Ortenberg, Germany). Two positive controls were
258 drawn: inoculated media without an antimicrobial agent and inoculated media with
259 1.2% (w/v) NaCl to see whether this concentration could inhibit the growth of the
260 microorganism as it mimics the salt concentration of the samples. Sterile MHB was used
261 as a negative control. Each condition was tested in triplicate.
262
263 *2.3.6. Determination of ACE-inhibitory effect*
264 The ACE-inhibitory effect of the different samples was determined using a plate
265 reader (Synergy H1, Vermont, USA) and fluorimetric assay described by Sentandreu and
266 Toldrá (Sentandreu et al., 2006), according to the modifications of Quirós et al. (2009)
267 in triplicate. For this purpose, the protein content of the samples was determined by

Kjeldahl. The inhibitory activity was represented as the peptide concentration needed to inhibit the initial ACE activity by 50% (IC_{50}).

2.3.7. Determination of antifreeze activity

The antifreeze activity of the different samples was determined using a colorimetric assay with gold nanoparticles (AuNPs). Citrate-stabilized AuNP (Cit-AuNP) was synthesized to generate mercaptosuccinic acid (MSA)-capped AuNPs (Grabar et al., 1995). In a round-bottom flask, a stock solution containing 1 mM $HAuCl_4.3H_2O$ diluted with deionized water to a final concentration of 300 nM, brought to a boil under vigorous stirring. Sodium citrate (30 mM, 29 µL/mL solution) was added to the solution, causing a color change from light yellow to red brown. The solution was cooled to room temperature (while stirring (200 rpm)) after 20 min of heating and MSA (30 mM, same volume as sodium citrate) was added to the Cit-AuNP solution and the solution left, under intense stirring (500 rpm), at room temperature for 1 h. Tetrachloroaurate, sodium citrate, and MSA had the following molar ratios: 1:200:200.

In a 96-well microplate (Nunc, Denmark), an adequate volume of the different samples was added to wells containing the MSA-AuNP solution (100 µL at 4 nM), adding deionized water to make a final volume of 200 µL. Bovine serum albumin (BSA; 0.1 mg/mL) was used as a negative control. The microplate was frozen at -20 °C for 60 min and thawed at 37 °C for 10 min. The extinction spectrum was achieved using microplate reader (Fluostar, Optima; BMG Labtech, Ortenberg, Germany) before and after the freezing/thawing cycle. The aggregation parameter (E520/E650) of the AuNP solution was evaluated.

2.4. Statistical analysis

The data was statistically analyzed using IBM SPSS Statistics v21.0.0.0 (New York, USA). To determine normality, the Shapiro-Wilks test was performed. Since the results were found to be normally distributed, the differences between them were assessed using One-Way ANOVA and Turkey's post-hoc test. For all statistical tests, the overall acceptable significance level of differences was set at $p < 0.05$.

3. Results and Discussion

3.1. Biochemical characterization of sardine cooking effluent

Sardine cooking effluent from the end of the canning process (with acorn shell extract) and pretreated sardine cooking effluent (the supernatant of centrifugation) were firstly characterized biochemically in terms of ash, NaCl, protein, and lipids content (see Fig. 2). The sardine cooking effluents (before and after centrifugation) contained a high concentration of NaCl, 36.80 ± 0.35 and 58.81 ± 0.37 g/100 g (DM), respectively, related to the high ash concentration values of 44.36 ± 0.28 and 67.38 ± 0.15 g/100 g (DM), respectively. Furthermore, analysis of this figure reveals that the protein content of sardine cooking effluents (before and after centrifugation) is 23.95 ± 0.89 and 29.46 ± 0.50 g/100g (DM), respectively. As this work focuses only on the proteins/peptides content with low NaCl content, the only samples to be characterized in terms of lipid content were the raw material sardine cooking effluent (with acorn shell extract) and lipid fraction separated by centrifugation from sardine cooking effluent with 25.29 ± 0.49 g/100 g (DM) and 83.20 ± 0.20 g/100 g, respectively.

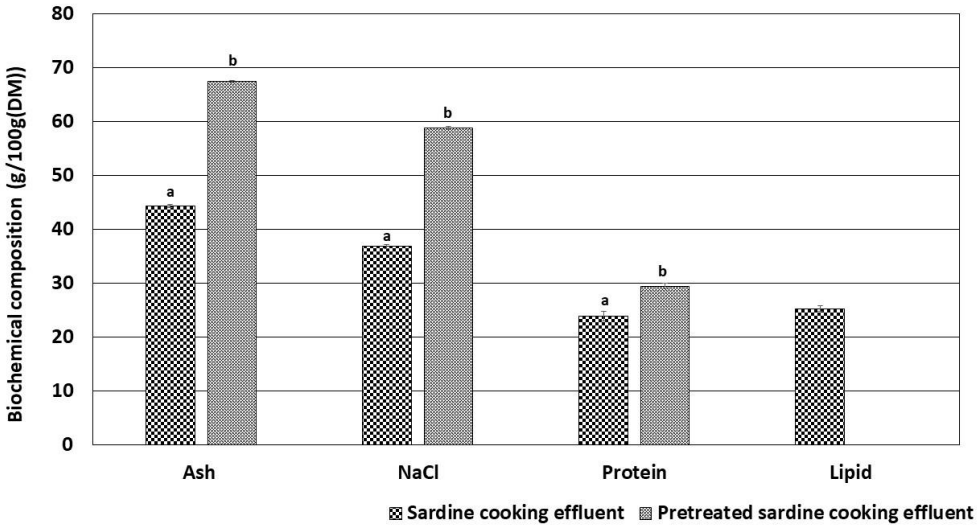


Fig. 2. Biochemical composition of sardine cooking effluent collected from the end of the canning process (with acorn shell extract) and pretreated sardine cooking effluent (the supernatant of centrifugation). Mean ± SD (Standard deviation) with different letters in the same column represent significant differences, as evaluated by ANOVA ($p < 0.05$).

NaCl (related to ash content) and lipids, which are present in significant amounts, must be eliminated. Centrifugation was used to remove lipids and solids from the sardine cooking effluent.

SEC was used to analyze the samples (sardine cooking effluent and pretreated sardine cooking effluent). Table 2 the chromatogram areas for the different MW ranges of different proteins/peptides (≥ 10 kDa and ≤ 1.2 kDa).

Table 2

SEC chromatogram areas of the peaks from the sardine cooking effluent and pretreated sardine cooking effluent. Results are reported in mL*mAU.

Molecular weight (kDa)	Sardine cooking effluent Area (mL*mAU)	Pretreated sardine cooking effluent Area (mL*mAU)
≥ 10	150.05 ± 3.15^{b}	153.49 ± 0.29^{b}

≤ 1.2	406.19 ± 2.90[a]	421.31 ± 2.21[a]

Mean ± SD with different letters in the same row represent significant differences, as evaluated by ANOVA ($p < 0.05$).

The SEC data showed that sardine cooking effluents (before and after centrifugation) exhibited a similar pattern in terms of the size distribution of their proteins and peptides, evaluated by their chromatogram area distribution with two distinct groups of protein/peptide: the group of protein/peptide with the highest MWs (≥ 10 kDa) and the group of peptides with the lowest MWs (≤ 1.2 kDa). The samples contained more peptides with the lowest MWs (≤ 1.2 kDa), which is consistent with the previous work (Ghalamara et al., 2020). In terms of protein/peptides characterization, the SEC characterization allows the selection of suitable membranes for separation of different fractions of protein/peptide, aligning the size of the target protein/peptide with the MWCOs/pore size of the membrane.

3.2. Production of protein/peptide fractions from sardine cooking effluent by UF and enzymatic hydrolysis

In this work, the various fractions enriched in protein/peptides were produced by membrane technology, namely UF, combined or not with enzymatic hydrolysis. The objective of using UF membrane technologies is to fractionate/purify bioactive protein/peptides from pretreated sardine cooking effluent. The selected UF membrane was a molecular weight cut-off (MWCO) of 3 kDa membrane, adequate to separate the protein/peptides fraction with the higher MWs/sizes (≥ 10 kDa), from the peptides fraction with the lower MWs (≤ 1.2 kDa). Also, the UF membrane has a hydrophilic behavior to enhance the permeation of proteins, as desired. The UF retentates

352 (hydrolyzed or not) and permeate of the pretreated sardine cooking effluent were
353 biochemically characterized in terms of ash, NaCl and total protein by Kjeldahl (Table 3)
354 and protein/peptide profile by SEC (Table 4).

355 **Table 3**

356 UF membrane processing of pretreated sardine cooking effluent: Biochemical
357 composition of the corresponding retentates and permeate after UF membrane
358 experiment. The results are given in g/100g(DM) of sample.

Membrane	Sample	Ash (g/100g(DM))	NaCl (g/100g(DM))	(Total) Protein (g/100g(DM))
UF	UF-H	47.09 ± 0.06[b]	39.83 ± 0.14[b]	52.07 ± 0.78[a]
	UF-NH	46.49 ± 0.1[b]	38.49 ± 0.93[b]	52.31±0.67[a]
	UF-PFG	82.38 ± 0.03[a]	77.10 ± 0.18[a]	16.32 ± 0.87[b]

359 Legend: UF fractions; UF-H Hydrolyzed retentate, UF-NH Non-hydrolyzed retentate, UF-
360 PFG Global Final Permeate. Mean ± SD with different letters in the same column
361 represent significant differences, as evaluated by ANOVA ($p < 0.05$).

362 When comparing the UF-H retentate with the UF-NH retentate, the values
363 remained the same, as expected. When relating the UF-PFG to UF-NH, no rejection to
364 ash and to NaCl was observed, maybe due to the smaller size of the relevant ions (mostly
365 mono-valent ions) compared to the pore size of UF membranes, lower charge density,
366 and the lack of a membrane surface charge (Mukherjee et al., 2015). The UF retentates
367 and permeate of the pretreated sardine cooking effluent were characterized by SEC (see
368 Table 4).

369 **Table 4**

370 SEC chromatogram areas of the peaks from UF corresponding retentates and permeate
371 obtained from pretreated sardine cooking effluent. Results are reported in mL*mAU.

	UF fractions		
	UF-H	UF-NH	UF-PFG

Molecular weight (kDa)	Area (mL*mAU)	Area (mL*mAU)	Area (mL*mAU)
≥ 10	166.74 ± 3.31[b]	368.52 ± 1.78[a]	–
≤ 1.2	692.28 ± 5.30[a]	502.495 ± 1.72[a]	265.35 ± 0.19[a]

Legend: UF fractions; UF-H Hydrolyzed retentate, UF-NH Non-hydrolyzed retentate, UF-PFG Global Final Permeate. Mean ± SD with different letters in the same row represent significant differences, as evaluated by ANOVA ($p < 0.05$).

When comparing the hydrolyzed UF-H retentate with the non-hydrolyzed UF-NH retentate, the H retentate had lower amounts (ca. 50%) of large proteins (≥ 10 kDa) and higher amount of small peptides (≤ 1.2 kDa), due to the hydrolysis. Table 5 shows the mass balance closure in relation to protein/peptide and the protein/peptide observed rejections both by Kjeldahl and SEC methods (in the latter case, a group of peptides with MWs ≤ 1.2 kDa and a group of protein/peptides with MWs ≥ 10 kDa) when processing the pretreated sardine cooking effluent.

Table 5

UF membrane processing of sardine cooking effluent: mass balance closure of total protein/peptides using the Kjeldahl and SEC methods, the observed rejection of total protein/peptides using Kjeldahl method, and the observed rejection of protein/peptides (with MWs ≤ 1.2 kDa and MWs ≥ 10 kDa) by the SEC method.

Kjeldahl		**SEC**		
Mass Balance Closure (%)	**R (%)**	**Mass Balance Closure (%)**	**R (≤ 1.2 kDa) (%)**	**R (≥ 10 kDa) (%)**
88 ± 3	64 ± 1.8	84 ± 1	34 ± 0	100

Regarding the Kjeldahl results, the mass balance closed within around 12%, assessing the good quality of the analytical data. Regarding the SEC results, using UF membrane, the mass balance closed within less than around 16%, which was still

391 reasonable, since some of the small peptides (≤ 1.2 kDa) could be more likely adsorbed
392 on the membrane than the larger protein/peptides and rejected. The rejection of the
393 peptides provided by the Kjeldahl method was higher than the rejection of the peptides
394 provided by the SEC method, as predicted and as in our last paper (Ghalamara et al.,
395 2020), since the latter only considered small peptides (≤ 1.2 kDa), which preferentially
396 permeated. As a result, the rejection of the peptides provided by the Kjeldahl method
397 was inadequate for analysis since it combined two groups of peptides (≥ 10 kDa and ≤
398 1.2 kDa).
399

400 ### *3.3. Removal of mono-valent salts from sardine cooking effluent and from*
401 ### *corresponding UF fractions by NF*

402 To reduce the amount of NaCl, the NF membrane treatment (MWCO of 120.37
403 g/mol) was conducted (each producing a retentate and a permeate) processing the
404 streams previously diluted (at a volumetric ratio of 1:3). Table 6 illustrates the
405 biochemical composition of RO retentate fractions of NF retentates from: water-diluted
406 sardine cooking effluent (sardine cooking effluent-NF-RO), water-diluted UF retentate
407 hydrolyzed (UF-H-NF-RO), water-diluted UF retentate non-hydrolyzed (UF-NH-NF-RO)
408 and water-diluted UF permeate (UF-PFG-NF-RO). The protein/peptides profile of
409 samples was achieved by SEC, as shown in Table 7.

410 **Table 6**

411 Biochemical composition of RO retentate fractions of NF retentates from water-diluted
412 sardine cooking effluent (sardine cooking effluent-NF-RO), water-diluted UF retentate
413 hydrolyzed (UF-H-NF-RO), water-diluted UF retentate non-hydrolyzed (UF-NH-NF-RO)

414 and water-diluted UF permeate (UF-PFG-NF-RO). The results are given in g/100g(DM) of
415 sample.

Membrane	Sample	Ash (g/100g(DM))	NaCl (g/100g(DM))	(Total) Protein (g/100g(DM))	Lipid (g/100g(DM))
NF	Sardine cooking effluent-NF-RO	33.47 ± 0.48[b]	28.33 ± 0.84[b]	36.84 ± 0.62[b]	25.29 ± 0.49
	UF-H-NF-RO	29.21 ± 0.01[c]	25.06 ± 0.98[c]	70.51 ± 0.68[a]	–
	UF-NH-NF-RO	27.26 ± 0.09[d]	22.19 ± 0.05[d]	72.68 ± 0.36[a]	–
	UF-PFG-NF-RO	66.66 ± 0.53[a]	59.15 ± 0.23[a]	32.82 ± 0.32[c]	–

416 Mean ± SD with different letters in the same column represent significant differences,
417 as evaluated by ANOVA ($p < 0.05$).

418 **Table 7**

419 SEC chromatogram areas of the peaks from RO retentate fractions of NF retentates from
420 water-diluted sardine cooking effluent (sardine cooking effluent-NF-RO), water-diluted
421 UF retentate hydrolyzed (UF-H-NF-RO), water-diluted UF retentate non-hydrolyzed (UF-
422 NH-NF-RO) and water-diluted UF permeate (UF-PFG-NF-RO). Results are presented in
423 mL*mAU.

	Sardine cooking effluent-NF-RO	UF-H-NF-RO	UF-NH-NF-RO	UF-PFG-NF-RO
Molecular weight (kDa)	Area (mL*mAU)	Area (mL*mAU)	Area (mL*mAU)	Area (mL*mAU)
≥ 10	105.55± 2.47[b]	153.89 ± 2.32[b]	305.46 ± 1.55[a]	–
≤ 1.2	383.65 ± 1.22[a]	552.12 ± 4.47[a]	463.55 ± 6.60[a]	278.64 ± 3.42[a]

424 Mean ± SD with different letters in the same row represent significant differences, as
425 evaluated by ANOVA ($p < 0.05$).

426 Table 6 shows that the total amounts of NaCl reduced in all of samples after the
427 NF step, particularly in the UF retentate fractions, as compared to the samples before
428 the NF (Fig. 2 and Table 4). NaCl reductions were of ca. 59, 73 and 30% for UF-H-NF-RO,
429 UF-NH-NF-RO and UF-PFG-NF-RO respectively. It can be observed that UF-PFG-NF-RO

still presents the highest amount of NaCl and the lowest amount of organic content (protein/peptides) and was therefore discarded.

The NF was highly efficient at lowering the amount of NaCl, which might result in partial protein/peptides loss. According to the SEC results (Table 7), the hydrolyzed UF-H retentate lost small peptides (≤ 1.2 kDa) after the NF step, implying that peptides with lower MW produced during hydrolysis were mostly eliminated in the permeate. Thus, RO retentate fractions of NF retentate from corresponding UF retentates (UF-H-NF-RO and UF-NH-NF-RO), were those selected for further evaluation, as they simultaneously present the higher potential for valorization and environmental impact.

3.4. Biological properties of sardine cooking effluent and corresponding UF retentates from NF membrane experiment

Based on the analysis previously performed (Tables 6 and 7), RO retentate fractions of NF retentate from water-diluted sardine cooking effluent (sardine cooking effluent-NF-RO) and corresponding water diluted UF retentates (UF-H-NF-RO and UF-NH-NF-RO) were selected, to evaluate their biological properties, namely, antioxidant capacity ($ABTS^{+}$ and ORAC), antimicrobial potential, antihypertensive and antifreeze activities. As a result, evaluating the biological properties of the products obtained by UF, NF membrane processing, combined or not with the enzymatic hydrolysis, will determine whether protein/peptide fractions demonstrate enhanced biological properties when compared to sardine cooking effluent after NF membrane experiments. If biological properties are enhanced in UF and NF treated products this may justify the

conversion of these fractions into possible high-value functional ingredients in food formulations or cosmetic/pharmaceutical products.

3.4.1. Antioxidant activity of purified protein/peptide fractions

Oxygen and nitrogen metabolism generates reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS can damage the body's cellular components. Through removing electrons from cellular macromolecules including proteins, lipids, and DNA, excessive levels of ROS, as in oxidative stress, cause oxidative damage. This starts a chain reaction that eventually leads in new radicals attacking and disrupting other cellular macromolecule components (Hew et al., 1988). Due to the enhanced free radical scavenging activity, metal chelation, and aldehyde adduction activity, peptides are assumed to be more potent food antioxidants than amino acids (AAs) (Mugnano et al., 1995; Zhou and Decker, 1999; Zhou et al., 1999). The exact structure antioxidant activity relationship of peptides has not been defined. However, the type, position, and hydrophobicity of AAs in peptides are supposed to be significant. Many reactive AAs in proteins have nucleophilic sulfur-containing side chains, such as taurine, cysteine, and methionine, or aromatic side chains, such as tryptophan, tyrosine, and phenylalanine (Elias et al., 2008).

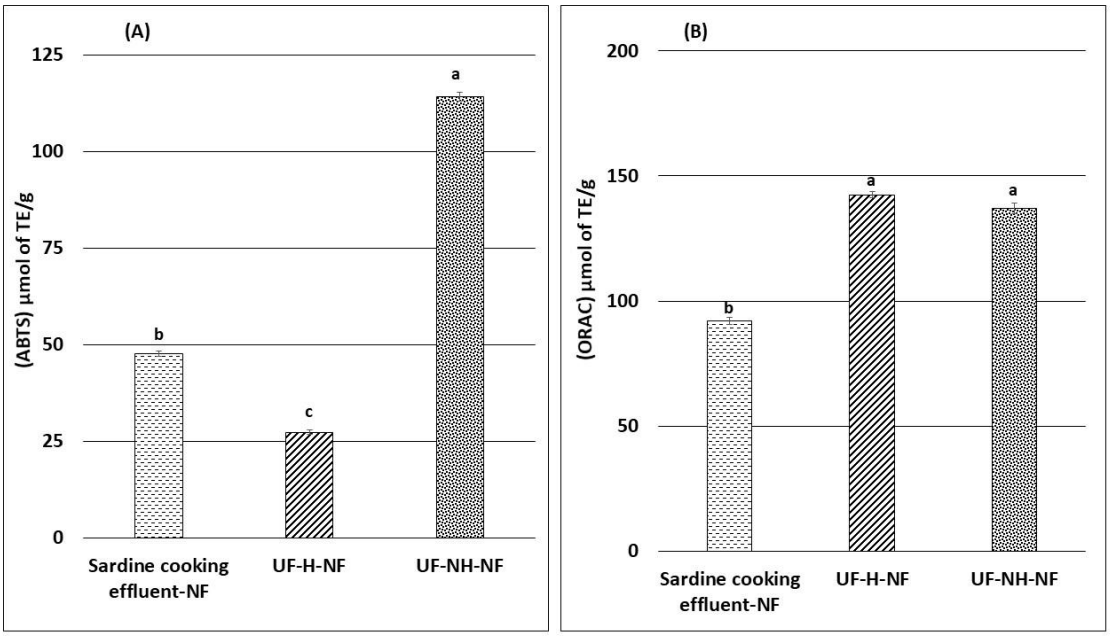
The antioxidant capacity of proteins and peptides is related to the structural properties such as MW, AA structure, sequence, and hydrophobicity (Zou et al., 2016). Low MW antioxidant peptides containing His and Ala have been reported from enzymatic hydrolysates of sardinella heads and viscera (*Sardinella aurita*) (Bougatef et al., 2010). Similarly, three antioxidant peptides (4–10 AA; with MWs of 584, 938, and 1305 Da) isolated from tuna (*Thunnus albacares*) cooking water hydrolysates possessed

476 antioxidant AAs in their sequences, such as His, Tyr, and Ala, as well as parts on
477 sequences that improve peptide antioxidant function, such as -His-Ala- and -Val-His-
478 (Hsu et al., 2009).

479 To evaluate the antioxidant activity of the purified protein/peptides, sardine
480 cooking effluent-NF-RO, UF-H-NF-RO, and UF-NH-NF-RO, two different assays were
481 used: ABTS and ORAC. A combination of tests is preferred since each assay measures a
482 specific type of antioxidant activity under assay-specific conditions rather than total
483 antioxidative activity (Huang et al., 2005).

484 In this work, the scavenging potential sardine cooking effluent-NF-RO, UF-H-NF-
485 RO and UF-NH-NF-RO, towards $ABTS^{+}$ radicals (Fig. 3A) was assessed. It was evident that
486 a lower activity was observed in the protein/peptide retentate fraction (43% decrease)
487 when hydrolysis was used compared to sardine cooking effluent-NF-RO. Conversely, in
488 the UF-NH-NF-RO fraction 140% increase in $ABTS^{+}$ radical scavenging activity was
489 observed when protein/peptide levels were raised in the retentate (UF-NH-NF-RO).

490 Using the ORAC method, the protein/peptide in the UF-H-NF-RO and UF-NH-NF-
491 RO fractions exhibited higher ORAC values than the sardine cooking effluent-NF-RO, by
492 55% and 49% respectively as shown in Fig. 3B.



493

494 **Fig. 3.** Antioxidant activities of RO retentate fractions of NF retentates from water
495 diluted sardine cooking effluent and corresponding water diluted UF retentates. (A)
496 $ABTS^{+}$ radical scavenging activity for sardine cooking effluent-NF-RO, UF-H-NF-RO and
497 UF-NH-NF-RO; (B) ORAC value for sardine cooking effluent-NF-RO, UF-H-NF-RO and UF-
498 NH-NF-RO. Mean ± SD with different letters in the same column represent significant
499 differences, as evaluated by ANOVA ($p < 0.05$).

500 In terms of the antioxidant capacity of protein/peptide in retentate fractions as
501 determined by ABTS and ORAC assays, the UF-NH-NF-RO consistently had the highest
502 antioxidant capacity, whereas the UF-H-NF-RO antioxidant activity differed between
503 assays: it exhibited the lowest antioxidant activity in ABTS but the highest antioxidant
504 activity in ORAC. This was due to the fact that these two methods are based on different
505 reaction mechanisms and target different free radicals. ORAC measures an antioxidant's
506 capacity to inactivate a free radical (ROO·) by releasing a hydrogen atom (Zou et al.,
507 2016; Huang et al., 2005; Wright et al., 2001). In contrast, ABTS method measures the
508 release of an electron to the (ROO·), converting it into an anion (ROO^{-}) (Wright et al.,

2001; Di et al., 2013; Lemańska et al., 2001). The above results show that when the UF retentate fraction is hydrolyzed, the proportion of small peptides and free amino acids increases, potentially resulting in a good hydrogen atom transfer but a loss of electrons transfer, which may correspond to peptides with low ABTS but high ORAC.

When the polarity of the peptides was evaluated in the RP-HPLC chromatograms (Fig. 4), it was observed that hydrolysis degraded more hydrophilic peaks (arrow 1), resulting in the appearance of more hydrophobic fragments (arrows 2-4). This might imply that UF-NH-NF-RO contains more hydrophilic peptides with a good capacity to transfer both electrons and hydrogen atoms, while in UF-H-NF-RO the ability to transfer electrons was lost, increasing the ability to transfer hydrogen atoms. This assumption should be investigated further in the future with peptide fraction sequencing.

Some findings have shown that when the protein in fishery by-products is extremely hydrolyzed, the elimination of the $ABTS^{+}$ radical is reduced, exposing the effect of the type and size of the peptides present. As the degree of hydrolysis is increased, soluble protein in stickwater derived from the processing of kilka-based fishmeal (*Clupeonella* sp.) appears to decrease its antioxidant activity, as measured with $ABTS^{+}$. This, according to the authors, is owing to differences in hydrophilicity associated with compounds that have higher $ABTS^{+}$ activity in general (Mahdabi and Hosseini Shekarabi, 2018). Furthermore, AA composition has already been shown to have a substantial effect on antioxidant activity; it has been confirmed that a high proportion of hydrophobic AAs (such as Ala) confers higher antioxidant activity (Zou et al., 2016). As a result, the approach used to concentrate protein fractions has an effect on antioxidant activity, and in terms of hydrolysis process, while it increased ORAC, the advantage was reduced, and we lost some AA that may be more significant for avoiding

oxidation in food products than as a biological function, system more related with ORAC technique.

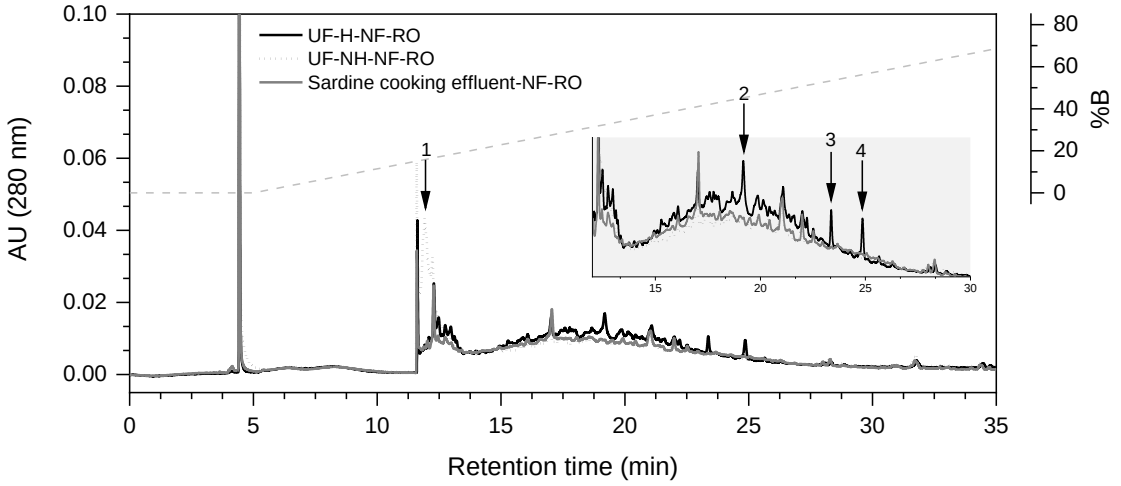


Fig. 4. RP-HPLC of purified peptides from sardine cooking effluent-NF-RO, UF-NH-NF-RO, and UF-H-NF-RO. The chromatogram shows the %B phase gradient (dashed grey line) and a chromatogram enlargement between 12 and 30 min. Arrows (1-4) indicate peptide fractions of increasing hydrophobicity.

3.4.2. Antimicrobial activity of purified protein/peptide fractions

Inhibition curves were drawn in order to obtain some insights into the effect of the fractions on the bacterial growth. Fig. 5 shows the inhibition curves found for *E.coli*, *S. aureus* and *C. albicans* when using sardine cooking effluent-NF-RO, UF-H-NF-RO and UF-NH-NF-RO. All the samples obtained from NF treatment at maximum concentration of 4% (w/v), interfered positively (inhibition effect) with all bacteria growth. Time inhibition curves for each microorganism in sardine cooking effluent-NF-RO, UF-H-NF-RO and UF-NH-NF-RO are shown in Fig. 5.

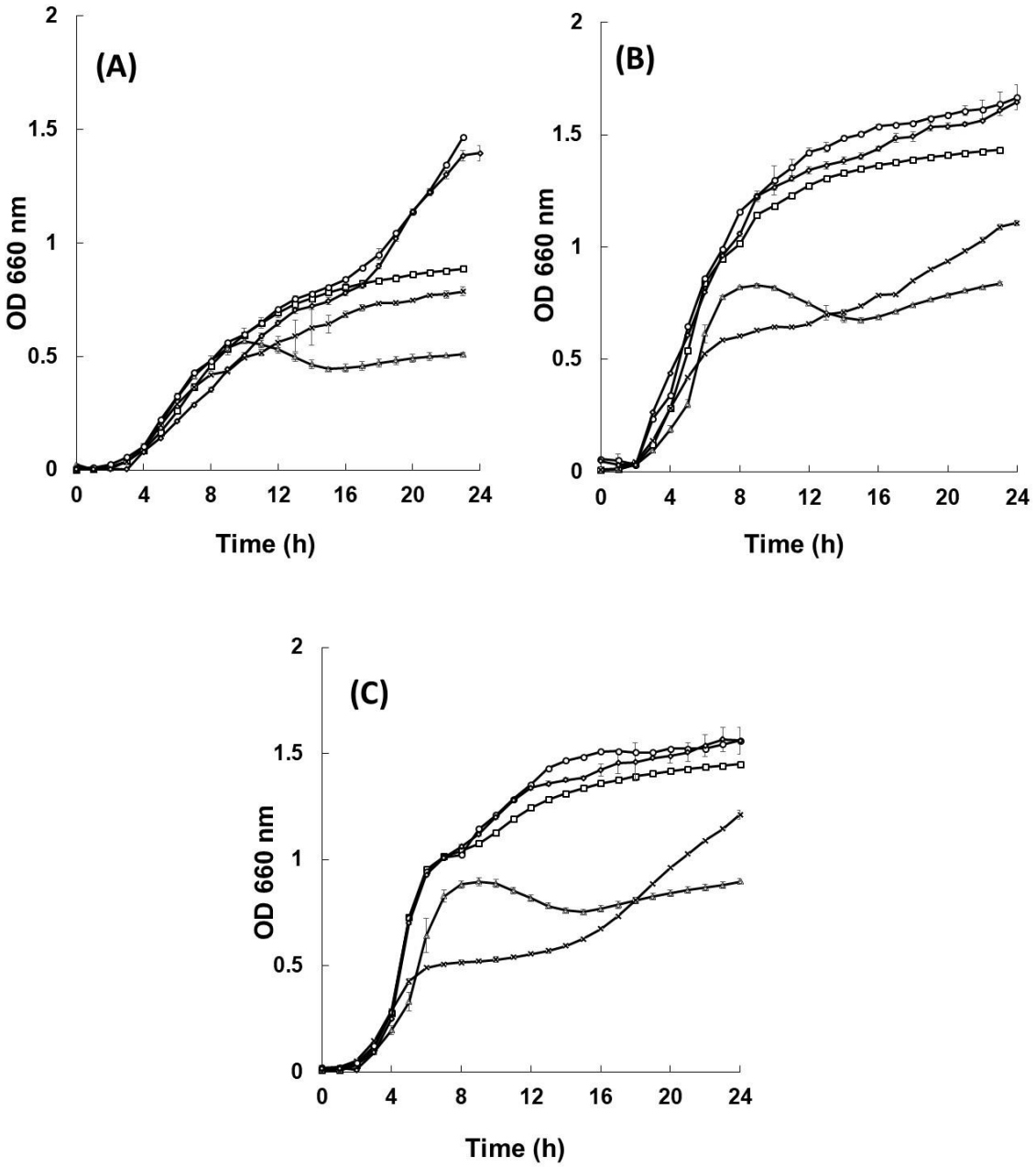


Fig. 5. Time inhibition curves drawn sardine cooking effluent-NF-RO, UF-H-NF-RO and UF-NH-NF-RO (○-positive control; □-sardine cooking effluent-NF-RO; Δ-UF-H-NF-RO; x-UF-NH-NF-RO; ◊-, 1.2% NaCl for, *E.coli* (A), *S. aureus* (B), *C. albicans* (C).

The purified protein/peptide seems to be effective against *E. coli*. According to the results presented in (Fig. 5A), despite the fact that a concentration of 40 mg/mL, could not completely inhibit the growth, but it could hinder microbial growth particularly in the UF-H-NF-RO. In fact, For *E. coli* the sardine cooking effluent as well as

558 the corresponding UF retentate fractions from NF membrane experiment appeared to
559 keep growing at a slow rate, and cause reduction of bacterial growth at the 24 h mark.
560 Considering *S. aureus* strains it is worth noting the parallel in behavior observed with *C.*
561 *albicans* (Figs. 5B, C). The UF retentate fractions from NF experiment (UF-H-NF-RO and
562 UF-NH-NF-RO) with a concentration of 40 mg/mL appeared to induce a reduction in OD
563 and bacterial growth, particularly in UF-H-NF-RO (Fig. 5B), while the sardine cooking
564 effluent-NF-RO exhibited an OD, after 24 h, similar to the one observed for the positive
565 and 1.2% NaCl controls although the maximum OD registered over the 24 h period was
566 slightly lower than the one registered for the controls (Fig. 5B).
567 For *C. albicans* (Fig. 5C), it can be seen that the corresponding UF retentate
568 fractions from NF membrane experiment caused a delay of the lag phase particularly in
569 UF-H-NF-RO fraction and cause reduction of bacterial growth at the 24 h mark.
570 Antimicrobial peptides (AMPs) are distinguished by their molecular sizes,
571 secondary structures, and net charges, allowing for a wide range of inhibition
572 mechanisms (Bechinger and Gorr, 2017). AMPs can translocate into the cytoplasm and
573 cause cell death by inducing apoptosis (Lee and Lee, 2014), inhibiting protein synthesis
574 (Roy et al., 2015; Gagnon et al., 2016), or interfering with cell wall formation, among
575 other mechanisms (Müller et al., 2016; Scocchi et al., 2016). Most AMPs act non-
576 specifically and exert their inhibitory effects through disruptive cytolytic or pore-forming
577 activities (Lee et al., 2015). AMPs are an excellent alternative to antibiotics because their
578 non-specific antimicrobial mechanism makes it impossible for bacteria to develop
579 resistance (Chan et al., 2006; Park et al., 2011).
580 Overall, it appeared that the UF-H-NF-RO fraction caused the highest reduction
581 in OD for all strains studied over the 24 h period, allowing to claim their better

582 antimicrobial activity, but not so significant compared with non-hydrolyzed that justify
583 the use of this approach with significant advantage.
584
585 *3.4.3. ACE-inhibitory activity of purified protein/peptide fractions*
586 Antihypertensive activity is based entirely on inhibiting the activity of
587 angiotensin-converting enzyme (ACE), a key regulator of blood pressure that, when
588 abnormally active, can raise blood pressure too high, resulting in hypertension (Mondorf
589 et al., 1998).
590 Many studies have shown that isolated peptides derived from a variety of fishes
591 have antihypertensive activity. The antihypertensive activity of peptides is affected by
592 their MW, molecular interaction, and chain length and specially presence of certain AA,
593 namely proline (Pro) and isoleucine (Ile). The antihypertensive potency of fish-derived
594 peptides is expressed as an IC50 value, which represents the half maximum inhibitory
595 concentration of peptides capable of inhibiting 50% of ACE activity (UG et al., 2019).
596 To better characterize the ACE inhibition by the sardine cooking effluent-NF-RO,
597 UF-H-NF-RO and UF-NH-NF-RO, the IC50 was determined to better understand the role
598 of membrane processing. As shown in Table 8, sardine cooking effluent and
599 corresponding UF retentates from NF membrane experiment showed no inhibition (IC50
600 > 1000 µg protein/mL) and (IC50 < 1000 µg protein/mL), respectively. In the current
601 study, the IC50 concentration was substantially high for all of the samples, indicating
602 that none of the samples had significant antihypertensive activity although other
603 authors published IC50 values for fish protein hydrolysates/peptides. Matsui et al.
604 (1993) indicate an IC50 of 260 µg/mL for sardine hydrolysates, Vieira et al. (2017)
605 identified IC50 values of 164 g/ml for hydrolysates derived from sardine sarcoplasmic

606 by-products and Carvalho et al. (2018) reported that low molecular weight fractions of
607 protein extracts (< 3 kDa) from sardine canning by-products had high ACE activity (IC50
608 = 51 μg/ml).

609 **Table 8**

610 Results of ACE inhibitory activity of RO retentate fractions of NF retentate from sardine
611 cooking effluent and corresponding UF retentates.

Sample	ACE-inhibitory activity (IC50, μg/mL)
Sardine cooking effluent-NF-RO	1266
UF-H-NF-RO	934
UF-NH-NF-RO	n.a.

612 Legend: Sardine cooking effluent-NF-RO RO of NF retentate from sardine cooking
613 effluent, UF-H-NF-RO RO of NF retentate from UF retentate hydrolyzed, UF-NH-NF-RO
614 RO of NF retentate from UF retentate non-hydrolyzed

615 n.a.: not analyzed

616

617 *3.4.4. Antifreeze activity of purified protein/peptide fractions*

618 Since antifreeze proteins (AFPs) preferentially bind to ice crystals, the ideal
619 potential AFP should be capable of impeding frozen aggregation of AuNPs, resulting in a
620 strong scattering peak in the extinction spectrum at around 520 nm. In contrast, AFP-
621 free conditions allow AuNPs to self-assemble during the freezing/thawing period.

622 To check the samples antifreezing potential, their effect upon the assembly of
623 AuNPs during one 1 h freezing cycle (at -20 °C) and 10 min thawing at 37 °C was
624 evaluated. In the current study, AuNP modified with MSA was used to increase particle
625 aggregation. As a result of the freezing/thawing procedure, the MSA-AuNP with BSA was
626 uniformly aggregated over the specified concentration of BSA (0.1 mg/mL). After the

freezing/thawing step, the protein-free AuNP solution was still aggregated and had changed to a blue color. Similarly, sardine cooking effluent and corresponding UF retenates from NF membrane experiment were evenly aggregated, and a blue color appeared over the defined concentration (1:15). In contrast, the MSA-AuNP did not change color as a protein/peptide having antifreeze activity (i.e. no freeze-induced aggregation). When the degree of aggregation was quantified by using extinction ratio (E520/E650) (Fig. 6A), there was a resemblance between the extinction ratios of BSA and various samples (Fig. 6B). As a result, it was assumed that no antifreeze activity was present.

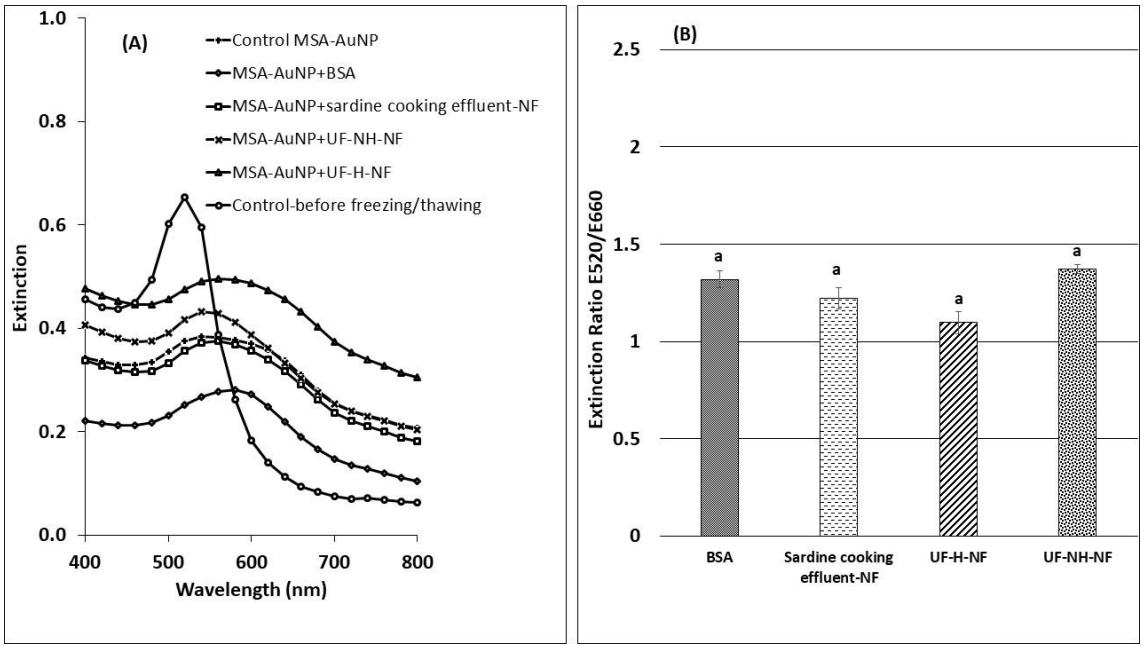


Fig. 6. AuNP-based colorimetric assay of RO fractions of NF retentate from sardine cooking effluent and corresponding UF retentates AFP activity. (A) Extinction spectra after freezing/thawing, MSA-AuNP (+), MSA-AuNP + BSA (◊), MSA-AuNP + sardine cooking effluent-NF-RO (□), MSA-AuNP + UF-H-NF-RO (Δ), MSA-AuNP + UF-NH-NF-RO (x), and the extinction spectrum of control MSA-AuNP solution before freezing/thawing (○). (B) The AuNP aggregation was quantified by the extinction ratio (E520/E650). Mean

643 ± SD with different letters in the same column represent significant differences, as
644 evaluated by ANOVA ($p < 0.05$).
645

646 4. Conclusions

647 The global amount of fishery-related by-products, among which stand the
648 considered effluent, represent an environmental concern. Therefore, the development
649 of effective strategies for their management is, by-itself, important. Moreover, assuming
650 the need for a shift in industrial practices toward a more circular model, the need lead
651 to effectively exploit them as a raw matter for attaining other products of relevance.
652 This work proposes the sustainable production of sardine cooking effluent fractions
653 enriched in bioactive protein/peptides, with enhanced biological properties compared
654 to the sardine cooking effluent. This process uses membrane technologies (UF, NF, and
655 RO) and enzymatic hydrolysis that allow obtaining fractions enriched in
656 protein/peptides with low content in NaCl. The sardine cooking effluent fractions
657 involving the fractionation of protein/peptides by UF, with and without enzymatic
658 hydrolysis, were selected based on its higher content in peptides and lower content in
659 NaCl, which resulted in two main fractions UF-H-NF-RO and UF-NH-NF-RO that were
660 compared with sardine cooking effluent-NF-RO. The selected fractions had revealed a
661 better antioxidant (except for hydrolyzed fraction measured by ABTS) and antimicrobial
662 activity than when the raw material was processed by membranes but with no UF step.
663 The application of NF membrane effectively reduce the NaCl content of sardine cooking
664 effluent and obtained UF fractions, particularly UF retentate fractions. However, given
665 the additional expenses of the enzymatic treatment, as well as the fact that it

significantly reduced the antioxidant activity assessed by ABTS, there will be no significant advantages for industrial application.

One of the main concerns of this work as it considered the use of a pilot scale approach to effectively transform one of the canning industry's effluents into added-value fractions that may be exploited as ingredients in a feasible manner, using adaptable processes and maximizing the desired characteristics (biological activity, salt, and protein/peptide content). Moreover, the application of this process could not only imply an additional revenue source but reduce costs as these effluents frequently require specialized treatments provided by a third-party. Future research, stemming from this work could expand to consider the use of other fishery effluents as well as the feasibility of incorporating the best performing fractions into product prototypes to assess their performance.

Abbreviations

UF: ultrafiltration; NF: nanofiltration; RO: reverse osmosis; IF: initial feed; FF: final feed; PFG: global final permeate; UF-R: UF retentate; UF-H: UF hydrolyzed retentate; UF-NH: UF non-hydrolyzed retentate; UF-PFG: UF global final permeate; sardine cooking effluent-NF-RO: RO retentate of NF retentate from water diluted sardine cooking effluent; UF-H-NF-RO: RO retentate of NF retentate from water diluted UF hydrolyzed retentate; UF-NH-NF-RO: RO retentate of NF retentate from water diluted UF non-hydrolyzed retentate; UF-PFG-NF-RO: RO retentate of NF retentate from water diluted UF permeate; PUFA: polyunsaturated fatty acids; PSF: polysulfone; TMP: transmembrane pressure; TFC-PSF: thin-film composite-polysulfone; VCF: volume concentration factor; SEC: size exclussion chromatography; RP-HPLC: reversed phase

690 high performance liquid chromatography; SPE: solid-phase extraction; HPLC: high-
691 performance liquid chromatography; DM: dry matter; ICP-OES: inductively coupled
692 plasma-optical emission spectrometry; MW: molecular weight; ORAC: oxygen radical
693 absorbance capacity; TE: trolox equivalent; MHB: mueller hinton broth; AuNPs: gold
694 nanoparticles; Cit-AuNP: citrate-stabilized AuNP; MSA: mercaptosuccinic acid; BSA:
695 bovine serum albumin; MWCO: molecular weight cut-off; ROS: reactive oxygen species;
696 RNS: reactive nitrogen species; AAs: amino acids; OD: optical density; AMPs:
697 antimicrobial peptides; ACE: angiotensin-converting enzyme; AFPs: antifreeze proteins;
698 Pro: proline; Ile: isoleucine.
699

700 ## Author contributions

701 S. Ghalamara: Formal analysis, Data discussion, Interpretation and Writing – original
702 draft; E.R. Coscueta: RP-HPLC analysis, Writing – review and Editing; S. Silva, C. Brazinha
703 and C.D. Pereira: Supervision, Writing – review and Editing; M. Pintado: Validation,
704 Supervision, Conceptualization, Writing – review and editing. All authors read and
705 approved the final manuscript.
706

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Declaration of competing interest

The authors declare that they have no competing interest.

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