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Serial fractionation of spent brewer's yeast protein hydrolysate by ultrafiltration: a peptide-rich product with low RNA content

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Abstract

Spent brewer's yeast (SBY) application as a new source of peptides depends on the development of proper downstream processes. This work aimed to separate yeast hydrolysate peptides from sugars and ribonucleic acids (RNA) by a 3-step designed fractionation process using ceramic ultra and nanofiltration following two sequences: (1) 50, 8 and 1 kDa and (2) 15, 8 and 1 kDa molecular weight cut-off (MWCO) membranes. In step one, 15 kDa MWCO membrane retained more components and increased performance of subsequent steps, when compared to 50 kDa. Peptide composition of the initial hydrolysate was gradually changed until 90% of the peptides smaller than 1 kg mol⁻¹ were found in the final permeate. Fractionation increased peptide purity regarding RNA and total sugars up to 1.7 and 2.7-fold, respectively. Innovative protein-rich ingredients from SBY residue with an RNA content as low as 1.4% were produced by membrane separation technology. High quality ingredients presented different peptide profiles and great potential in food and pharmaceutical industries applications.

Keywords: Peptide fractionation, low ribonucleic acids content, *Saccharomyces cerevisiae*, Protein hydrolysates separation, Ceramic membranes

1. Introduction

2 Alternative sources of protein have become an important subject of research that has gained considerable attention in the last decade. This effect has been driven by the interest in the transition to a more sustainable

Abbreviations: MW: molecular weight; MWCO: molecular weight cut-off; RNA: ribonucleic acids; SEC: size-exclusion chromatography; d.w.: dry weight; SBY: spent brewer's yeast; SCP: single cell protein; T50, T15, T8 and T1: ceramic membranes of 50, 15, 8 and 1 kg mol⁻¹ molecular weight cut-off, respectively; UF: ultrafiltration; VRF: volumetric reduction factor;

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5 food production and an increase in market demand for protein, entailing innovative and economically viable
6 solutions [1–3]. Proteins from plants (specially from pulses), insects, fungi and algae have been intensively
7 investigated [4]. Although plant-based proteins typically present a higher consumer acceptance and have been
8 studied for a longer period [2], some features hinder their use as alternative protein: (1) their extraction
9 process is complex and may involve harsh conditions that can damage protein quality and functionality
10 (organic solvents, extreme pHs and high temperatures); (2) low yields are typically obtained compared
11 to some other sources, decreasing the economical viability of the process; (3) these proteins contain anti
12 nutritional factors (e.g. phytic acids, tannins, alkaloids, lectin and other protease inhibitors) and an amino
acids composition with a lower content of essential amino acids than that of animal-based sources [1, 3, 5].

13 Proteins from yeasts have been explored mainly for single cell protein (SCP) production, which is the
14 usual term for microbial protein, but the use of yeast protein as an alternative source is still under de-
15 velopment, with new research exploring ways to valorise both yeast and yeast by-products [6–8]. Spent
16 brewer's yeast is a widely available, underutilised and protein-rich by-product from the brewing industry
17 with great potential for the production of bioactive peptides and protein-based ingredients. The production
18 of yeast protein hydrolysates was successfully done using enzymatic hydrolysis and currently, one of the
main challenges for its application is the separation of yeast peptides from the hydrolysate matrix [9].

20 The choice of a downstream separation process depends on the composition of the hydrolysate and
21 the development of targeted downstream technologies, that should be optimised based on each source.
22 Different protein and peptides properties and structures result in important changes in protein physico-
23 chemical characteristics (e.g. size range, solubility and reactivity), thus affecting separation [5]. Moreover,
24 a fairly important consideration is that protein ingredients are supposed to achieve a minimum purity level
25 in order to be successfully applied as a food industry ingredient or as a nutraceutical [10]. Separation of
26 protein hydrolysates have been successfully achieved using membrane separation technologies, that allow
27 the recovery of enriched bioactive fractions following fractionation [11]. Recent works report mainly the
use

28 of ultrafiltration (UF) to recover bioactive peptides from several matrices such as milk [12, 13], corn [14],
29 flaxseeds [15], soybeans [16], algae [17].

30 The SBY protein hydrolysate is reported to be a complex matrix that contains high amounts of peptides
31 (covalently bonded amides with two or more amino carboxylic acid molecules) [18] and other components
32 such as polysaccharides, ribonucleic acids (RNA), vitamins and minerals [19, 20]. Among these compounds,

33 the RNA content represents great challenge because they are often extracted with proteins and if the intake
34 of protein-rich yeast products is high, RNA molecules are degraded into uric acids than can then progress 35
to hyperuricemia, ultimately linked to kidney stones or gout [21, 22].

36 Separation of RNA molecules from proteins and peptides for biological purposes is typically done by
37 the use of precipitation using complex and sometimes toxic solvents that may denature proteins (phenols,
38 trichloroacetic acid, acetone, ethanol, ammonium compounds, sodium dodecyl sulphate, chloroform, etc.)
39 [23, 24]. These techniques are too expensive and complex, inappropriate for scaling up and for food industry
40 processing. Reduction of ribonucleic acid content in SCP and yeast protein concentrates is typically done
41 using alkali hydrolysis, acid precipitation and heat-shock treatments [25–27], resulting in the denaturation
42 of proteins. The use of naturally present endogenous nucleases to solubilise nucleic acids and then separate
43 them from insoluble protein by centrifugation was also reported [28]. However, the starting yeast material
44 must have sufficient nuclease to promote the enzymatic hydrolysis of nucleic acids [28], which is strain-
45 dependent [21]. Moreover, the reduction of RNA content achieved after hydrolysis using RNases may not
be
46 applicable to yeast residues that are exposed to high temperatures and other treatments applied during
beer
47 production/fermentation that may inactive these enzymes. Although the reduction of nucleic acid content
48 in yeast protein concentrates has been explored, there is a lack of technologies adapted to the processing of
49 residual yeasts [25], specially for high added-value ingredients, such as bioactive peptides.

50 The separation of RNA from complex matrices using membranes is a novel field of research and requires
51 further study. Few reports are available on the fractionation of peptides from SBY protein hydrolysates by
52 UF [19, 20, 29], with very limited information on the separation of peptides from other components such as
53 RNA and polysaccharides in a cascade fractionation [20, 30]. In a previous work of our group, polymeric
54 UF membranes of 30 kDa MWCO of polyethersulfone and regenerated cellulose were used to study the
55 separation and fouling during the filtration of SBY protein hydrolysate. Hydrophilic membranes resulted
56 in a better performance and less fouling, and thus may be envisaged for the separation of SBY protein 57
hydrolysates. However, the retention of RNA was not achieved at the studied conditions [31].

58 The objective of this work was to design a membrane fractionation process that could separate peptides
59 from RNA and polysaccharides and result in fractions containing different size ranges of peptides, appro-
60 priate for different applications. Two different UF sequences, 50-8-1 an 15-8-1 kDa molecular weight cut-
off

61 (MWCO) membranes, were proposed using hydrophilic ceramic membranes and fractions were analysed re-

62 garding their composition and molecular weight distribution. Depending on the sequences used, fractions of

63 different peptide purity and peptide profiles could be obtained, indicating outset application opportunities

64 in food and pharmaceutical industries.

65 2. Material and Methods

66 2.1. Materials

67 2.1.1. Reagents

68 Azocasein (A2765), Folin reagent (2 N), bovine serum albumin, d-(+)-glucose, insulin, substance P

69 1-7, leupeptin, triglycine, glycine and RNA from *S. cerevisiae* yeast were purchased from Sigma-Aldrich

70 (Steinheim, Germany). 3,5-dinitrosalicylic acid was purchased from Prolabo. Enzymes Protamex™ and

71 Alcalase™ were provided by Novozymes (Denmark), and Brauzyn® was provided by Prozyn (Brazil). All

72 other reagents were of analytical grade.

73 2.1.2. Spent brewer's yeast (SBY) hydrolysate

74 SBY from Ale beer production (*Saccharomyces cerevisiae*, SafAle™ HA-18, Fermentis, France) was col-

75 lected after 11 days of beer maturation without repitching at Brasserie La Singuli`ere (S`ete, France). The

76 collected material had about 10% dry weight, a pH value at 20 °C of 4.8 ± 0.1 and a specific mass of 1.017 g

77 mL^{-1} . The production of the protein hydrolysate followed the procedure developed by Marson *et al.* (2020)

78 [19], with modifications. Temperature of the heat treatment prior to hydrolysis was adjusted to $85 \pm 2 \text{ }^\circ\text{C}$ for

79 30 min, to ensure the inactivation of remaining glucoamylase used during brewing. A total amount of 2000

80 U of protease $\text{g}_{\text{protein}}^{-1}$ divided in an equal proportion of enzymes Brauzyn®, Protamex™ and Alcalase™ was

81 used. Proteolytic activity of each enzyme preparation was determined using azocasein as substrate [32, 33]

82 and were, 11,700; 83,300 and 256,500 U mL^{-1} , respectively. SBY protein hydrolysate was produced in a 7 L

83 capacity jacketed glass reactor connected to a recirculating water bath (Haake S30, Thermo Fisher Scientific,

84 USA) and an automatic titrator (TitroLine Alpha plus, Schott Instruments, Germany). Hydrolysis took

85 place at 50 °C and pH 7.0 for 2 h. Mechanical agitation at 1000 rpm was needed to ensure a well-mixed

86 system. Enzymes inactivation was done at $95 \pm 1 \text{ }^\circ\text{C}$ for 20 min. Reaction mixture was centrifuged at 10,000

87 × g for 15 min at 4 °C (3-16KL Sigma, Germany) separating the protein hydrolysate from yeast cell debris.

88 The degree of hydrolysis was determined as previously described, using pH stat method [34]. A protein
89 hydrolysate with a degree of hydrolysis of 7.5% was obtained.

90 2.1.3. Ultrafiltration membranes

91 Commercial ceramic membrane disks with a diameter of 90 mm (Inside Disram™ of Tami Industries,
92 France) were used. Ultrafiltration (UF) membranes of 50 and 15 kDa MWCO were made of a support of
93 α -Al₂O₃ and a filtering layer of ZrO₂. Ultrafine UF membrane of 8 kDa was made of the same support but
94 a filtering layer of TiO₂. A nanofiltration (NF) membrane (N001) of 1 kDa from the same supplier was also
95 used, with a support layer of Al₂O₃, TiO₂ and ZrO₂, and an active layer of TiO₂ [35]. These membranes
96 were autoclavable and supported transmembrane pressures up to 4 bar. Other membrane characteristics
are

97 presented in Table 1. Further comments on these data are presented in section 3.2.1.

Table 1: Characteristics of flat ceramic membranes used for the fractionation of spent brewer's yeast protein hydrolysate.

| Membrane | T50 | T15 | T8 | T1 |
|--|---------|--------|--------|--------|
| MWCO ^a (kg mol ⁻¹) | 50 | 15 | 8 | 1 |
| pH range ^a | 0-14 | 0-14 | 0-14 | 2-14 |
| Initial water permeation ^a (L m ⁻² h ⁻¹ bar ⁻¹) | 100-150 | 80-100 | 60-80 | 20-30 |
| Initial water permeation ^b (L m ⁻² h ⁻¹ bar ⁻¹) | 242 ± 2 | 69 ± 3 | 46 ± 8 | 88 ± 6 |

MWCO: molecular weight cut-off. ^aManufacturer data, at 25 °C. ^bInitial water permeation determined under the conditions evaluated in this work (0.5-2.0 ± 0.2 bar of transmembrane pressure, 20 °C, using deionised water) in a cross-flow module for ceramic disk membranes.

98 2.2. UF fractionation

99 2.2.1. Experimental procedure

100 Two fractionation sequences were carried out using UF and NF membranes, as presented in Figure 1.

101 Membrane experiments were performed in a stainless steel cross-flow membrane system (Spiralab®, Tami
102 Industries, France) of 52.7 cm² of effective permeation area. In this module, the feed is introduced in
103 the centre of the cartridge perpendicularly to the membrane surface and then flows tangentially along the
104 surface guided by a coil-shaped (spiral) support. Experiments were done at controlled temperature, using
a

105 recirculating water bath. Pristine membranes were firstly left 12 h embedded in deionised water, and then
106 conditioned following the cleaning procedure proposed by the manufacturer (a two-step process involving
107 successively 1.5% (m/v) NaOH solution and 1.5% HNO₃ (v/v) at 60 °C for 15 min in recirculation mode
108 and 5 min under pressure, up to 0.5 bar). Deionised water was used between steps to wash out the cleaning

109 solutions, until the pH of permeate and retentate was 7.0. Before initial water permeation measurement
 110 at 20 °C, conditioned membranes were left in recirculation mode for 10 min. Initial water permeation was
 111 conducted at 20 ± 1 °C, at transmembrane pressures of 0.5, 1.0, 1.5 and 2.0 bar.

112 Experiments (Figure 1) were performed at pH 7.0, 20 ± 1 °C, 2.0 ± 0.2 bar and a feed flow of 0.01054
 113 m³/s (i.e. cross-flow velocity of about 2 m/s) until a volumetric reduction factor (VRF = ratio between feed

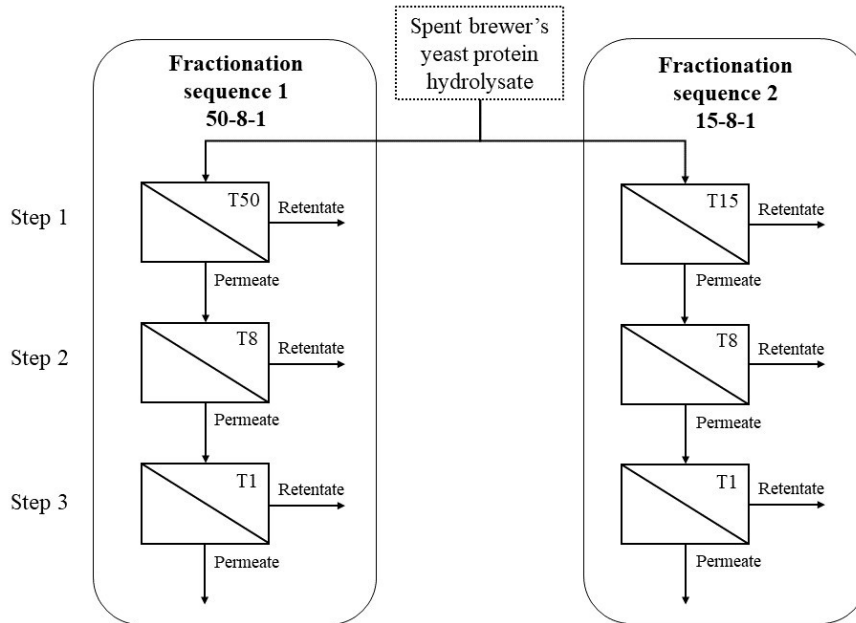


Figure 1: Experimental scheme of UF sequences and steps used in the fractionation of spent brewer's yeast protein hydrolysate using ceramic membranes of 50 (T50), 15 (T15), 8 (T8) and 1 (T1) kg mol⁻¹ molecular weight cut-off, respectively.

114 and retentate volume) of 4.0 for the first UF step and 2.5 for the others was reached. The experiments took
 115 from 3 h 30, using membrane T50, to 5 h, using T15, in the first step. The second and third steps, using
 116 T8 and T1 were carried out up to 6 h. A mass of 400 g of feed was used in the first while for the others it
 117 was 200 g.

118 2.2.2. Permeate flux

119 The collected mass of permeate was registered automatically as a function of time. Permeate volumetric
 120 flux was calculated at a given instant Δt , every 10 s, according to Equation 1, where J_p is the volumetric
 121 flux of permeate (L m⁻² h⁻¹); Δm_p is the permeate mass (kg); ρ is the specific mass of the hydrolysate (kg
 122 L⁻¹); t is the time (h) and A_p is the effective permeation area (m²). The specific mass of hydrolysate and
 123 fractions was determined using a pycnometer at 20 ± 1 °C. All fractions were kept frozen at -20 °C until
 124 analysis.

$$J_p = \frac{\Delta m_p}{\rho \Delta t A_p} \quad (1)$$

2.2.3. Membrane hydraulic resistances

The water flux before and after the filtration of the hydrolysate (or the permeate from the previous UF step) was measured, following the same procedure for initial water permeation, at transmembrane pressures

of 0.5-2 bar. Permeate fluxes of hydrolysate and water were used for the calculation of mass transfer resistances, using the resistance-in-series model. Intrinsic membrane resistance (R_M , m^{-1}) was calculated using initial water flux (J_0), water dynamic viscosity data under the experiment conditions applied (μ_w), as shown in Equation 2. Total resistance (R_T , m^{-1}) was calculated considering the permeate flux of hydrolysate (J_{hyd}) and the dynamic viscosity of the permeate under operation conditions ($\mu_{hyd} = 0.00123 \text{ kg m}^{-1} \text{ s}^{-1}$) (Equation 3). Finally, resistances related to concentration polarisation in the boundary layer and fouling were obtained considering the water flux after the hydrolysate filtration and rinsing step (J_w') and their difference to the total resistance, as shown by Equations 4 and 5. In these two mathematical expressions, parts of total resistance that are and are not eliminated by water rinsing represent, respectively, the reversible

R_{pl} (m^{-1}) and irreversible fouling R_I (m^{-1}) [36, 37]. In Equations 2, 3, 4 and 5, transmembrane pressure (ΔP) is in $\text{kg m}^{-1} \text{ s}^{-2}$, dynamic viscosity in $\text{kg m}^{-1} \text{ s}^{-1}$ and flux of permeate in $\text{m}^3 \text{ m}^{-2} \text{ s}^{-1}$.

$$R_M = \frac{\Delta P}{\mu_w J_0} \quad (2)$$

$$R_T = \frac{\Delta P}{\mu_{hyd} J_{hyd}} \quad (3)$$

$$R_I = \frac{\Delta P}{\mu_w J_w'} - R_M \quad (4)$$

$$R_{pl} = R_T - R_M - R_I \quad (5)$$

2.3. Analytical methods

2.3.1. Proximal composition

Proximal composition of SBY protein hydrolysate was determined using a different protocol than that of UF fractions. Prior to the determination of total sugars in the hydrolysate, the protein hydrolysate was

143 submitted to a polysaccharide precipitation protocol [38] with modifications. Briefly, to 10 mg of yeast
144 hydrolysate, 2 mL of ethanol were added (12 h at 4 °C). Samples were centrifuged (5000 × g for 10 min
145 at 4 °C), washed with 2 mL of ethanol and left to dry in a bath at 70 °C to remove the residual solvent.
146 Pellet was then redissolved in 2 mL of deionised water at 60 °C and mixed. Total sugars content of this
147 solution was estimated by the Phenol-sulfuric acid assay [39] measuring the absorbance at 490 nm. Results
148 were expressed in g 100 g_{d.w.}⁻¹. Peptides concentration in the hydrolysate (g 100 g_{d.w.}⁻¹) were measured by
149 far-UV absorbance at 214 nm (2800, Unico, United States) [40]. Dry weight and RNA concentration were 150
determined as for UF fractions.

151 For all UF fractions, dry weight (% m/m) was determined gravimetrically at 105 °C for 12 h using
152 an incubator (UE 400, Memmert, Germany), an analytical balance (XT 120A, Precisa, Hong Kong) and
153 a glass desiccator [41]. Protein content was determined by the Lowry method (g 100 L⁻¹_{sample}) measuring
154 absorbance of the reaction mixture at 750 nm using a bovine serum albumin standard curve for reference
155 (UV-2401 PC, software UV Probe (version 2.21), Shimadzu, Japan) [42]. Ribonucleic acid content (RNA)
156 was determined spectrophotometrically (260-290 nm) following trichloroacetic acid hydrolysis (75 µL of
70%
157 acid per 1 mL of sample) at 90 °C for 30 min. A standard curve using RNA from *S. cerevisiae* was used
158 at 260 nm, and results were expressed in g (100 g_{d.w.})⁻¹ for the initial hydrolysate, and for the fractions, in
159 mg L⁻¹_{sample} [43–45]. Total reducing sugars mg (100 L⁻¹_{sample}) were determined in dried samples using the
160 Somogyi-Nelson method measuring absorbance at 500 nm [39] after hydrolysis with concentrated
sulphuric 161 acid and centrifugation at 3800 × g for 5 min at 4 °C [46]. Reducing sugars in samples (g (100
g_{d.w.})⁻¹) were 162 determined by the DNS method [47]. Sugar results considered d-(+)-glucose standard
curves.

163 2.3.2. Anions determination in the hydrolysate by ion-exchange chromatography

164 Ion concentrations in the SBY feed prior to UF were determined by ion-exchange chromatography. Anion
165 determinations were performed in a Dionex ICS1000 and ICS900 systems (ThermoFisher Scientific, USA),
166 respectively, composed of an eluent producer, a suppressor system (ADRS-600 for anions and CERS 500
for
167 cations) to reduce background eluent conductivity and a conductivity detector. Dionex AS19 and Dionex

168 CS12A columns (4x250 mm) (Thermofisher Scientific, USA) were used for anions and cations, respectively.
169 Elution of anions was done by an aqueous solution containing KOH at 10 mM (10 min) followed by a
170 gradient for 20 min until 45 mM and then 10 mM (10 min), at a flow rate of 1.0 mL min⁻¹. Cations
171 elution was carried out using a 20 mM methanesulphonic acid solution, at the same flow rate. All samples
172 were filtered with 0.22 μm polytetrafluoroethylene (PTFE) syringe filters prior to analysis. The amount
173 of sample injected was 25 μL. Data were collected using the Chromeleon™ Chromatography Data System
174 (CDS) Software v. 7.2.9.11323 (Thermo Fisher Scientific, USA). Anions and cations concentration in yeast
175 samples was calculated using calibration curves relating amount of analyte (Cl, NO₂, NO₃, ClO, Br, SO₄,¹⁷⁶
PO₄, Na, NH₄, K, Mg, Ca) and peak area.

177 2.3.3. Molecular weight distribution of SBY peptides by size-exclusion chromatography (SEC)

178 Molecular weight distribution of peptides in non-treated and heat-treated SBY as well as in the protein
179 hydrolysate and UF fractions was determined using the column Superdex Peptide GL 10/300 (GE Health-
180 care, USA) with a fractionation range of 100-7000 g mol⁻¹ in a chromatography system (Thermo Fisher
181 Scientific, USA) including a pump system Dionex (ICS1000), a UV detector (Ultimate 3000) and an auto-
182 sampler (AS40). Size-exclusion chromatography was performed using a 50 mM sodium phosphate buffer
183 as eluent (ionic strength of 0.5 M, pH 7.0) at 20 °C, at a constant flow rate of 0.5 mL min⁻¹ for 70 min,¹⁸⁴
monitored at 214 nm. The ionic strength of the eluent was selected considering a 14 fold more important
185 ionic strength than in the most concentrated sample (35 mM) (section 2.3.2). All samples were filtered with
186 0.22 μm polytetrafluoroethylene (PTFE) syringe filters prior to analysis. Volume of injected sample was 25
187 μL. A calibration curve using peptidic standards (bovine serum albumin, aprotinin, insulin, cyanocobalamin,
188 substance P 1-7, leupeptin, triglycine and glycine) was used to determine the molecular weight distribution
189 of fractions (log of molecular weight versus retention volume). Instrument was controlled and data were
190 generated by the same software used for ion-exchange chromatography (section 2.3.2). Definite integral
191 values were determined by a numerical integration method (trapezoid rule) after baseline correction using
192 a developed Python script for this purpose. The retention of peptides (R_p) was calculated using the inte-
193 grated peaks of feed (S_f) and permeate (S_p) for the different molecular weight (MW) ranges, as presented¹⁹⁴
in Equation 6.

$$R_p (\%) = \left(1 - \left(\frac{S_p}{S_f}\right)\right) \times 100 \quad (6)$$

196 Experiments were performed in triplicate and all analyses were determined at least in triplicate. Results
 197 were expressed as average values \pm standard deviation and were submitted to analysis of variance
 (ANOVA)

198 one and two way, followed by the comparison of means by Tukey HSD test. ANOVA assumptions were
 199 checked through analysis of the residues, data distribution (Ryan-Joiner's and Shapiro Wilk's tests) and
 200 homogeneity of variances (Bartlett's and Levene's tests). Differences were considered significant at a level
 201 of 5% for all statistical analysis.

202 3. Results and Discussion

203 3.1. SBY protein hydrolysate initial composition and molecular weight distribution

204 Proximal composition of the protein hydrolysate is presented in Table 2. SBY hydrolysate consisted of
 205 7% dry weight, about 76% (d.w.) protein (Far-UV detection of peptide bonds at 214 nm), 6% (d.w.) RNA,
 206 6% (d.w.) total sugars (Phenol-sulphuric acid assay), and 13% of other compounds such as fibre and ashes
 207 (d.w.). The molecular weight distribution of protein fractions and peptides in the hydrolysate indicated the
 208 presence of protein chains and peptides of 1-7 kg mol⁻¹ (Table 2). The mass yield of hydrolysate obtained
 209 from non-treated SBY was 75 \pm 5% (m/m).

Table 2: Proximal composition in dry weight (d.w.) of the spent brewer's yeast protein hydrolysate and the molecular weight (MW) distribution of the protein fractions present in kg mol⁻¹.

| Content (% d.w.) | SBY protein hydrolysate |
|---------------------|-------------------------|
| Protein | 76% |
| MW > 7 | 8% |
| 4 < MW \leq 7 | 5% |
| 1 < MW \leq 4 | 18% |
| 0.3 < MW \leq 1 | 20% |
| 0.1 < MW \leq 0.3 | 11% |
| MW \leq 0.1 | 14% |
| RNA | 6% |
| Total sugars | 6% |
| Other | 12% |

210 The confirmation of protein hydrolysis is shown in the chromatograms generated by size-exclusion chro-
 211 matography (SEC) of non-treated material, the spent yeast after the heat treatment and after protein hy-
 212 drolysis, given in Figure 2. In the chromatograms, peaks in the range of higher molecular weight molecules
 213 (> 7000 g mol⁻¹) are abundantly present in the non-treated and heat-treated yeast materials, but are found

214 in much less amounts in the hydrolysate. The yeast protein hydrolysate is rich in peptides in the whole 215
 range of the column (from 7000 to 1000 g mol⁻¹), confirming that yeast proteins were cleaved into peptides.
 216 Smaller peptides and amino acids are also present (1000-100 g mol⁻¹) in the hydrolysate.

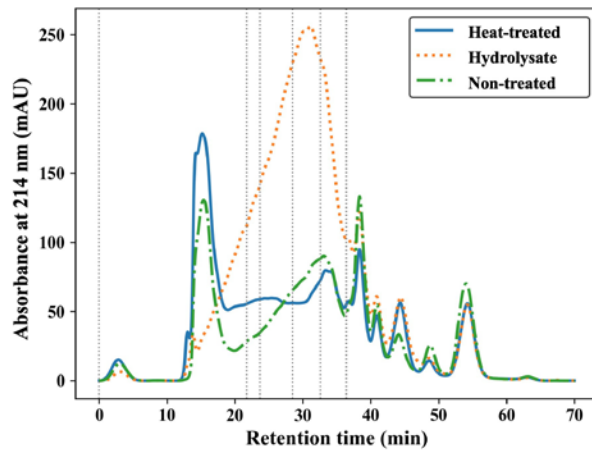


Figure 2: Size-exclusion chromatographs of non-treated, after heat-treatment and hydrolysed spent brewer’s yeast. Vertical grey dashed lines represent the molecular weight limits. From the left to the right: MW > 7000 g mol⁻¹, 4000 - 7000 g mol⁻¹, 1000 - 4000 g mol⁻¹, 300 - 1000 g mol⁻¹ and 100 - 300 g mol⁻¹ (t = 36.4 min corresponds to the total column volume and t = 14.2 min corresponds to the void column volume). Absorbance units in mAU.

217 Table 3 shows the concentration of anions and cations in the SBY protein hydrolysate. Mineral com-
 218 position indicated a higher representation of cations with potassium being the main component, with 11.5
 219 mEq L⁻¹, followed by phosphate, at 8.3 mEq L⁻¹. These two minerals are reported to be the most represen-
 220 tative of spent yeast materials [48]. The estimated total ionic strength of the hydrolysate used in UF was
 221 approximately 35 mM.

Table 3: Mineral composition of spent brewer’s yeast (SBY) protein hydrolysate determined by ion-exchange chromatography. Other undetermined ions = 5 mEq/L.

| Minerals (mEq L ⁻¹) | SBY protein hydrolysate |
|--|-----------------------------|
| Ammonium (NH ₄ ⁺) | 1.9 |
| Calcium (Ca ²⁺) | 0.2 |
| Magnesium (Mg ²⁺) | 1.1 |
| Potassium (K ⁺) | 11.5 |
| Sodium (Na ⁺) 4.4 | Chloride (Cl ⁻) |
| 5.4 Nitrate (NO ₃ ⁻) | 0.1 |
| Phosphate (PO ₄ ²⁻) 8.3 | |
| Sulphate (SO ₄ ²⁻) | 0.3 |
| P Cations | 19.1 P Anions 14.1 |
| Total ions | 33.2 |

222 3.2. UF fractionation performance: process

223 3.2.1. Flux of permeate

224 Table 1 shows initial water permeation for 50, 15, 8 and 1 kDa MWCO inorganic membranes. At
225 the conditions evaluated in this study, a decrease in water permeation values as the membranes MWCO
226 decreased was observed, with an exception for T1 membrane. For this membrane, the water permeability
227 was higher than that of T15 and T8 membranes. For manufacturer data, measured at 25 °C, this effect
228 was not observed. Higher water permeation compared to manufacturer values were detected for T50 and
229 T1 while smaller values were seen for T15 and T8. The discrepancies could be related to characteristics of
230 the water used in the experiment, measurement conditions (cross-flow velocity, membranes pre-
231 conditioning treatment, water properties) and, consequently membranes surface properties before
and during filtration.

232 Flux of permeate graphs for each step and each membrane tested are shown in Figure 3. The flux
233 of permeate in T50 membrane was 1.8 fold higher ($16.0 \pm 1.9 \text{ L m}^{-2} \text{ h}^{-1}$) than that observed with T15
234 membrane ($8.9 \pm 1.9 \text{ L m}^{-2} \text{ h}^{-1}$). Figure 3a shows the rapid decrease of permeate flux in the first step
235 of filtration followed by the stabilisation at higher VRFs for both membranes, suggesting that a surface
236 deposition of molecules occurred. The accumulated layer played the role of a secondary membrane that
237 prevented smaller particles and molecules from blocking membrane pores. In this fouling mechanism, often
238 reported for membrane filtration of food and biological fluids, there is limited risk of internal clogging and
239 total blockage of the membranes in concentration mode, a great feature for industrial processing [49].

240 For the second step of fractionation using 8 kg mol⁻¹ MWCO membrane (Figure 3b), very similar per-
241 meate fluxes were observed (about $5 \text{ L m}^{-2} \text{ h}^{-1}$) for both sequences 50-8 and 15-8. In this step, the flux
242 decreased continuously at a slower rate than that observed in the first step, suggesting that an internal pore
243 clogging fouling mechanism played a role [49].

244 Different permeate fluxes were seen for sequences 1 and 2 in the third fractionation step using 1 kg
245 mol⁻¹ MWCO membrane (Figure 3c). In the case of sequence 1 (50, 8, 1 kDa) the permeate flux decreased 246
gradually as VRF increased (until reaching 9.2 ± 0.5 at VRF 2.5), suggesting that, at least to some extent,
247 an internal pore clogging mechanism took place. On the contrary, for sequence 2 (15, 8, 1 kDa) the flux 248
decreased rapidly and then stabilised at a 79% higher value (16.5 ± 0.8). These results may be related to the

249 difference in feed composition between the two filtration sequences. Indeed, the composition

250 of a permeate is related to the MWCO membrane used but also, in the case of biological solutions, to the

d nami laye forme du in fi tr tio whic clearl d pend o th co p itio o th feed Ther fore a
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feeds of the second filtration were different, it is possible that the two permeates obtained after filtration on

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th T me bran ha di fe en co p ition an le t di fe en fou in whe fi tere o th T me brane
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253

In particular, it is possible that small MW molecules that were not retained by T50 membrane during the

254

first step, were still present in the permeate of T8 membrane and thus contributed to the subsequent internal

255

pore clogging of the T1 membrane in this sequence. In sequence 2, as a more important cake layer seems to

256

have been formed during filtration with T15 membrane, these small MW molecules could have been trapped

257

during this first step and thus, no further fouling occurred in the next steps.

258

3.2.2. Hydraulic resistances of the membranes

259

In practice, more than one fouling mechanism act simultaneously during the UF of heterogeneous mix-

260

tures [50], such as the SBY protein hydrolysate. In contrast to concentration polarisation, which is an

261

inherently reversible phenomena, fouling may cause irreversible losses on membrane permeability. Both

262

polarisation and fouling induce hydraulic resistances which can be defined here as "reversible" (R_{pl}) or

263

"irreversible" (R_I) as they can or cannot be removed by simple rinsing, respectively.

264

All components of hydraulic resistances of the ceramic membranes used in the study are presented in

265

Table 4. The value of total resistance observed for the T8 membrane, used in step 2 of both sequences, is

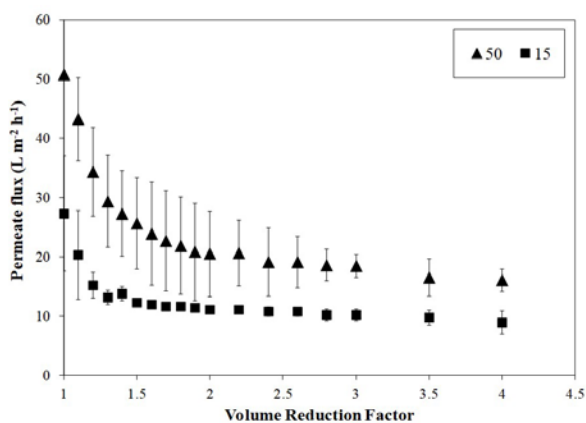
266

significantly higher compared to those observed for membranes T50, T15 and T1, used in the other filtration

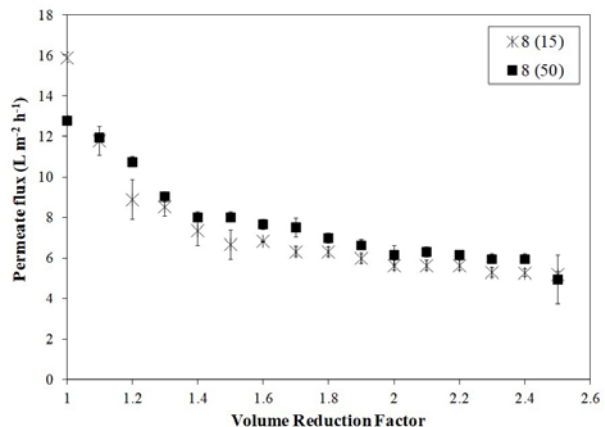
267

steps, regardless of sequence. For this membrane, the higher value of intrinsic membrane resistance compared

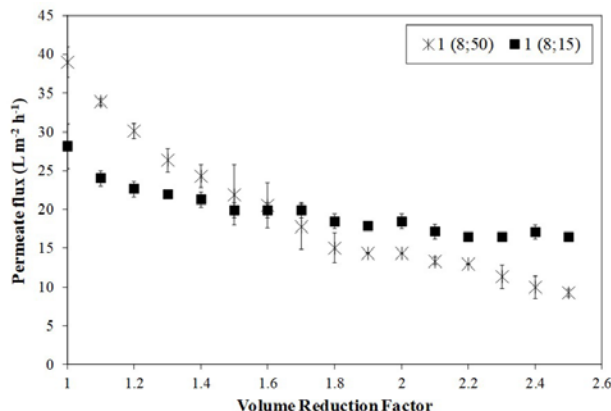
268



(a)



(b)



(c)

Figure 3: Permeate flux of spent brewer's yeast hydrolysate at fractionation steps 1 (50 or 15 kg mol⁻¹ molecular weight cut-off, MWCO) (a), 2 (8 kg mol⁻¹ MWCO) (b) and 3 (1 kg mol⁻¹ MWCO) (c)

Experiments were performed at pH 7.0, 20 ± 1 °C, 2.0 ± 0.2 bar and a feed flow of 0.01054 m³/s (i.e. cross-flow velocity of about 2 m/s) until a volumetric reduction factor (VRF = ratio between feed and retentate volume) of 4.0 for the first UF step and 2.5 for the others was reached.

to those of other membranes is probably responsible for the slowing down of mass transfer, as evidenced

by the lower flux detected for T8 membranes. Solute accumulation near the membrane surface was thus

more important, leading to more pronounced polarisation phenomena. This effect is confirmed by the value

of reversible resistance, which was higher for T8 membrane than for the others. In the step 3, the only

difference observed was that reversible resistance among T1 membranes was almost 2 fold higher for T1

membrane of sequence 1.

All membranes showed similar distributions of the components of the total resistance to mass transport,

irrespective of the sequence considered. The reversible resistance R_{pl} is the main part of the total resistance,

contributing to 78-92% of it, whereas the irreversible resistance represented only 3-11% of the total. The

filtration conditions used in this work were very unfavourable (cross-flow mode), and an optimisation of

hydrodynamic conditions (i.e. such occurs when tangential mode is chosen) will surely lead to an improve-

ment of membrane performance at industrial scale. The small proportion of irreversible resistance observed

suggests that accumulated material onto the surface or in the pores were easily removed by simple water

rinsing, not requiring extensive chemical cleaning.

Table 4: Membrane hydraulic resistances: Intrinsic membrane resistance (R_M), reversible (R_{pl}) and irreversible fouling (R_I) and total resistance (R_T) for the two fractionation sequences using 50, 15, 8 and 1 kDa molecular weight cut-off membranes.

| Sequence | Membrane | R_M (m ⁻¹) | R_{pl} (m ⁻¹) | R_I (m ⁻¹) | R_T (m ⁻¹) |
|----------|-----------|--------------------------|-----------------------------|--------------------------|--------------------------|
| 1 | 50 | 1.49×10^9 | 3.38×10^{10} | 1.29×10^9 | 3.66×10^{10} |
| 1 | 8 (50) | 7.81×10^9 | 10.85×10^{10} | 3.18×10^9 | 11.95×10^{10} |
| 1 | 1 (50, 8) | 4.08×10^9 | 5.60×10^{10} | 3.51×10^9 | 6.36×10^{10} |
| 2 | 15 | 5.21×10^9 | 5.71×10^{10} | 3.49×10^9 | 6.58×10^{10} |
| 2 | 8 (15) | 7.81×10^9 | 10.16×10^{10} | 3.11×10^9 | 11.26×10^{10} |
| 2 | 1 (15, 8) | 4.08×10^9 | 2.86×10^{10} | 3.87×10^9 | 3.66×10^{10} |

3.3. UF fractionation performance: selectivity

Dry weight content in the fractions of fractionation sequences 1 and 2 are shown in Figure 4, in g per 100 g of wet sample. The initial hydrolysate had a dry weight of 0.98 g per 100 g_{w.s.}. In the graph, the dry weight of samples increased after filtration by 47% and 29% in the first retentate, for sequences 1 and 2, respectively. Then, as the fractionation steps were carried out and compounds were retained, dry weight went from 0.98 g per 100 g_{w.s.} in the initial feed to about 0.4 g per 100 g_{w.s.} for both sequences.

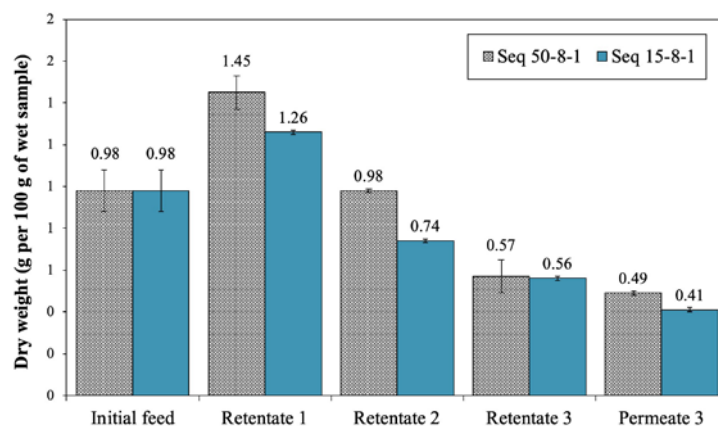


Figure 4: Evolution of dry weight composition in the initial feed, retentates and permeates from the first, second and third step of ultrafiltration, for the fractionation sequences 1 and 2, using the sequence of membranes 50-8-1 and 15-8-1 kDa, respectively.

The evolution of composition of proteins, RNA, total reducing sugars in g per 100 g of dry weight, in the three retentates and the last permeate obtained after the two fractionation sequences, is given in Figures 5a and 5b, respectively. Protein, RNA and total sugars represented 23 to 80% of total solids in these fractions. The SBY protein hydrolysate contained other components that were not quantified, such as fibres, ashes, lipids as well as polysaccharides and peptides that were not detected by the analytical methods used in this work. For instance, in the first retentate, the percentage of other compounds consisted probably of fibres and other high molecular weight polysaccharides. In the last permeate, on the other hand, salts are probably the main component. From the ion concentration measurements, the salt content in the feed of the first UF was estimated as 10.87 g per 100 g of dry weight (Table 3, considering a specific mass of 1.017 g mL⁻¹ and a dry weight of 0.98 g per 100 g of wet sample). Indeed, salt content is reported to represent about 10 g of salt per 100 g of dry sample in non-fractionated yeast hydrolysates [51], a similar value as the one found in this work. From the first to the last step of UF fractionation, an increase in salt content is expected, as these small components are not retained by UF membranes.

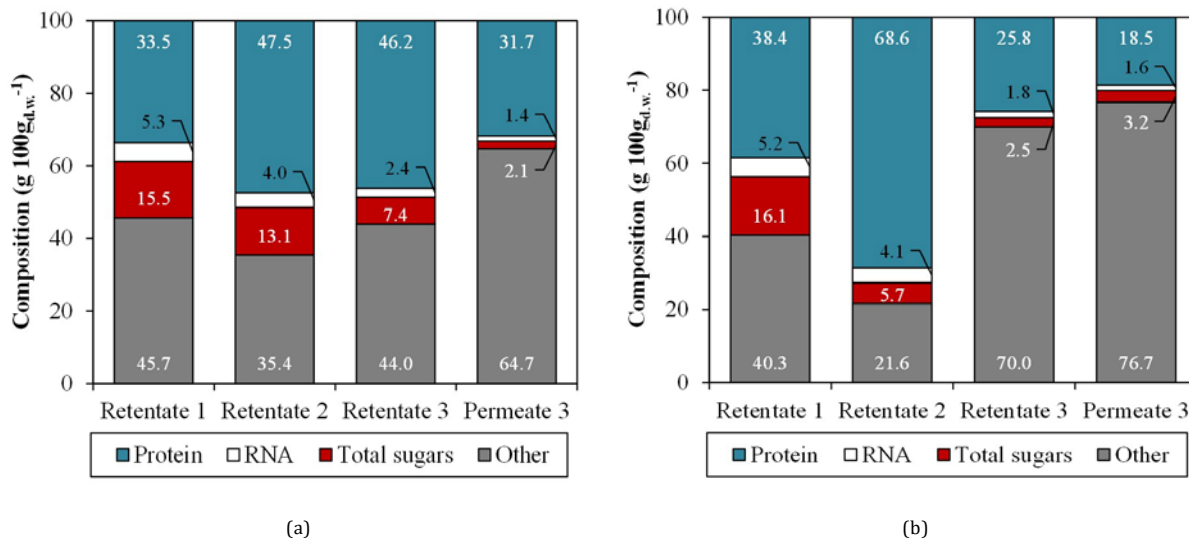


Figure 5: Distribution of protein (Lowry), RNA, total reducing sugars (after hydrolysis by DNS) for UF fractions obtained after the fractionation sequences 1 (50-8-1) (a) and 2 (15-8-1) (b). Standard deviation for all analyses was smaller than 15%.

In these composition charts (Figures 5a and 5b) it can be observed that the total sugars and RNA, the main compounds to be separated from the protein hydrolysate, had their contents decreased in fractions from step 1 to 3, regardless of fractionation sequence. Both components were partially retained in the three steps, reaffirming the need of different MWCO membranes to promote their separation. Regarding RNA, the

natural presence of RNases in yeast that could act before the hydrolysis could be responsible for the different

MW RNAs found in the SBY protein hydrolysate, as previously reported [21, 28, 52]. Three factors can be related to the activation and increased ribonuclease activity in yeasts: 1) heat treatments can activate RNases; 2) the presence of sodium chloride and phosphate; and 3) higher aeration intensity [21]. These conditions could have been met during SBY processing, specially during the heat treatment with constant mixing prior to proteolysis. Also, SBY's mineral composition data (Table 3) clearly confirms the presence of considerable amounts of sodium, chloride and phosphate.

The separation of peptides was not the same for the two tested sequences (Figures 5a and 5b). It seems that in sequence 1, the proportion of protein was smaller for the first two retentates but higher for retentate 3 and permeate 3, when compared to sequence 2. These differences in composition happen because protein hydrolysates are mixtures of peptides of different sizes, structures and physicochemical properties, that influence their separation.

Retention factors of peptides presented by molecular weight ranges achieved in each fractionation step for both tested sequences (50-8-1 and 15-8-1) are presented in Figures 6a, 6b and 6c. It is important to note that

320 peptides concentration in the SBY hydrolysate fractions chromatograms was measured by far-UV
absorbance

321 at 214 nm, that detects peptide bonds. This is a widely used methodology that predominantly identifies
322 peptides, but other compounds can be detected at neighbouring wavelengths, influencing the
measurement.

323 Starting from Figure 6a, a greater retention of peptides by membrane T15 is seen when compared to the
324 T50 membrane. This finding is in accordance with the MWCO of the membranes. For sequence 15-8-1, the
325 first step seemed to remove most of non-protein and other sample contaminants, increasing the
performance
326 of the subsequent steps, where a higher proportion of protein, for instance in retentate 2, in comparison to
327 sequence 1.

328 In the second step (Figure 6b) differences in the retention for peptides smaller than 7% were not detected
329 considering the 15% standard deviation of the analysis. This finding is coherent because membranes were
330 of the same MWCO, and even if the feeds were different (i.e. permeates of 50 and 15 kDa membranes,
331 respectively), the hydrodynamic deposits formed during filtration were not fundamentally different (i.e. the
332 hydraulic resistances observed were of the same order of magnitude). It is thus not surprising to observe
333 similar retention.

334 In the last step of filtration, a more elevated retention for peptides was observed in Figure 6c for sequence
335 1. This result is related to the different composition of the permeates of the previous steps between the two
336 sequences. In the first sequence, 50 kDa membrane presented a smaller retention of smaller molecules

337 (MW < 1kDa) compared to the 15 kDa membrane (Figure 6c and Table 5). The resulting permeate, which also

338 the feed of the second filtration step was the more concentrated in low MW peptides. According to Figure

339 6b, these small molecules were poorly retained by the 8 kDa membrane, and then altered the reversible

340 resistance of the subsequent step using the T1 membrane. The change in the polarisation layer of T1 did

341 not only affected the flux of permeate, but have also modified the membrane selectivity, as seen in Figure

6c.

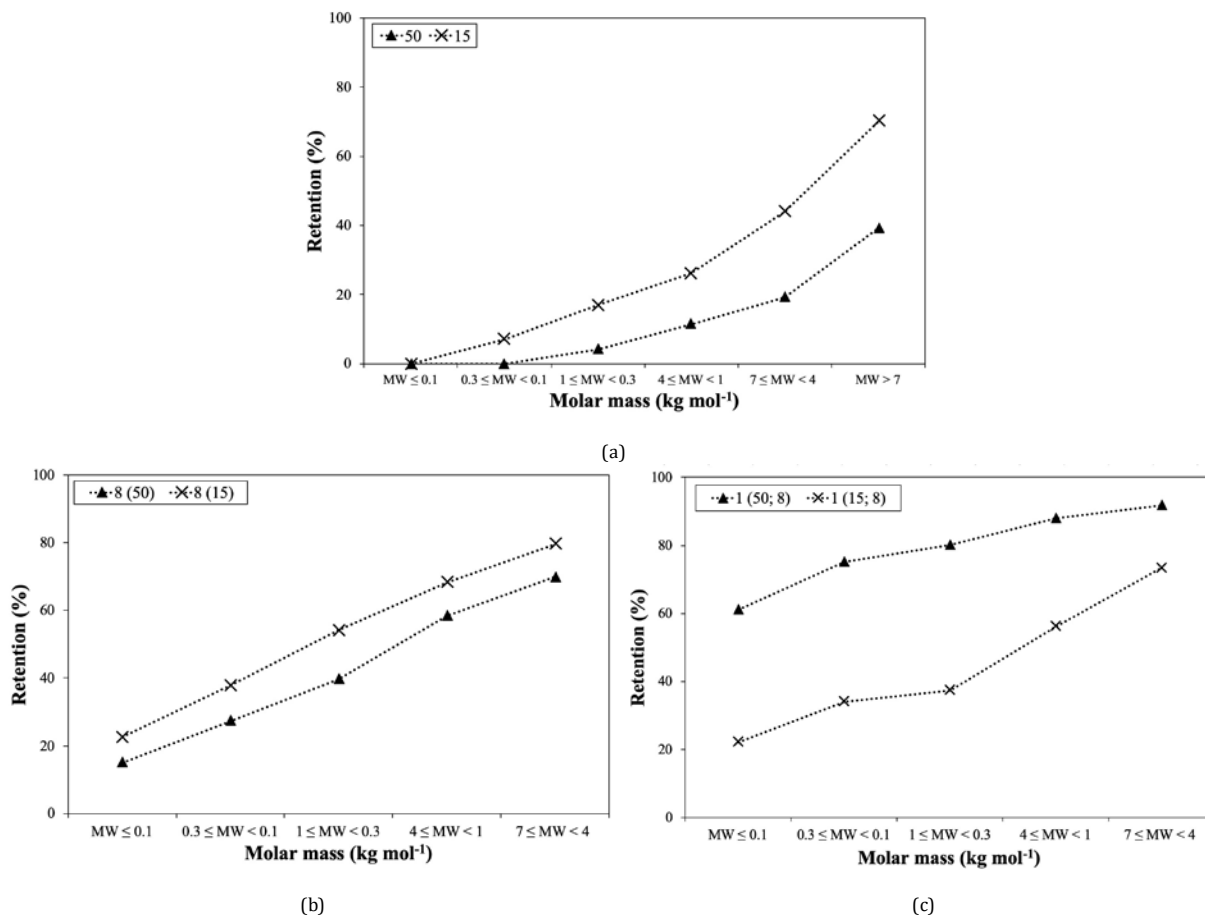


Figure 6: Retention versus molar mass of peptide fractions during UF fractionation in steps 1 (a), 2 (b) and 3 (b) using 50, 15, 8 and 1 kg mol⁻¹ MWCO membranes.

Standard deviations smaller than 15%.

343 Briefly, selectivity results confirm that each step of the fractionation was important to separate peptides
 344 from RNA and total sugars. As a general rule, a higher retention of the T15 membrane was observed for
 345 all components, difference that affected the composition of fractions and separation performance on the
 346 subsequent steps. The last step was still important as it retained proteins in the hydrolysate. Finally, the
 347 mass balance distribution of dry weight, RNA, total sugars and protein for fractions of sequences 1 (50-8-
 1) 348 and 2 (15-8-1) confirmed the discussed trends observed for concentration and retention
 coefficients of the
 349 components of SBY protein hydrolysate (Figures S1a and S1b of Supplementary Material). Additional data
 350 comprising the concentration and mass for feed, retentate and permeate for both sequences are also available
 351 in the Supplementary Material, Tables S1 and S2).

3.4. SBY peptide fractions as ingredients: molecular weight distribution and purity

352 Molecular weight distribution considering the peptide ranges smaller than 1 kg mol⁻¹, between 1 and 7
 353 kg mol⁻¹ and higher than 7 kg mol⁻¹ for both fractionation sequences is given in Table 5. Very small changes
 354 between sequences were detected in the molecular distribution of fractions, but the molecular distribution of
 355 of

356 the hydrolysate was gradually changed with further fractionation. The composition of the initial
 hydrolysate
 357 was changed with the fractionation until the peptides smaller than 1 kg mol^{-1} represented about 90% of the
 358 peptides in the permeate of the third step. Higher molecular weight peptides were retained by the first step,
 359 representing 17-19% of the composition. These results indicate that UF fractionation sequences were able
 360 to produce fractions with different peptide compositions.

Table 5: Molecular weight (MW) distribution (%) of peptides recovered after sequences 1 (50-8-1) and 2 (15-8-1) divided in three regions: smaller than 1 kg mol^{-1} , between 1 and 7 kg mol^{-1} and higher than 7 kg mol^{-1} .

| Protein fractions molecular weight distribution (%) | | | |
|---|----------|---------------------|-----------------|
| | MW < 1 | $1 < \text{MW} < 7$ | $7 < \text{MW}$ |
| <i>Initial hydrolysate</i> | 59.0 | 30.1 | 11.2 |
| <i>Sequence 50-8-1</i> | | | |
| Retentate 1 | 50.0 | 30.8 | 19.2 |
| Retentate 2 | 62.3 | 29.6 | 8.1 |
| Retentate 3 | 79.0 | 19.0 | 2.0 |
| Permeate 3 | 91.7 | 7.0 | 1.3 |
| <i>Sequence 15-8-1</i> | | | |
| Retentate 1 | 51.0 | 32.2 | 16.8 |
| Retentate 2 | 66.0 | 28.7 | 5.3 |
| Retentate 3 | 80.3 | 17.1 | 2.6 |
| Permeate 3 | 88.7 | 7.9 | 3.4 |

Molecular weight ranges in kg mol^{-1} . Standard deviations smaller than 15%.

361 Peptide-rich fractions should attain a certain separation level (that varies depending of product applica³⁶²
 tion and country legislation) from the other components so that their use as ingredients or nutraceuticals
 363 can be envisaged [10]. Figures 7a and 7b show the evolution of protein purity with regard to RNA, to-
 364 tal sugars (Somogyi-Nelson) and reducing sugars (DNS) as fractionation sequences 50-8-1 and 15-8-1 were
 365 carried out. Protein purity for the initial hydrolysate was $12.7 \text{ g}_{\text{protein}}/\text{g}_{\text{RNA}}$ and $12.6 \text{ g}_{\text{protein}}/\text{g}_{\text{total sugars}}$.
 366 Reducing sugars were not determined for the initial feed. Relative protein purity concerning RNA in the
 367 sequence 1 of fractionation increased 3.4 fold (comparison between the first retentate and the last permeate).
 368 Highest protein purity regarding RNA for this sequence was obtained for the permeate obtained after the
 369 T1 membrane ($22.1 \text{ g}_{\text{protein}}/\text{g}_{\text{RNA}}$). In the 15-8-1 fractionation sequence, protein purity with regard to RNA
 370 was the highest for the retentate obtained from the T8 membrane ($16.6 \text{ g}_{\text{protein}}/\text{g}_{\text{RNA}}$), and was 30% higher
 371 in comparison to the last recovered permeate. Protein purity of fractions regarding total sugars (Somogyi-
 372 Nelson) showed roughly the same tendency as RNA for both fractionation sequences, as seen previously.

373 Higher purity for sequences 50-8-1 and 15-8-1 was achieved for the retentate of T8 for both membranes,
 374 around 31-34 g_{protein}/g_{total sugars}. For the first fractionation sequence, the purity regarding total sugars was
 375 also high for the retentate of T1 membrane.

376 The decrease in protein purity regarding total sugars, RNA and reducing sugars in the permeate of mem-
 377 brane T1 of sequence 2 is related to its smaller relative protein concentrations and higher salt
 concentration.

378 Indeed, an increase in protein purity after the first and second filtration steps is observed for all
 components

379 (RNA, total sugars and reducing sugars) regardless of the fractionation sequence also because protein con-
 380 centration was increased from retentate 1 to retentate 2 (Figures 5a and 5b). These results corroborate the
 381 differences observed in the concentrations and mass balances of fractions, where more important
 differences

382 were observed for the first membranes (T50 and T15). These differences then affected the following UF
 383 fractionation stages in relation to the composition and purity of fractions.

384 Even though protein and total sugars content in the initial hydrolysate was determined by different

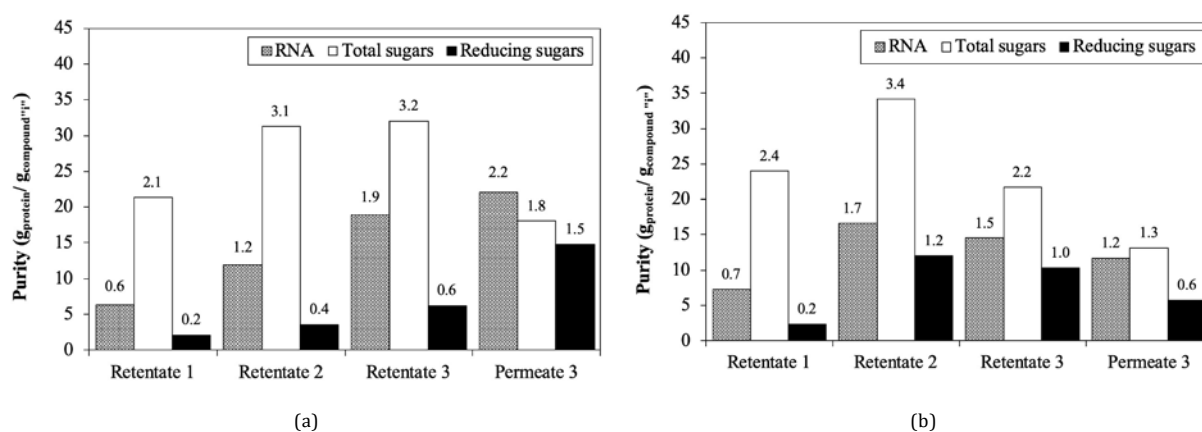


Figure 7: Evolution of fractions purity (g of protein in comparison to g of compound "i") in the first, second and third fractionation steps for sequence 1 (50, 8, 1 kDa) (a) and 2 (15, 8, 1 kDa) (b). Compound "i" is either RNA, total sugars or reducing sugars

385 methods, it can be seen that the protein purity was considerably increased after the UF process. For the
 386 first

387 time, fractions of SBY peptides rich in peptides could be produced with RNA contents as low as 1.4% (dry
 388 weight) by membrane separation technology, without the use of toxic chemicals, solvents and the addition

of NaCl. Up to 22.1 g of protein per g of RNA was achieved, using a multi-stage fractionation process. In

389 the cases where brewer's residual cells are used to produce protein-rich ingredients without treatments to

390 reduce RNA content, RNA levels can easily reach 8% [48]. Considering the great influence of RNA upon

391 serum uric acid levels in humans, the introduction of RNA from SCP-based products is recommended to be

392 less than 2 g per adult, per day [53]. European Commission recommendations for infant formulas establish
393 a limit of added nucleotides of maximum 5 mg/ 100 kcal, which would result in maximum 0.1 g per adult,
394 per day [54]. According to these recommendations, the maximum consumption of SBY protein-rich
material
395 with 1.4% (d.w.) of RNA would be 142 g and 7 g of dry SBY protein hydrolysate per day for an adult and
396 an infant, respectively.

397 Previous work reported some level of RNA reduction in *Saccharomyces* sp. yeast cells, but rarely for
398 residual yeasts from brewing. Heat shock treatment of *Saccharomyces cerevisiae* cells with 3% NaCl to
399 activate intracellular RNases resulted in a final RNA concentration of 2% [52]. RNA in *Saccharomyces* 400
cerevisiae cells achieved a minimum of 1% after precipitation at pH 9.0 at 90 °C using NaCl 10%. However,
401 this method reported the decrease of available lysine after treatment [27]. A RNA reduction strategy
402 activating endogenous RNases was applied to *Saccharomyces cerevisiae* and reached a minimum of 2.1%
403 RNA [28]. However, when spent yeast recovered from brewer's wort (*Saccharomyces carlsbergensis*) was
404 tested, a minimum level of 5.8% RNA was achieved, limited by the low endogenous RNases present in
405 this yeast material. These strategies, although sometimes efficient in the reduction of RNA, employ toxic
406 compounds and conditions that are not adapted for the production of bioactive peptides. Indeed, the
407 molecular structure of such peptides should not be damaged in view to preserve all their bioactive
potential.

408 Fractions obtained by the fractionation process proposed in this work could be used for many applications.
409 For instance, if the interest lies in an ingredient enriched in very small peptides (<1 kg mol⁻¹) and the
presence
410 of salts is not a limitation, the permeate 3 could be considered. The retentate of the T8 membrane is also
411 interesting as it contains a peptide composition that is within the typical range of bioactive peptides
(around
412 5 kDa and smaller) [55], with 30% of peptides between 1 and 7 kg mol⁻¹ and about 65% of peptides smaller
413 than 1 kg mol⁻¹. This fraction is highly pure regarding both RNA and sugars, and could also be an option 414
for the pharmaceutical or food industries.

415 4. Conclusions

416 Peptide fractions from SBY protein hydrolysate were produced from a brewing by-product through a
417 green process using biocatalysis and membrane fractionation. Membrane performance and selectivity
results

418 suggest that differences in the performance of 15 and 50 kDa MWCO membranes affected the fractionation
419 of subsequent stages. These membranes presented a profile compatible with cake fouling whereas smaller
420 MWCO membranes presented internal pore clogging as well. The MWCO chosen between 15 and 1 kDa
421 seemed to be adapted to the SBY hydrolysate molecules size range. Our results have shown that the 15
422 kDa MWCO membrane could efficiently retain higher MW compounds, increasing performance of the next
423 separation steps. Following UF using 8 and 1 kDa MWCO were important to fractionate protein
424 hydrolysate. The designed process was able to increase protein purity regarding RNA and total sugars up
425 to 1.7 and 2.7 fold, thus improving fractions quality. Fractions with higher protein purity and different
426 MW peptide range were obtained and may be exploited as new peptide-rich ingredients for the food and
427 pharmaceutical industries. Bioactive and functional potentials of these peptides are of interest to add value
428 to SBY protein fractions, and should be evaluated. Nevertheless, and although the results obtained are
429 promising, further experiments will be needed to study the effect of transmembrane pressure, tangential
430 flow velocity, feed concentration, diafiltration conditions, etc.) in order to optimise the fractionation process
431 and the purity of peptide fractions.

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442 None.

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