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# Reduction in flavor-intense components in fish protein hydrolysates by membrane filtration

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**Abstract:** Enzymatic protein hydrolysates based on side stream materials from the fish-filleting industry are increasingly explored as food ingredients. However, intense sensory properties, and high salt contents, are often a limiting factor. Most of the sensory attributes, such as fish flavor and salty taste, can be ascribed to low-molecular-weight, water-soluble components, whereas bitterness is associated with small hydrophobic peptides. In this study, protein hydrolysates based on head and backbone residuals from Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) were produced using two different enzymes. The effects of micro- and nanofiltration on the chemical composition, protein recovery, and sensory properties of the final products were investigated. The choice of raw material and enzyme had negligible effects, whereas nanofiltration caused a considerable reduction in metabolites, ash, and the intensity of several sensory attributes. The intensity of bitterness increased after nanofiltration, indicating that small peptides associated with bitter taste were retained by the membrane. Total protein yield after microfiltration was 24%–29%, whereas 19%–24% were recovered in the nanofiltration retentate.

**Practical Application:** Enzymatic protein hydrolysates can be included in food products to increase the protein content, and as a nutritional supplement and/or functional ingredient; however, unpalatable and intense flavors limit applications. This study investigated the use of membrane filtration to improve flavor quality and reduce salt content in fish protein hydrolysates. Although some protein loss is unavoidable in micro- and nanofiltration, this study demonstrates the production of fish protein hydrolysates with >90% protein and peptide content, which is suitable for inclusion in foods.

## 1 | INTRODUCTION

Enzymatic protein hydrolysis is a promising approach for valorization of side stream materials, such as heads and backbones, from the fish filleting industries (Aspevik et al., 2017). Fish protein hydrolysates are rich in essential amino acids and may have beneficial functional and bioactive properties, such as antioxidative or antihypertensive activity, making them valuable food additives (Liaset & Espe, 2008; Zamora-Sillero et al., 2018). After the primary separation of solids and fat from the crude hydrolysate, further refining is necessary to obtain a palatable product. This includes removal of fine particles, traces of fat, salt, bitter

tasting peptides, and small molecular volatiles that cause a stale and unpleasant fishy flavor.

Peptides, free amino acids, minerals, and other water-soluble molecules and metabolites contribute to the overall sensory profile of a protein hydrolysate (Aspevik et al., 2021; Steinsholm et al., 2020). Bitter taste is a major challenge in the production of protein hydrolysates and is associated with the formation of small peptides containing hydrophobic amino acids (Kim & Li-Chan, 2006). Bitter peptide formation is mostly dependent on enzyme specificity and degree of hydrolysis, whereas other flavor-contributing compounds can be associated with raw material–specific components. Different fish species, such as cod and salmon, have several small water-soluble metabolites in common, but at varying levels, resulting in hydrolysates with different sensory profiles (Steinsholm et al., 2020). Trimethylamine oxide, an osmolyte found in mollusks, crustaceans, and fish, can be degraded to dimethylamine (DMA) and trimethylamine (TMA) by enzymatic activity (Wu & Bechtel, 2008). High levels of DMA and TMA are associated with an unpleasant "fishy" flavor of stale fish–based products. Furthermore, marine side stream materials are high in polyunsaturated fatty acids that are susceptible to oxidation (Halldorsdottir et al., 2014), which may cause rancid flavor in the final product (Ladikos & Lougovois, 1990).

A variety of approaches have been suggested to reduce and remove taste and flavor from marine protein hydrolysates. The focus is often on debittering by masking or removal of small hydrophobic peptides (Fu et al., 2019; Idowu & Benjakul, 2019). The formation of bitter taste may also be significantly reduced by proper choice of enzyme and processing conditions (Aspevik et al., 2016). However, these approaches do not provide a reduction in other flavor-contributing compounds, so further refining is necessary to obtain a taste-neutral hydrolysate.

Membrane filtration enables fractionation of compounds based on molecular size (Castro-Munoz et al., 2020). Microfiltration (MF) can remove suspended fine particles, emulsified fat, and microorganisms, and gives a typically light colored and clear permeate of soluble compounds. Nanofiltration (NF) membranes will retain most of the peptides while letting monovalent ions and small organic molecules (which may contribute to unpleasant flavors) through, potentially improving the overall sensory profile of the retentate. NF also removes water, reducing downstream processing costs associated with dewatering and/or drying of the hydrolysate (Petrova et al., 2018). Diafiltration techniques (i.e., dilution of the retentate by addition of water) can be applied in both MF and NF to either improve process yield or product purity. However, diafiltration adds to the processing cost and need to be evaluated relative to effects on yield and product properties.

Several studies have explored crossflow membrane filtration of hydrolysates based on side stream products from agro-industrial sources (Beaulieu et al., 2009a, 2009b; Castro-Munoz et al., 2020; Picot et al., 2010; Vandanjon et al., 2007). However, the focus is often limited to recovery of bioactive molecules, and to our knowledge, there are no studies assessing the effects of NF on compositional and sensory properties of fish protein hydrolysates. In this study, we aimed to (1) assess protein recovery (PR) and chemical composition after MF and NF of fish protein hydrolysates based on Atlantic cod and Atlantic salmon, (2) elucidate the effects of NF and diafiltration on sensory profiles and metabolite composition of protein hydrolysates based on different raw materials and enzymes, and (3) evaluate whether product variation, due to the choice of raw material and enzyme, influences membrane filtration performance.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

Heads and backbones of Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) were kindly provided by Sotra Fiskeindustri AS (Øygarden, Norway) and Hopen Fisk AS (Kabelvåg, Norway), respectively. The salmon was farmed on the south-west coast of Norway and filleted on slaughter day. The cod was caught by seine outside Lofoten (Norway) and delivered to the fish-filleting facility the same day. The salmon raw material was collected immediately after filleting, transported on ice, milled on a Comitrol 1700 industrial mill (Urschel laboratories, Chesterton, IN) the same day, and stored at −23°C until processing (<1 week). The cod residuals were frozen after filleting, shipped, and stored at −23°C until processing (<1 week). The cod raw material was thawed overnight and milled on the Comitrol 1700 immediately prior to hydrolysis. Bromelain BR1200 (EC 3.4.22.32) protease was provided by Enzybel (Waterloo, Belgium) and FoodPro PNL (E.C. 3.4.24.28) was provided by DuPont (Wilmington, DE, USA). Peptide standards were purchased from Sigma Aldrich (Oslo, Norway) except lysozyme (Fluka biochemicals, Buchs, Switzerland) and Alberta standards (Alberta Peptide Institute, Department of Biochemistry, University of Alberta, Edmonton, AB, Canada). All chemicals used were of analytical grade.

### 2.2 | Chemical analyses

Analysis of nitrogen (N) was performed according to the Kjeldahl method (ISO Official Method 5983-2 [ISO, 2009])

and crude protein was estimated by multiplying by a factor of 5.2 and 5.3 for salmon and cod, respectively (Steinsholm et al., 2020). Ash was determined by sample combustion at 550°C (ISO Official Method 5983-2 [ISO, 2002]). Fat content was analyzed by the Bligh and Dyer method (Bligh & Dyer, 1959). Dry matter (DM) was determined by drying at 103°C (ISO Official Method 6496-2 [ISO, 1999]). Sodium content was measured by ICP-OES (ISO-11885). Estimation of molecular weight distribution (MWD) was performed by size exclusion chromatography (1260 series HPLC Agilent Technologies, Santa Clara, CA, USA) as described by Oterhals and Samuelsen (2015).

## 2.3 | Enzymatic protein hydrolysis

Downstream processing was performed at Nofima pilot-scale facilities in Bergen, Norway. Raw material and water were added 1:1 (w:w) to a temperature controlled 200-L reactor with continuous stirring at 80–90 rpm. The temperature was raised to 50°C. Enzyme was added to achieve equal enzyme activity level among the different hydrolysis processes (10 U/g protein) (Steinsholm et al., 2020), and the hydrolysis was run at 50°C for 50 min. The reaction was terminated by raising the temperature to 90°C for approximately 15 min. Coarse bone fragments were removed with a 2-mm sieve. The crude salmon hydrolysates were separated on a three-phase decanter centrifuge (Z23-3; Flottweg, Vilsbiburg, Germany) to remove oil and sediment, and all hydrolysates were filtered on a Jesma VS 20/65 Roto-Fluid sieve (Jesma, Velje, Denmark) with a 10-μm sieve net before membrane filtration.

## 2.4 | Membrane filtration

The aqueous phase was purified by crossflow MF using a 0.1-μm ceramic filter apparatus (MT Separation, Flekkefjord, Norway), to remove remaining suspended solids and lipids. The MF permeate was further refined by NF using a 200-Da spiral membrane (MT separation, Flekkefjord, Norway). The retentate was concentrated to the dead volume of the apparatus (approximately 20 L). Diafiltration was then performed in two steps by the addition of 2 × 30 kg of tap water. A simplified process flow diagram is given in Figure 1. PR was calculated for the MF and NF steps to evaluate the retention of peptides in the filtration processes:

$$\text{PR}\ (\%) = \frac{\text{Total protein in product}\ (g)}{\text{Total protein in raw material}\ (g)} \times 100. \quad (1)$$

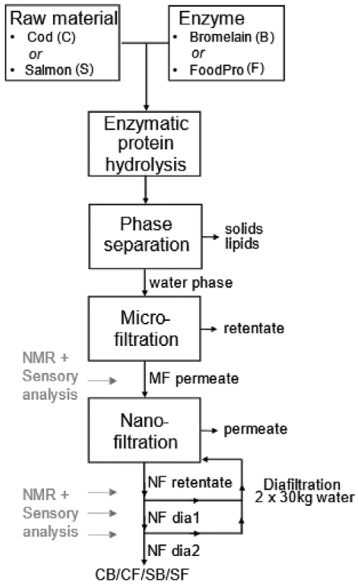


**FIGURE 1** Simplified process flow diagram of the hydrolysis and membrane filtration steps

## 2.5 | Sensory analysis

The hydrolysates were diluted to 2% DM with tap water and assessed by a sensory panel of 10 assessors at Nofima (Ås, Norway). The assessors are regularly trained and tested in accordance with ISO 8586, which includes continuous training with references of the basic tastes (Table 1), in addition to other attribute references where applicable. The panel is also regularly provided with a wide range of hydrolysates to obtain consensus on hydrolysate sensory attributes. A generic descriptive analysis was performed as described by Lawless and Heymann (2010) and in accordance with ISO Official Method 13299 (ISO, 2016). The evaluated attributes, typical for the hydrolysate samples to be tested, and their descriptions are listed in Table 1. The assessors were calibrated on samples that were considered the most different in a pretest session. Samples were served in plastic glasses (20 ml) with a lid at room temperature (18 ± 2°C) under green light, coded with a three-digit number in a full balanced design (ISO Official Method 8589 [ISO, 2007]). The attributes were evaluated on an unstructured 15-cm line scale with labeled end points from *no intensity* (1) to *high intensity* (9) and registered in a computer system for direct recording of data (EyeQuestion, Software Logic8 BV, Utrecht, the Netherlands). Tap water and unsalted crackers were available for palate cleansing during the assessment.

## 2.6 | NMR spectroscopy

1D $^{1}$H spectra with solvent suppression (Bruker, noesygppr1d pulse program) were acquired of hydrolysates

**TABLE 1** Sensory attributes and descriptions used in the sensory evaluation of cod and salmon protein hydrolysates by the sensory panel

| Attribute | Definition |
|---|---|
| Total intensity | The intensity of all different flavors present in the sample |
| Sweet taste | Describes the basic taste produced by diluted aqueous solutions of various substances such as sucrose |
| Salty taste | Describes the basic taste produced by diluted aqueous solutions of various substances such as sodium chloride |
| Acidic taste | Describes the basic taste produced by diluted aqueous solutions of most acid substances (i.e., citric acid) |
| Bitter taste | Describes the basic taste produced by diluted aqueous solutions of various substances such as quinine and caffeine |
| Umami taste | Describes the basic taste of umami |
| Metallic flavor | Related to aroma and taste of iron sulphate ($FeSO_4$) |
| Marine flavor | Related to the aroma of fresh, salty sea |
| Fish flavor | Related to the aroma and taste of fresh fish |
| TMA flavor | The taste of TMA, ammonia, and a sharp sensation |
| Rancid flavor | The intensity of all rancid flavors (gras, hay, stearin, paint) |
| Flavorless | Describes the perception of taste neutrality in a product |
| Fullness (mouthfeel) | Mechanical texture property related to the flow resistance, a rich sensation of the same sample in the mouth |
| Astringent (mouthfeel) | A complex feeling, followed by contractions, dryness sensation, puckering of the skin or mucus membranes in the mouth |
| Fatness (mouthfeel) | Surface textural property related to perception of fat in a product |

*Note*: Descriptions are based on the Norwegian standard NS-ISO 5492.

diluted to 1% DM with 100 mM sodium phosphate buffer (pH 6.8) containing 10% $D_2O$ and 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Acquisitions were made at 300 K with four dummy scans, 32 real scans, 4-s relaxation delay, 25-Hz pre-saturation field strength, 96k time-domain points, and a spectral width of 29.8 ppm using a Bruker AVANCE NEO ultrashielded 600 MHz spectrometer equipped with a QCI cryoprobe (Karlsruhe, Germany). Dimethyl sulfone (10.16 mM; $DMSO_2$, TraceCert(R), Sigma Aldrich) was used as an internal quantification reference. The NMR spectra were processed using TopSpin (v. 4.0.7, Bruker BioSpin, Karlsruhe, Germany). The free induction decay (FID) was zero filled to 128k points and an exponential line broadening of 0.3 Hz was applied before Fourier transformation. Quantification was achieved by deconvolution of peaks for area determination using MestReNova (v. 14.1.2-25024, Santiago de Compostela, Spain). Quantification of the DSS peak was used to verify the data processing. Concentration ($C_{met}$) of metabolites was calculated as:

$$C_{met} = \frac{A_{met}}{A_{ref}} \frac{n_{ref}}{n_{met}} \times C_{ref}, \quad (2)$$

where the subscripts "met" and "ref" denote the metabolite in question and the $DMSO_2$ reference, respectively. $A$ is the total area of peak, $n$ is the number of protons assigned to the peak ($n_{ref} = 6$), and $c$ is the molar concentration ($c_{ref} = 10.16$ mM).

## 2.7 | Statistics

ANOVA of the sensory data was performed using Minitab (v.19.2020.1, Pennsylvania State University, PA, USA). Two-way mixed effects ANOVA modeling was applied to assess differences between sensory attributes for all products, and to evaluate the individual fixed effects of raw material, enzyme, and NF on the sensory attributes. Assessor was set as random variable. Tukey's pairwise comparison was applied where significant ($p \leq 0.05$) differences were found. Principal component analysis (PCA) was performed using Unscrambler (v. 10.4.1, Camo, Oslo, Norway). All variables were unit-variance scaled, centered, and cross validated. Nondetected or nonquantifiable variable values were set to zero.

# 3 | RESULTS AND DISCUSSION

## 3.1 | Overview of hydrolysate variation

The associations between chemical composition (Tables 2–4) and sensory properties (Table 5) of the hydrolysates before (MF permeate) and after NF (NF retentate, before to and after diafiltration) were evaluated using PCA (Figure 2). PC1 and PC2 explained 39% and 34% of the variation, respectively, whereas PC3 (not shown) explained 10%. The PCA loading plot (Figure 2a)

**TABLE 2** Dry matter (DM)[a], protein, ash, and sodium content of hydrolysates based on cod (C) and salmon (S) heads and backbones with FoodPro PNL (F) and Bromelain (B) as an effect of membrane filtration technology

| Product | DM (%) | Protein(g•kg$^{-1}$ DM) | Ash(g•kg$^{-1}$ DM) | Na(g•kg$^{-1}$ DM) |
|---|---|---|---|---|
| CF MF-perm | 3.5 | 867 ± 48 | 104 ± 9 | 26 ± 6 |
| CF NF | 10.8 | 882 ± 17 | 38 ± 4 | 11 ± 2 |
| CF NF-dia1 | 11.5 | 910 ± 1 | 27 ± 3 | 7 ± 1 |
| CF NF-dia2 | 11.6 | 911 ± 1 | 28 ± 5 | 6 ± 1 |
| CB MF-perm | 3.5 | 797 ± 14 | 77 ± 17 | 26 ± 5 |
| CB NF | 12.7 | 900 ± 25 | 32 ± 0 | 9 ± 2 |
| CB NF-dia1 | 13.0 | 917 ± 8 | 25 ± 3 | 6 ± 1 |
| CB NF-dia2 | 12.6 | 912 ± 2 | 22 ± 2 | 5 ± 1 |
| SF MF-perm | 3.8 | 794 ± 13 | 81 ± 0 | 19 ± 4 |
| SF NF | 10.5 | 860 ± 13 | 44 ± 4 | 9 ± 2 |
| SF NF-dia1 | 10.2 | 878 ± 2 | 22 ± 5 | 6 ± 1 |
| SF NF-dia2 | 9.7 | 872 ± 11 | 22 ± 6 | 4 ± 1 |
| SB MF-perm | 4.3 | 779 ± 8 | 84 ± 21 | 18 ± 4 |
| SB NF | 11.7 | 848 ± 2 | 38 ± 1 | 8 ± 2 |
| SB NF-dia1 | 11.4 | 862 ± 13 | 28 ± 1 | 6 ± 1 |
| SB NF-dia2 | 11.0 | 871 ± 18 | 26 ± 3 | 4 ± 1 |

Abbreviations: dia1, diafiltered once; dia2, diafiltered twice; MF-perm, membrane filtration permeate; NF, nanofiltration retentate.
[a]Dry matter (DM) duplicate variation <0.1%.

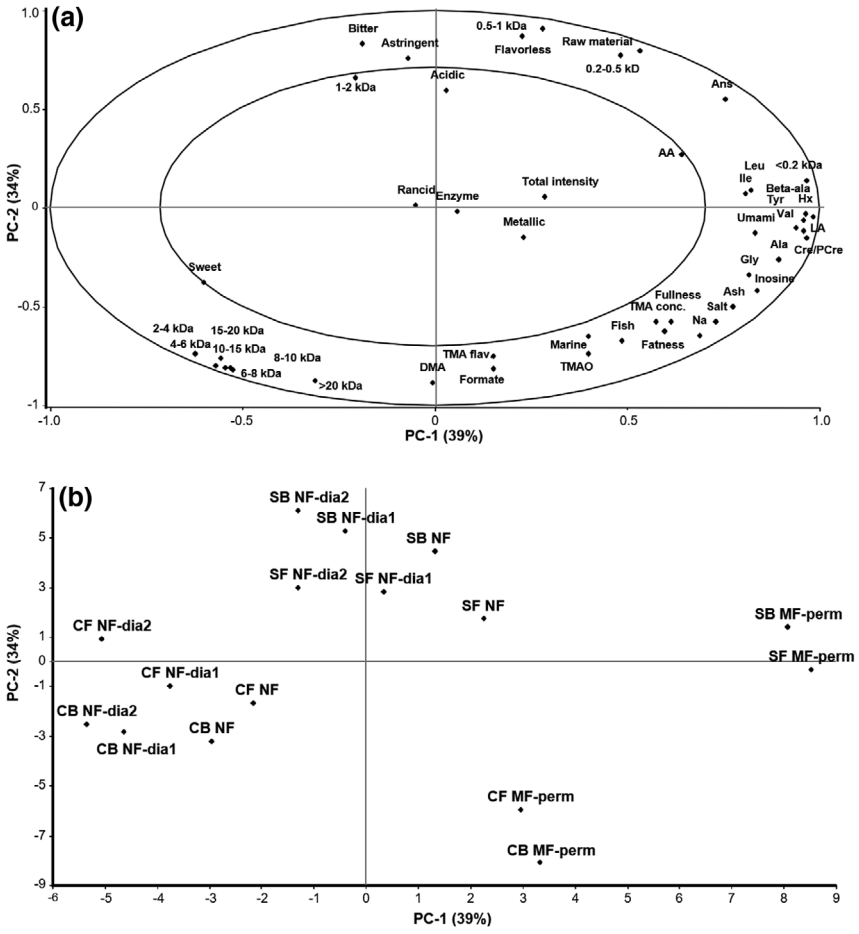


**FIGURE 2** (a) Loading plot and (b) score plot. PCA biplot of data collected from hydrolysates based on salmon (S) and cod (C) heads and backboned with FoodPro PNL (P) and Bromelain (B). AA, acetic acid; Ala, alanine; Ans, anserine; $\beta$-ala, beta-alanine; Cre/PCre, creatine/phosphocreatine; dia1, diafiltered once; dia2, diafiltered twice; DMA, dimethylamine; Gly, glycine; Hx, hypoxanthine; Ile, isoleucine; Ino, inosine; LA, lactic acid; Leu, leucine; MF-perm, microfiltration permeate; Na, sodium; NF, nanofiltered; TMA flav/conc, trimethylamine flavor/concentration; TMAO, trimethylamine-oxide; Tyr, tyrosine; Val, valine

**TABLE 3** Protein mass balance (%) through the main process steps during hydrolysis and separation of hydrolysates based on cod and salmon heads and backbones with the enzymes FoodPro PNL and Bromelain

| | Cod FoodPro PNL | | | Cod Bromelain | | | Salmon FoodPro PNL | | | Salmon Bromelain | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Wet weight (kg) | Protein (kg) | Mass balance (%) | Wet weight (kg) | Protein (kg) | Mass balance (%) | Wet weight (kg) | Protein (kg) | Mass balance (%) | Wet weight (kg) | Protein (kg) | Mass balance (%) |
| Raw material | 79 | 9.6 | 100 | 79 | 9.6 | 100 | 81 | 9.7 | 100 | 79 | 9.4 | 100 |
| Bone fragments | 17 | 2.6 | 27 | 16 | 2.5 | 26 | 7 | 1.2 | 13 | 10 | 1.8 | 19 |
| MF feed | 117 | 5.6 | 58 | 127 | 5.5 | 57 | 107 | 4.6 | 48 | 99 | 4.4 | 46 |
| MF retentate | 34 | 2.7 | 28 | 28 | 2.1 | 22 | 31 | 2.1 | 22 | 24 | 1.9 | 20 |
| MF permeate | 83 | 2.6 | 27 | 99 | 2.8 | 29 | 76 | 2.4 | 24 | 75 | 2.6 | 27 |
| NF retentate[a] | 20 | 1.9 | 20 | 20 | 12.3 | 24 | 20 | 1.8 | 19 | 16 | 2.0 | 21 |

[a]Wet weight (kg) based on mean volume collectable when filtration is run and estimated uncollectable volume.

demonstrated a considerable change in metabolite composition and MWD by NF. PC1 mainly separated the hydrolysates based on filtration parameters, whereas PC2 reflected the raw material variation, with salmon samples in the upper part and cod in the lower part of the plot (Figure 2b). The NF retentates were negatively correlated with ash and high metabolite concentration, reflecting that a large proportion of these molecules was removed in the NF process (Figure 2). Furthermore, many of the sensory attributes were negatively correlated with the NF retentates confirming that NF can be used to reduce hydrolysate sensory intensity. Of the attributes outside the 50-percentile line in the loading plot (Figure 2a), only bitterness and astringency were positively associated with the NF retentates, indicating that bitter-tasting peptides were retained by the NF membrane. Bitterness correlated with peptides of 0.5–1 kDa ($R^2 = 0.77$, $p < 0.001$) and 1–2 kDa ($R^2 = 0.56$, $p = 0.025$), and astringency ($R^2 = 0.89$, $p < 0.001$), in agreement with previous studies and the general consensus of peptide size and bitterness causation (Aspevik et al., 2016; Fu et al., 2019; Kim & Li-Chan, 2006; Steinsholm et al., 2020). There was a clear effect of diafiltration within each raw material/NF group, displacing the samples leftward on PC1 after a first and second diafiltration step. However, the score plot groupings were conserved (Figure 2b), confirming that the removal of flavor-contributing metabolites did not override the primary effects of raw material and enzyme.

## 3.2 | Raw material, mass balance, and PR

The cod and salmon raw materials had similar protein levels (Table 6), whereas the higher DM level in the latter raw material is an effect of the lipid content. The elevated ash levels in the cod raw material reflect the difference in bone content between the raw materials. After protein hydrolysis, MF, NF, and diafiltration led to an increase in protein on DM basis due to removal of nonprotein DM over the NF membrane (Table 2). A protein level >90% was only obtained for the cod hydrolysates (Table 2), being an effect of the larger MF permeate volumes due to varying water contents in the raw material. DM in the NF retentates reflects net removal of water in the process. A considerable decrease in ash from MF to NF was observed, in agreement with other studies applying NF membranes (Beaulieu et al., 2009a; Picot et al., 2010; Vandanjon et al., 2007). A further reduction of ash content was obtained by diafiltration, although to a lesser extent due to the low dilution ratio (1.5) compared to the concentration factor of 4–5 in the initial NF step. The higher level of reduction in the first diafiltration step compared to the second likely reflects the difference in ash and sodium contents. A sub-

**TABLE 4** Molecular weight distribution of protein hydrolysates from Cod (C) and Salmon (S) heads and backbones with FoodPro PNL (F) and Bromelain (B)

| Product | >10 | 8–10 | 6–8 | 4–6 | 2–4 | 1–2 | 0.5–1 | 0.2–0.5 | <0.2 |
|---|---|---|---|---|---|---|---|---|---|
| CF MF-perm | 1.0 | 1.1 | 3.5 | 10.5 | 23.9 | 20.6 | 15.2 | 12.7 | 11.4 |
| CF NF | 1.0 | 1.2 | 3.7 | 11.2 | 25.6 | 22.0 | 15.9 | 12.1 | 7.2 |
| CF NF-dia1 | 1.0 | 1.2 | 3.7 | 11.4 | 26.2 | 22.4 | 16.1 | 11.9 | 6.1 |
| CF NF-dia2 | 1.1 | 1.2 | 3.7 | 11.4 | 26.4 | 22.6 | 16.2 | 11.8 | 5.7 |
| CF NF-perm | 0.0 | 0.0 | 0.0 | 0.1 | 0.3 | 1.7 | 7.9 | 26.4 | 63.6 |
| CB MF-perm | 1.3 | 1.5 | 4.3 | 12.4 | 29.8 | 22.6 | 11.5 | 5.3 | 11.2 |
| CB NF | 1.1 | 1.5 | 4.6 | 13.1 | 31.9 | 25.0 | 12.8 | 5.5 | 4.4 |
| CB NF-dia1 | 1.4 | 1.7 | 4.9 | 13.7 | 32.7 | 24.7 | 12.3 | 5.0 | 3.7 |
| CB NF-dia2 | 1.4 | 1.7 | 4.9 | 13.9 | 33.0 | 24.9 | 12.2 | 4.8 | 3.2 |
| CB NF-perm | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 2.1 | 5.3 | 11.2 | 81.0 |
| SF MF-perm | 0.4 | 0.6 | 1.8 | 5.2 | 17.1 | 20.2 | 17.3 | 16.2 | 21.2 |
| SF NF | 0.5 | 0.7 | 2.0 | 6.1 | 19.7 | 23.1 | 19.4 | 16.2 | 12.3 |
| SF NF-dia1 | 0.5 | 0.7 | 2.1 | 6.3 | 20.6 | 24.1 | 20.0 | 15.8 | 9.7 |
| SF NF-dia2 | 0.5 | 0.7 | 2.2 | 6.4 | 21.0 | 24.7 | 20.5 | 15.7 | 8.2 |
| SF NF-perm | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.8 | 4.2 | 23.5 | 71.4 |
| SB MF-perm | 0.2 | 0.1 | 0.6 | 2.7 | 15.2 | 25.3 | 21.7 | 16.2 | 17.9 |
| SB NF | 0.1 | 0.2 | 0.7 | 3.1 | 17.4 | 28.7 | 24.3 | 16.0 | 9.5 |
| SB NF-dia1 | 0.1 | 0.1 | 0.6 | 2.8 | 16.1 | 28.1 | 25.3 | 16.9 | 10.0 |
| SB NF-dia2 | 0.1 | 0.1 | 0.6 | 2.9 | 16.2 | 28.3 | 25.5 | 16.8 | 9.5 |
| SB NF-perm | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 1.2 | 4.5 | 24.6 | 69.5 |

Abbreviations: dia1, diafiltered once; dia2, diafiltered twice; MF-perm, membrane filtration permeate; NF, nanofiltration retentate; NF-perm, nanofiltration permeate.

**TABLE 5** Metabolites ($g \cdot kg^{-1}$ dry matter) present in protein hydrolysates of heads and backbones from cod (C) and salmon (S) hydrolyzed with FoodPro PNL (P) and Bromelain (B) as determined by NMR spectroscopy

| Product | Leu | Ile | Val | Tyr | Ala | Gly | DMA | TMA | TMAO | LA | AA | Cre | Ans | β-ala | Hx | Ino | FA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CF MF-perm | NQ | 2.1 | 2.7 | 1.1 | 5.4 | 2.1 | 3.6 | 0.7 | 15.6 | 22.1 | 0.4 | 17.4 | ND | 3.1 | 2.2 | 4.4 | 0.5 |
| CF NF | NQ | 3.5 | 1.7 | 0.6 | 2.3 | 0.7 | 1.7 | 0.3 | 6.0 | 9.2 | 0.1 | 6.5 | ND | 1.5 | 1.0 | 2.6 | 0.2 |
| CF NF-dia1 | NQ | NQ | NQ | NQ | 1.3 | 0.4 | 1.1 | 0.2 | 3.0 | 4.8 | 0.1 | 3.5 | ND | NQ | 0.5 | 1.6 | 0.1 |
| CF NF-dia2 | NQ | NQ | NQ | NQ | 0.9 | NQ | 0.9 | 0.1 | 1.7 | 3.0 | ND | 1.8 | ND | NQ | 0.2 | 0.9 | ND |
| CB MF-perm | 7.5 | 3.3 | 4.4 | 2.7 | 11.8 | 5.9 | 5.0 | 0.4 | 11.6 | 21.2 | 0.4 | 17.2 | ND | 3.2 | 2.3 | 3.5 | 0.6 |
| CB NF | 2.4 | 1.3 | 1.3 | 0.9 | 3.2 | 1.5 | 1.9 | 0.1 | 3.1 | 6.2 | ND | 5.3 | ND | 1.4 | 0.9 | 1.5 | 0.2 |
| CB NF-dia1 | NQ | 1.0 | 0.7 | 0.4 | 1.5 | 0.6 | 1.4 | 0.1 | 1.3 | 2.6 | ND | 2.5 | ND | 1.0 | 0.4 | 0.9 | 0.1 |
| CB NF-dia2 | ND | NQ | 0.4 | NQ | 0.7 | 0.3 | 1.1 | 0.1 | 0.6 | 1.2 | ND | 1.0 | ND | NQ | 0.1 | 0.2 | 0.0 |
| SF MF-perm | 15.4 | 7.8 | 7.9 | 3.6 | 11.3 | 3.7 | 0.1 | 0.4 | 4.4 | 42.9 | 1.1 | 30.2 | 30.0 | 6.1 | 5.0 | 5.4 | ND |
| SF NF | NQ | 6.2 | 4.4 | 1.6 | 4.9 | 1.5 | 0.1 | 0.3 | 1.6 | 18.5 | 2.2 | 12.4 | 18.0 | 2.8 | 2.7 | 1.7 | ND |
| SF NF-dia1 | 4.4 | 5.8 | 3.2 | 1.0 | 2.4 | 0.8 | 0.1 | 0.1 | 0.8 | 10.3 | 0.7 | 7.4 | 15.9 | 1.9 | 1.3 | 1.3 | ND |
| SF NF-dia2 | NQ | 1.4 | 1.0 | 0.5 | 1.1 | 0.5 | 0.1 | 0.1 | 0.4 | 3.7 | 0.4 | 3.2 | 9.8 | 1.0 | 0.6 | 0.6 | ND |
| SB MF-perm | 17.8 | 5.1 | 7.0 | 4.1 | 15.2 | 5.8 | 0.1 | 0.3 | 4.2 | 44.5 | 1.0 | 29.8 | 21.9 | 6.0 | 4.9 | 4.2 | ND |
| SB NF | 9.2 | 2.3 | 2.7 | 1.5 | 5.3 | 2.3 | 0.1 | 0.2 | 1.6 | 15.6 | 0.2 | 12.6 | 16.0 | 2.6 | 2.3 | 1.6 | ND |
| SB NF-dia1 | 4.9 | 1.7 | 1.5 | 0.9 | 2.4 | 1.0 | 0.1 | 0.1 | 0.8 | 8.3 | 0.7 | 7.2 | 12.3 | 1.7 | 1.2 | 1.1 | ND |
| SB NF-dia2 | NQ | 1.4 | 1.0 | 0.5 | 1.1 | 0.5 | 0.1 | 0.1 | 0.4 | 3.7 | 0.4 | 3.2 | 9.8 | 1.0 | 0.6 | 0.6 | ND |

Abbreviations: AA, acetic acid; Ala, alanine; Ans, anserine; β-ala, beta-alanine; Cre, creatine/phosphocreatine; dia1, diafiltered once; dia2, diafiltered twice; DMA, dimethylamine; FA, formic acid; Gly, glycine; Hx, hypoxanthine; Ile, isoleucine; Ino, inosine; LA, lactic acid; Leu, leucine; MF, microfiltered; ND, not detected; NF, nanofiltered; NQ, not quantifiable; TMA, trimethylamine; TMAO, trimethylamine-oxide; Tyr, tyrosine; Val, valine.

**TABLE 6** Proximate composition of cod and salmon heads and backbones

| | Protein (%)[a] | DM (%)[b] | Ash (%) | Lipids (%) |
|---|---|---|---|---|
| Cod | 12.2 ± 0.1 | 24.5 ± 1.3 | 8.4 ± 0.2 | 1.0 ± 0.1 |
| Salmon | 12.0 ± 0.3 | 43.8 ± 2.5 | 4.1 ± 1.9 | 21.5 ± 0.4 |

[a]Cod protein N×5.3, salmon protein N×5.2.
[b]Dry matter.

stantial fraction of the remaining ash is likely to be divalent ions retained by the NF membrane (Van der Bruggen et al., 2004).

Total PR after mechanical separation and MF of the hydrolysates was calculated to 19%–24% (Table 3). The highest losses were due to separation of bones (13%–27%), separated solids and residual liquid in the decanter centrifuge (not quantified), and the MF retentate (20%–28%). The discrepancy between PR of the raw material and sum of bone fragments and MF feed in Table 4 is due to losses in the hydrolysis reactor, pumps and pump hoses, sieves, and/or decanter centrifuge. The lower recovery of protein in the MF feed from the salmon trials (Table 3) compared to the cod trials can be attributed to the use of a three-phase decanter centrifuge to remove fat in the former process. A relatively high proportion of bones (containing up to 27% of the total protein) were recovered from the processes, which may have potential for further valorization by extraction of collagen or use as a calcium source (Toppe et al., 2007). Another option is to combine the MF retentate to produce a low-protein, mineral-rich fish meal with possible applications within the feed industry (Toppe et al., 2006; Wei et al., 2017).

Only 24%–29% of the total raw material protein were recovered in the MF permeates, but relative to the MF feed, 46%–59% were recovered (Table 3). The high loss in the MF process and relatively low MF permeate yields underline the need for further optimization of the MF process, either by use of diafiltration and/or membranes with a higher molecular weight cutoff (MWCO). Such trials were, however, outside the scope of this study. Compared to the NF feed (the MF permeate), a PR of approximately 75% was obtained in the NF retentates, reflecting a high level of low-MW compounds in the hydrolysates being lost in the NF process. The PR of the NF retentates, relative to the crude hydrolysates (MF-feed), was approximately 40%, which is higher than that reported by Beaulieu et al. (2009a) (10% DM). There were small changes in protein levels on DM basis by the consecutive NF diafiltration steps. The first round of diafiltration gave a 2%–3% increase in protein, whereas the second round gave even less change (±1%). There is a potential for increased PR in the filtration processes by applying membranes with higher and lower MWCO in the MF and NF operations, respectively.

## 3.3 | Effect of downstream processing on MWD and metabolite composition

MWD of the hydrolysates was evaluated throughout the filtration process (Table 4). All products were mostly composed of peptides <4 kDa, with some variation in distribution depending on raw material and enzyme, in agreement with previous studies (Aspevik et al., 2016; Steinsholm et al., 2020). The main compositional effect of NF on MWD was the considerable decrease of <0.2-kDa molecules after the NF process. This was also evident from the NF permeates that mostly contained molecules <0.2 kDa and, to a lower degree, molecules in the 0.2–0.5 kDa range (Table 4).

The compositional change in hydrolysate metabolites through the filtration sequence was determined using $^{1}$H-NMR. The spectra provide a visual overview of the metabolite composition, and the changes in composition caused by NF and diafiltration (Figure 3). In addition to the removal of free amino acids and small peptides, NF resulted in a reduction in the concentration of small, flavor-contributing metabolites, such as TMA, DMA, organic acids, anserine, and inosine (Table 5). The decline in TMA and DMA was particularly promising for lowering the intensity of fish flavor in the products. Furthermore, it is likely that the concentration of other fish flavor–contributing metabolites, such as volatile alcohols and carbonyls (Josephson & Lindsay, 1986), undetected in the NMR spectra, was also reduced. Simultaneously with the decrease of smaller metabolites, an integral increase of some spectral areas, as for instance, 0.8–0.1, 1.1–2.5, and 3.9–4.6 ppm, could be observed (Figure 3). These areas correspond to an increase in methyl, aliphatic, and H$\alpha$ signals (Rule & Hitchens, 2006) from peptides of various amino acid combinations. This makes specific assignments by NMR impractical but shows an increased concentration of peptides in the retentate by NF and diafiltration. The greatest reduction in metabolite concentrations (two- to threefold) was provided by the first round of NF, after which only a relatively small decrease could be observed in the consecutive diafiltration steps (Table 5). This suggests that diafiltration only gives marginal additional improvement of product quality with the volumes applied in this study. Additional studies on the relationship between concentra-

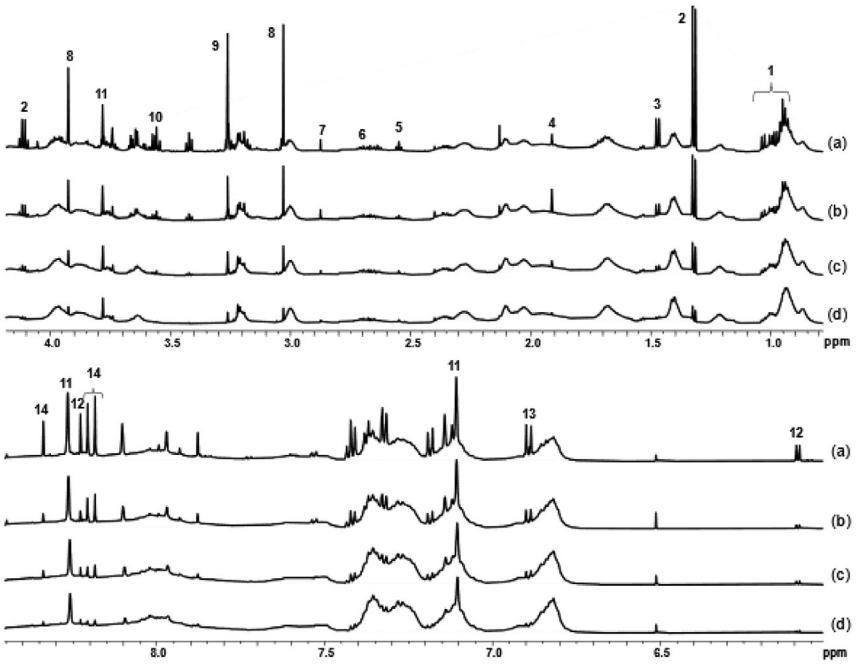


**FIGURE 3** 600 MHz $^1$H NMR spectra of enzymatic protein hydrolysate after microfiltration (a), nanofiltration (b), diafiltered once (c), and diafiltered twice (d), illustrated with samples from the filtration cascade of the salmon FoodPro PNL product. Upfield and downfield regions of the spectra are scaled differently. 1, leucine, valine, isoleucine, and various peptides; 2, lactate; 3, alanine; 4, acetate; 5, beta-alanine; 6, DMA; 7, TMA; 8, phosphocreatine/creatine; 9, TMAO; 10, glycine; 11, anserine; 12, inosine; 13, tyrosine: 14, hypoxanthine

tion factor and retentate composition are needed but were outside the scope of this study.

## 3.4 | Effect of NF and diafiltration on sensory profiles

Descriptive sensory profiles were obtained for all hydrolysates following the downstream processing. Significant product differences ($p \leq 0.05$) were found for all attributes except sweet taste, rancid flavor, and astringency (Table 7). Sweet taste was previously found to be associated with the presence of lactate, alanine, and anserine (Steinsholm et al., 2020); however, the reduction of these compounds in the NF retentates (Table 4) did not influence this attribute's intensity in this study. The low intensity of rancidity indicates negligible lipid oxidation in the raw material. Astringency of protein hydrolysates is poorly understood but has been found to correlate with bitter taste sensation (Aspevik et al., 2016; Steinsholm et al., 2020). The intensity of bitter taste and flavorlessness increased throughout NF processing (Table 7; Figure 4a), confirming an up-concentration of bitter peptides in the NF retentate, in agreement with a typical molecular size of bitter-tasting peptides in the 0.5–2 kDa range (Figure 2; Aspevik et al., 2016). Furthermore, omission of lactate and inosine/IMP from food products has been suggested to enhance bitterness (Schlichtherle-Cerny & Grosch, 1998; Steinsholm et al., 2020), and may add to the increased bitter perception of NF retentates where these metabolites were reduced. The intensity trend of astringency was similar to that of bitterness (Table 7), but was not significantly affected by NF. Except for bitter taste and flavorlessness, the intensity of the remaining, statistically significant attributes decreased when the hydrolysates were nano- and diafiltrated (Table 7).

ANOVA was performed to evaluate the individual effects of filtration, fish species, and enzyme on the sensory attributes (Figure 4). The MF permeates were significantly less bitter than the NF retentates, possibly explained by the reduction of other flavors, intensifying this attribute, and the omission of lactate and inosine/IMP, as discussed above. Furthermore, hydrolysates based on salmon were significantly more bitter as compared with cod (Figure 4b), whereas no significant effects of choice of enzyme on bitterness were observed in this study (Figure 4c). The attributes salt, umami taste, marine, fish, TMA, fullness, and fatness were significantly reduced by NF for all hydrolysates (Figure 4a), reflecting the reduction of small metabolites responsible for these tastes and flavors. The significant reduction in salty taste (Table 7) was likely due to the substantial decrease in sodium and ash (Table 2). No clear pattern of differences was observed in the diafiltrated products most likely due to small changes in metabolite composition. Umami, fullness, and fatness have previously been found to be positively correlated to lactate, alanine, and anserine (Steinsholm et al., 2020), all of which decreased with NF and contribute to explaining the decrease in the attributes' intensity. However, other studies (Aspevik et al., 2016; Fu et al., 2018, 2019) suggest that enzyme and processing conditions are the main determinators for the development of bitter taste, and judicious choice of these parameters must be met to obtain hydrolysates of low bitterness.

**TABLE 7** Mean attribute intensity values[a] in hydrolysates based on cod (C) and salmon (S) heads and backbones hydrolysates with Bromelain (B) and FoodPro PNL (P) as an effect of membrane filtration technology

| Product | Sweet taste | Salty taste | Acidic taste | Bitter taste | Umami taste | Metallic flavor | Marine flavor | Fish flavor | TMA flavor | Rancid flavor | Flavorless | Total intensity | Fullness | Astringency | Fatness |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CF MF-perm | 2.4 | $3.9^{ab}$ | $2.2^{d}$ | $4.7^{b}$ | $3.4^{abc}$ | $4.5^{a}$ | $1.4^{ab}$ | $4.6^{ab}$ | $5.1^{ab}$ | 2.0 | $1.8^{c}$ | $6.2^{ab}$ | $3.2^{ab}$ | 3.7 | $2.5^{abc}$ |
| CF NF | 2.7 | $2.3^{ef}$ | $2.4^{bcd}$ | $5.4^{ab}$ | $2.6^{bc}$ | $4.0^{ab}$ | $1.3^{ab}$ | $3.7^{bcd}$ | $4.5^{abc}$ | 1.6 | $2.1^{bc}$ | $5.7^{ab}$ | $2.9^{abc}$ | 4.0 | $2.2^{bc}$ |
| CF NF-dia1 | 2.6 | $2.1^{ef}$ | $2.5^{bcd}$ | $5.0^{a}$ | $2.7^{bc}$ | $4.0^{ab}$ | $1.35^{ab}$ | $3.9^{abcd}$ | $4.2^{abc}$ | 1.3 | $2.1^{abc}$ | $5.3^{b}$ | $2.7^{bc}$ | 3.7 | $2.2^{bc}$ |
| CF NF-dia2 | 2.5 | $1.9^{f}$ | $2.3^{cd}$ | $5.6^{ab}$ | $2.5^{c}$ | $3.8^{ab}$ | $1.1^{b}$ | $3.1^{d}$ | $3.9^{abc}$ | 2.0 | $2.3^{abc}$ | $5.4^{b}$ | $2.3^{c}$ | 4.2 | $2.0^{c}$ |
| CB MF-perm | 2.7 | $4.1^{a}$ | $2.3^{cd}$ | $4.4^{b}$ | $3.6^{ab}$ | $4.2^{ab}$ | $1.6^{a}$ | $4.5^{ab}$ | $5.2^{a}$ | 1.8 | $1.9^{c}$ | $6.1^{ab}$ | $3.6^{a}$ | 3.5 | $2.8^{a}$ |
| CB NF | 3.0 | $2.5^{def}$ | $2.4^{bcd}$ | $4.5^{b}$ | $3.1^{abc}$ | $3.8^{ab}$ | $1.2^{ab}$ | $4.2^{abcd}$ | $4.6^{abc}$ | 1.4 | $2.2^{abc}$ | $5.4^{b}$ | $3.3^{ab}$ | 3.6 | $2.4^{abc}$ |
| CB NF-dia1 | 3.0 | $2.2^{def}$ | $2.4^{bcd}$ | $5.0^{b}$ | $3.0^{abc}$ | $3.9^{ab}$ | $1.5^{ab}$ | $4.3^{abc}$ | $4.3^{abc}$ | 1.6 | $1.9^{c}$ | $5.8^{ab}$ | $2.8^{bc}$ | 3.7 | $2.1^{bc}$ |
| CB NF-dia2 | 3.0 | $2.3^{def}$ | $2.2^{d}$ | $4.6^{b}$ | $2.7^{bc}$ | $4.0^{ab}$ | $1.5^{ab}$ | $4.0^{abcd}$ | $4.2^{abc}$ | 1.5 | $2.5^{abc}$ | $5.2^{b}$ | $3.0^{abc}$ | 3.6 | $2.0^{bc}$ |
| SF MF-perm | 2.3 | $3.2^{bcd}$ | $2.4^{bcd}$ | $4.8^{b}$ | $4.0^{a}$ | $4.2^{ab}$ | $1.4^{ab}$ | $4.9^{a}$ | $4.4^{abc}$ | 1.5 | $2.5^{abc}$ | $5.8^{ab}$ | $3.4^{ab}$ | 4.0 | $2.5^{ab}$ |
| SF NF | 2.9 | $2.8^{cde}$ | $3.1^{ab}$ | $5.2^{ab}$ | $3.4^{abc}$ | $4.2^{ab}$ | $1.3^{ab}$ | $3.7^{bcd}$ | $4.9^{abc}$ | 2.1 | $3.1^{abc}$ | $6.2^{ab}$ | $3.1^{abc}$ | 3.6 | $2.1^{bc}$ |
| SF NF-dia1 | 2.6 | $2.7^{cdef}$ | $3.3^{a}$ | $5.3^{ab}$ | $3.4^{abc}$ | $4.1^{ab}$ | $1.3^{ab}$ | $3.5^{bcd}$ | $4.1^{abc}$ | 1.8 | $3.1^{abc}$ | $5.8^{ab}$ | $3.2^{ab}$ | 3.9 | $2.3^{abc}$ |
| SF NF-dia2 | 2.6 | $2.5^{cdef}$ | $3.0^{abc}$ | $5.5^{ab}$ | $3.4^{abc}$ | $4.5^{ab}$ | $1.3^{ab}$ | $4.0^{abcd}$ | $4.2^{abc}$ | 2.0 | $3.4^{a}$ | $6.0^{ab}$ | $2.9^{abc}$ | 4.1 | $2.3^{abc}$ |
| SB MF-perm | 2.4 | $3.4^{bcd}$ | $2.4^{bcd}$ | $5.0^{b}$ | $3.4^{abc}$ | $3.8^{ab}$ | $1.5^{ab}$ | $4.2^{abcd}$ | $3.8^{abc}$ | 1.3 | $3.1^{abc}$ | $5.4^{ab}$ | $3.2^{ab}$ | 3.9 | $2.4^{abc}$ |
| SB NF | 2.4 | $2.3^{ef}$ | $2.4^{bcd}$ | $5.6^{ab}$ | $3.4^{abc}$ | $3.9^{ab}$ | $1.3^{ab}$ | $3.6^{bcd}$ | $3.7^{abc}$ | 1.4 | $3.2^{ab}$ | $5.6^{ab}$ | $2.8^{bc}$ | 4.0 | $2.1^{bc}$ |
| SB NF-dia1 | 2.5 | $2.2^{ef}$ | $2.5^{abcd}$ | $5.5^{ab}$ | $3.0^{abc}$ | $3.7^{b}$ | $1.2^{ab}$ | $3.5^{bcd}$ | $3.6^{abc}$ | 1.3 | $3.1^{abc}$ | $5.5^{ab}$ | $2.7^{bc}$ | 4.1 | $2.0^{bc}$ |
| SB NF-dia2 | 2.4 | $2.2^{ef}$ | $3.0^{abcd}$ | $6.5^{a}$ | $2.7^{bc}$ | $4.4^{ab}$ | $1.2^{ab}$ | $3.3^{cd}$ | $4.2^{abc}$ | 2.2 | $3.2^{ab}$ | $6.6^{a}$ | $2.6^{bc}$ | 4.5 | $2.1^{bc}$ |
| *p* (product) | 0.133 | <0.001 | <0.001 | <0.001 | <0.001 | 0.004 | 0.024 | <0.001 | <0.001 | 0.082 | <0.001 | 0.001 | <0.001 | 0.263 | <0.001 |

*Note*: Different letters within each column indicates significant ($p < 0.05$) products differences as determined by Tukey's post hoc test.

Abbreviations: dia1, diafiltered once; dia2, diafiltered twice; MF, microfiltered; NF, nanofiltered.

[a]Mean values based on duplicate sample evaluation by eight panelists on a 15-cm unstructured line ranging from 1 (*no intensity*) to 9 (*high intensity*).

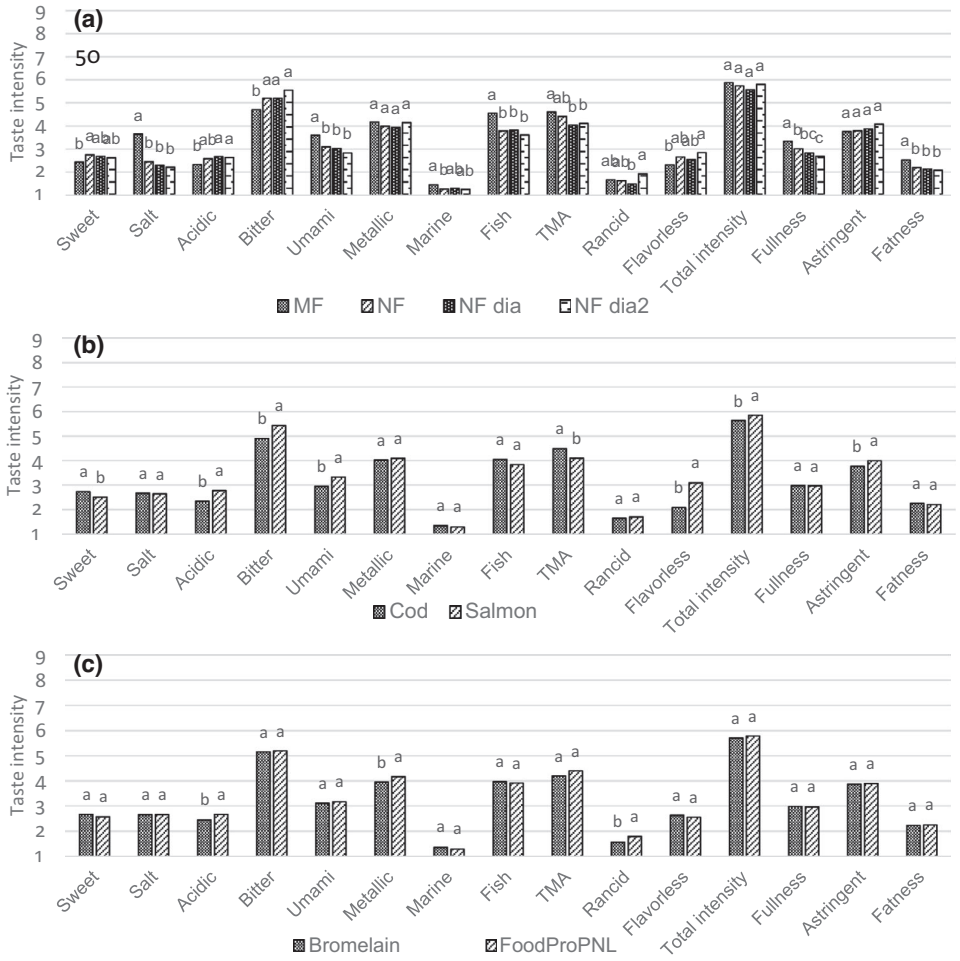


**FIGURE 4** Significance of filtration parameters (a), raw material (b), and enzyme (c) on the development of sensory attributes based on ANOVA. Different letters for each parameter within each attribute indicate significant differences ($p < 0.05$) based on Tukey's post hoc test.

Two-factor interactions (data not shown) were included in the mixed-model ANOVA model. A significant ($p < 0.05$) interaction between raw material and filtration process was found for salty taste and metallic and TMA flavor, total intensity, and fatness. The salty taste was the highest in the cod MF permeates (Table 7), explained by the higher sodium content of these products (Table 2). The difference in salty taste of the four products that were diafiltered twice (NF dia2) was not significant, indicating that the initial sodium concentration in hydrolysates may be of negligible importance for the final perception of the attribute. For the attributes fatness, TMA, and metallic the raw material and filtration interaction effects showed that the cod hydrolysates changed from having the highest to the lowest attribute intensity compared to the salmon products. Again, as with salty taste, there were no significant differences between products of the same degree of filtration (i.e., NF and diafiltration; Table 2), suggesting that the effect of NF was not product dependent.

## 4 | CONCLUSION

NF of fish protein hydrolysates significantly decreased concentration of flavor-contributing metabolites, leading to lower intensity of the majority of tested sensory attributes. However, the perception of bitterness increased in the NF retentates due to the retention of small peptides, with MW in the range of 0.5–2 kDa, associated with bitter taste. This suggests that NF is a promising tool in the flavor reduction of protein hydrolysates. Bitter taste, however, remains a challenge. No significant effect of diafiltration on the sensory profile was observed by the initial four- to five-fold concentration by NF, and there was no obvious effect of product variation (i.e., different raw material and enzymes) on membrane performance. PR after NF was only 19%–24%, with the highest protein losses ascribed to removal of bones and solids in the crude hydrolysate and the MF step. Additional research should be performed to improve protein yield

throughout the downstream processing, particularly after microfiltration.

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## AUTHOR CONTRIBUTIONS

T. Aspevik, Å. Oterhals, L. Thoresen, and S. Steinsholm conceptualized the study. T. Aspevik, Å. Oterhals, J. Underhaug, and S. Steinsholm helped in methodology. Å. Oterhals and K. Kousoulaki acquired funding. S. Steinsholm helped in formal analysis and visualized the study. Å. Oterhals, S. Steinsholm, and K. Kousoulaki provided resources. L. Thoresen and S. Steinsholm performed the experiments. T. Aspevik, Å. Oterhals, and J. Underhaug supervised the study. T. Aspevik administered the project. S. Steinsholm wrote the original draft. T. Aspevik, Å. Oterhals, L. Thoresen, J. Underhaug, S. Steinsholm, and K. Kousoulaki reviewed and edited the manuscript.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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