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Reduction of proteins and products of their hydrolysis in process of cleaning post-production herring (*Clupea harengus*) marinating brines by using membranes

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Abstract. The molecular weight of proteins and protein hydrolysis products (PHP) in the fractionated post-production marinating brines left after herring marination process was determined by the HPLC. The proteins and PHP retention was evaluated in the three-stage purification process with the usage of polypropylene bag (25 μm) and ceramic membranes with the cut-off of 150 and 1 kDa. It was found that the process of marination contributes to high participation of compounds in the post-production marinating brines. Those are characterised by low molecular weight, formed as a result of protein hydrolysis. Each stage of the scavenging process was reducing the content of proteins and PHP. The lowest retention was observed in the stage at which a PP bag was used, while the highest in the UF process, with the usage of 150 kDa membrane. The total retention of proteins and PHP differed according to the type of post-production marinating brines and reached the level of 16-22%.

Keywords: herring (Clupea harengus); ceramic membrane; ultrafiltration

1. Introduction

Salting and marinating herrings (*Clupea harengus*) provides the examples of technological processes deeply rooted in the tradition of Northern Europe (Osman *et al*. 2015). The marinating solutions put in use contain, among many, acetic acid, that reduces the pH, and brines that are characterised by a high concentration of NaC1. The existence of the abovementioned components influences the change in the fish meat structure and causes activation of endo- and exopeptidase class enzymes and muscle cathepsin (Christensen *et al*. 2011, Gringer *et al*. 2014, Taheri *et al*. 2014). The biochemical transformations contribute, among others, to the protein hydrolysis and transition into organic compounds solutions that burden the sewage. Those process waters are difficult to purify and therefore, some alternative methods for its disposal are being searched for. The solution could be used to treat processed waters with the usage of membrane separation and reuse the treated medium in technological processes (Ripperger and Grein 2007, Kuca and Szaniawska 2009, Gringer *et al*. 2014, Drost *et al*. 2014). According to e.g., Afonso *et al*. (2004),

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Amado *et al*. (2013), Benhabiles *et al*. (2013) or Dumay *et al*. (2008), while processing shrimps, sepia, sardines or fish flour the process of ultrafiltration is able to recover from the processed waters 60-97% of proteins and 90% of lipids.. For instance, the water used in the production of surimi, and then added to surimi again, does not have any negative influence on the quality of the final product (Stine *et al*. 2012). Thus, as e.g., Osman *et al*. (2015) indicate, the retentate received in the process of post-production sewage treatment with the usage of membrane separation can be used e.g. in feed production and the permeate can be reused in the food production.

In the research carried out on membrane separation in sewage treatment process in the fish industry, organic matter is usually evaluated by general rates, such as BOD_5 and COD_{Cr} (Kuca and Szaniawska 2009, Drost *et al*. 2014, Osman *et al*. 2015). The rate does not provide neither information concerning the quantitative composition or macromolecular weight of the separated organic compounds, nor the macromolecular nitrogen compounds or the protein hydrolysis. What is more, according to e.g., Szymczak and Kołakowski (2012) the protein hydrolysis in the process of fish marinating/brining contributes compounds creation of different molecular weight. A knowledge of compounds, together with detailed scopes of molecular weight in permeate and retentate, can be useful in developing new technologies facilitating the usage of permeate and retentate.

In the food industry are often used of polymer membranes, e.g., polysulone (Esfandian *et al*. 2016). In recent years the usage of new ceramic materials produced on the basis of the aluminium oxide, titanium and zirconium has increased, both in the scientific research and the industry, including the food one. Those membranes are characterised by high tolerance to chemical and physical conditions, including extreme pH values. They can, therefore, be used in the fish industry. For example, Osman *et al*. 2015, Gringer *et al*. 2015, 2016, Drost *et al*. 2014, Kuca and Szaniawska 2009, Szaniawska *et al*. 2014 have filtrated process waters after herring marination. Those process waters did not contain acetic acid, even though it is a common ingredient of brines. This acid, among others, reduces the pH and influences protein hydrolysis (Szymczak and Kołakowski 2012). The marinating process that applies to such brines is short, lasts about 7 days.

According to our knowledge, marinating brines containing acetic acid have not been tested in membrane kind of processes. Taking the above into consideration the aim of the present research was to: (i) determine the scope of molecular weight of proteins and their hydrolysis products (PHP) in post-production marinating brines which include acetic acid (ii) estimate the retention level of those compounds in a cascade system of membrane separation with the usage of ceramic membranes of diverse cut-off.

2. Materials and methods

2.1 Research material

The raw materials used for research, industrial brines produced in the marination process of fresh and frozen herring, were acquired from fish processing plants located in Poland. As a result of this diversity of raw material (skinning of fresh or frozen-thawed Baltic and Atlantic herring) and the way of marinating (one- or two-degree marinating) four kinds of post-production marinating brines were determined (Table 1). The percentage share of basic components of the marinating brines (acetic acid and NaC1) used in the technological process and the way of herring marination is presented in Table 1.

Table 1 The division of post-production marinating brines (MB-A÷MB-D) by the fish material and the % share of acetic acid and sodium chloride in the marinating brines used to marinate herring in industrial plants

Name of post-production marinating brine	Kind of fish stock	Initial chemical composition of marinating brines	Composition of post-production marinating brine
MB-A	Fresh, skin-on fillet of Baltic herring (*Clupea harengus membras*) - single-stage marinating	4% CH_3COOH 6% NaCl Period of marination: 7 days Temperature about 6°C	1.78% CH_3COOH 4.31% NaCl
MB-B	Fresh, skin-on fillet of Atlantic herring (*Clupea harengus*) - single-stage marinating	4% CH_3COOH 6% NaCl Period of marination: 7 days temperature about 8°C	1.84% CH_3COOH 3.72% NaCl
MB-C	Frozen-thawed, skin-on fillet of Atlantic herring (*Clupea harengus*) - single-stage marinating	4% CH_3COOH 6% NaCl Period of marination: 7 days Temperature about 5°C	1.39% CH_3COOH 3.79% NaCl
MB-D	Frozen-thawed, skin-on fillet of Atlantic herring (*Clupea harengus*) - double-stage marinating	1°-10% solution of NaCl 2°-3% CH_3COOH, 1.6% NaCl Period of salting: 3 days Period of marination: 4 days Temperature about 6°C	1.47% CH_3COOH 3.96% NaCl

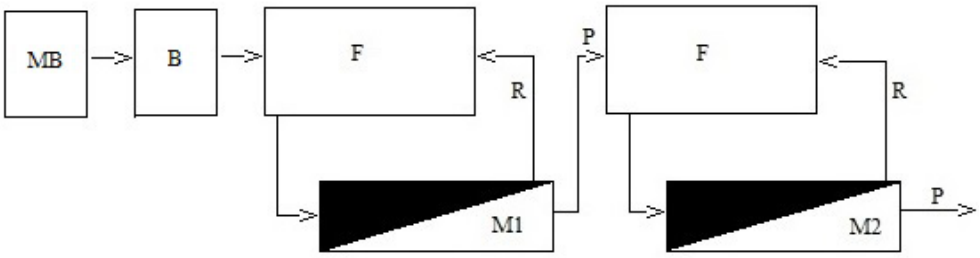

Fig. 1 Scheme of the three-stage process of purifying/scavenging post-production marinating brines (B – polypropylene sack of 25 μm, F - feed container, P - permeate, R - retentante, MB - post-production marinating brine, M1 - membrane module equipped with 150 kDa membrane, M2 - membrane module equipped with 1 kDa membrane)

2.2 Treatment of marinating brines

The post-production marinating brines used in the research were drawn directly after finishing the process of marinating fish. The acquired marinating brines went through a cleaning process in three-stage system with the usage of filtration unit (the scheme is presented in Fig. 1). The first stage of filtration was conducted on a polypropylene bag (PP) with the porosity of 25 μm (WT Techologie, Sosnowiec, Poland). The aim of this separation stage was to carry out a preliminary elimination of such particulate as torn particles of fillet or skin shreds. Two further stages of

separation were conducted with the usage of ultrafiltration ceramic membranes with the cut-off of 150 kDa and 1 kDa. The ceramic membranes of 23-channel, manufactured from $Al_2O_3/TiO_2/ZrO_2$ (TAMI Industries, Nyons, France), were used in the process. The linear velocity of feed in membrane's channel was u = 4 m/s. The permeate acquired after ultrafiltration in membrane with the cut-off of 150 kDa, constituted the feed for ultrafiltration in the membrane with the cut-off of 1 kDa. The transmembrane pressure for the 150 and 1 kDa membrane amounted to 0.2 and 0.1 MPa respectively.

Analytical methods

The pH was measured with the pH-meter (CP 103, by Elmetron, Zabrze, Poland). The total content of nitrogen converted into protein and the product of its hydrolysis (PHP) was determined by the Kjeldahl method according to AOAC (1990). The molecular weight of proteins and products of their hydrolysis were determined by high-performance liquid chromatography (HPLC) with the usage of the Knauer Smartline Pump 1000 chromatograph equipped with UV detector (S-2500, Knauer, Berlin, Germany). The division took place in Protein column KW 802.5 Protein (Shodex), in 23°C at the flow of isocratic eluent (50 mM phosphate buffer pH 7.0/0.3 M NaCl) on the level of 1 ml/min. Due to the amount of dry matter > 5%, the samples were diluted 10 times by the mobile phase, before being put to HPLC system. To the column 10µl of the tested sample or the external standard, used for column calibration, was introduced. The standard consisted of a mixture of proteins with the following molecular weights: 670 kDa, 158 kDa, 44 kDa, 17 kDa and 1.35 kDa (Gel Filtration Standard, Bio-Rad, catalogue number 151-1901). The analysis of molecular weight distribution as well as measurement of the peak surface was conducted with the usage of a computer program (ChromGate, Knauer, Berlin, Germany).

Statistical analysis

Results were analyzed statistically, using 1-way analysis of variance (ANOVA) with StatSoft Statistica 10.0 (Statsoft, Tulsa, Okla., U.S.A.). The ANOVA P value was set at 0.05, and the differences between treatments were examined using the post hoc test Tukey's honestly significant differences ($P < 0.05$).

3. Results and discussion

3.1 The scopes of molecular weight in the post-production marinating brines

Chromatographs have shown that the post-production marinating brines include proteins and PHP of wide range of molecular weight, from ca. 100 Da to over 500 kDa (as an example chromatograph for MB-A, Figs. 2 and 3, was presented). Proteins and PHP of low molecular weight are dominant. The value of peak area for the molecular weight in the scope of 5-45 kDa ranged from 5100 for MB-D to 176000 for MB-C. As far as higher molecular weight is concerned, the value of peak area ranged from 21000 for MB-C to 52000 for MB-B (Table 2). In the marinating brines MB-A and MB-B two main scopes of molecular weight can be isolated, while in brines MB-C and MB-D three (Table 2).

Proteins and PHP of all scopes of molecular weight were present in majority of fractions received on each filtration steps, majority were stopped by a membrane with the cut-off of 150 kDa. Retentante after this stage, was characterised by the highest increase in protein concentration and PHP of lower molecular weight (Table 2). Such a discrepancy in the matter content in the

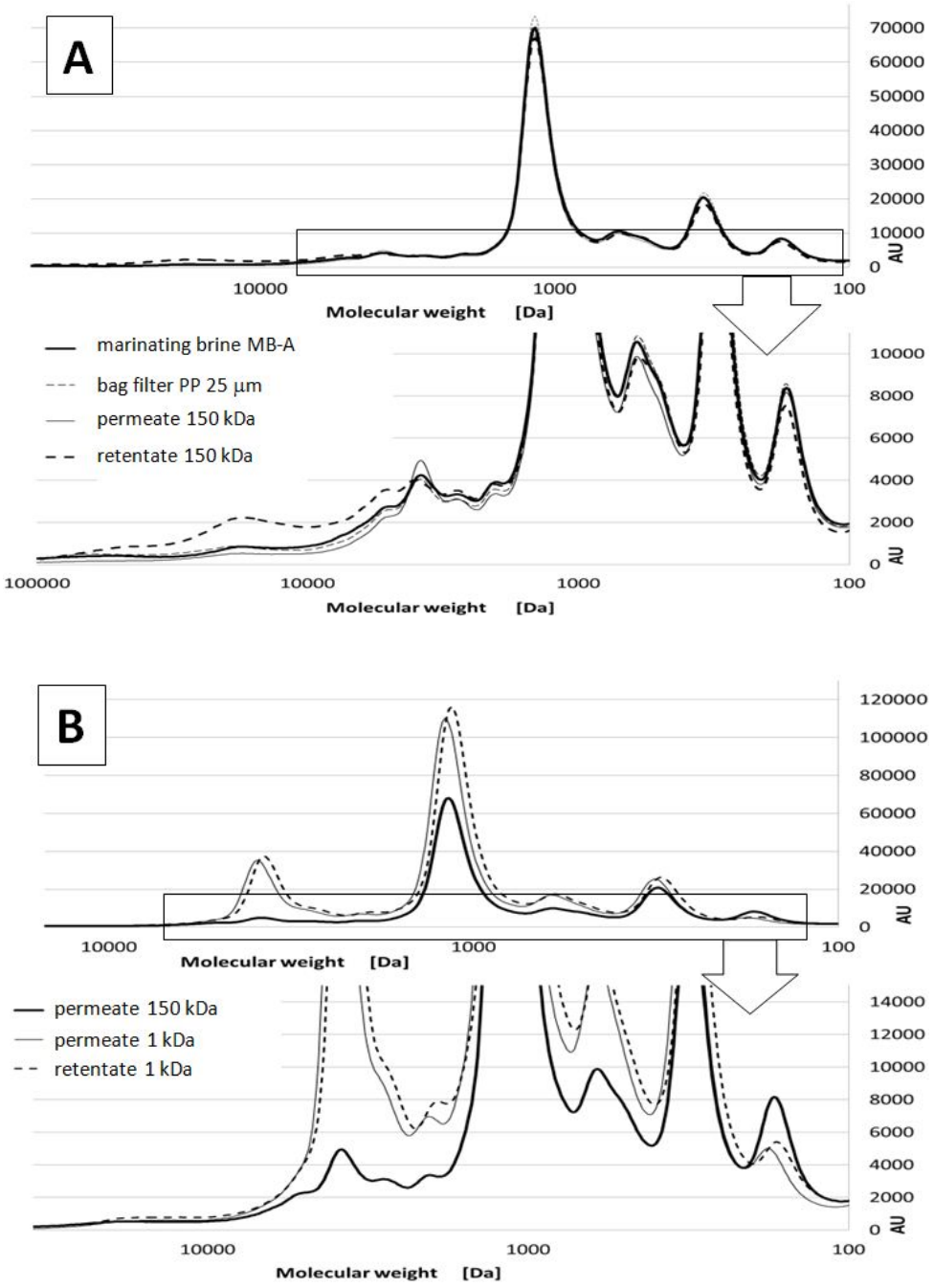

Fig. 2 The chromatogram for the post-production marinating brine MB-A in the range of high molecular weight; (A) MB-A marinating brine and the solutions received after the first (bag filter of 25 μm) and the second degree of purifying, with the usage of the ceramic membrane of 150 kDa (permeate 150 kDa and retentate 150 kDa), (B) the third degree of separation with the usage of 1kDa membrane (permeate 1 kDa and retentate 1 kDa) and the chromatogram for 150 kDa permeate that was used as a feed for the membrane separation

particular scope can be reasoned by the kind of matter put under the process of marinating. MB-A and MB-B were taken from technological process of fresh fish stock. MB-C and MB-D, however, derived from technological process of frozen-thawed fish (Table 1). With references to Szymczak (2011) who proved that frozen-thawed fish, while marinating, is more susceptible to weight decrement and tissue component diffusion to the solution, than the fresh one. What is more, the compounds that diffuse to solution have low molecular weight (Szymczak *et al*. 2012). According to Shenderyuk and Bykowski (1990), the majority of nitrogen compounds that diffuse to marinating brines are those with molecular weight below 15 kDa.

3.2 Membrane separation

No significant changes of pH in solutions, received after each stage of the membrane separation, was noticed (Table 3). It is typical for UF processes due to the sieve type of the suspension

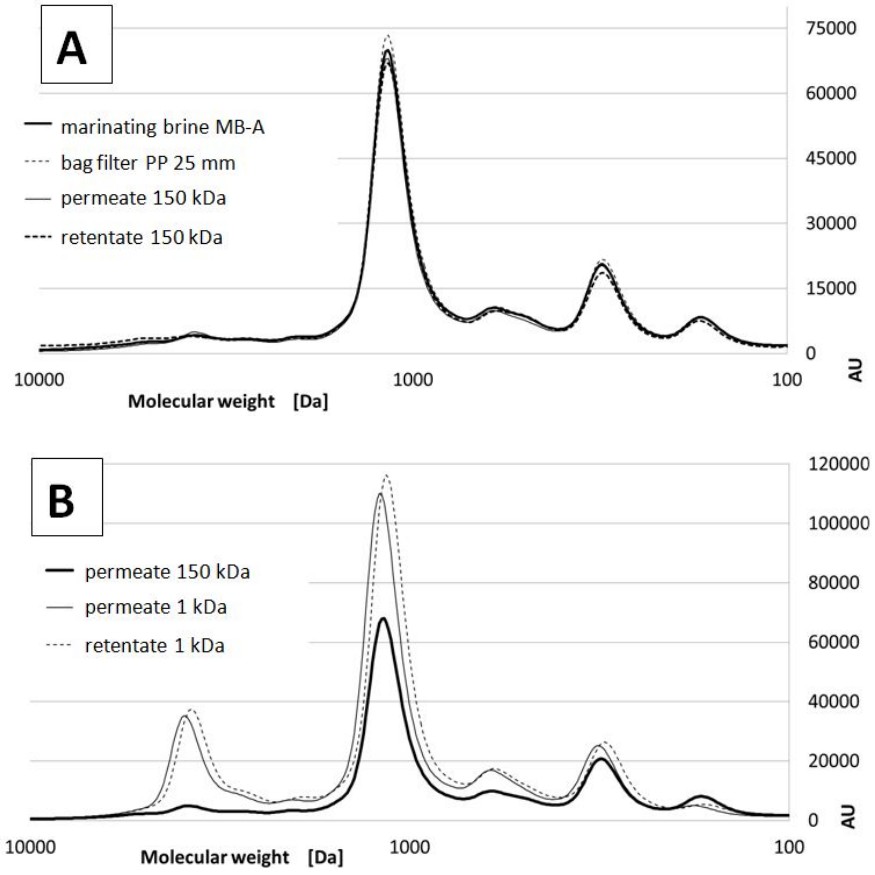

Fig. 3 The chromatogram for the post-production marinating brine MB-A in the range of lower molecular weight; (A) MB-A marinating brine and the solutions received after the first (bag filter of 25 μm) and the second degree of purifying, with the usage of ceramic membrane of 150 kDa (permeate 150 kDa and retentate 150 kDa); (B) the third degree of separation with the usage of 1kDa membrane (permeate 1 kDa and retentate 1 kDa) and the chromatogram for 150kDa permeate that was used as a feed for the membrane separation

Table 2 Comparison of peak area for the main peaks, given by the four samples types and their permeates/retentates during HPLC analysis

	MB-A		**MB-B**	
	Main peaks (molecular weight range kDa)			
	115-24	24-9	100-20	20-8,5
Marinating brine	37800	60000	52000	150000
Bag PP 25 μm	34000	40000	39000	142000
Permeate 150 kDa	8200	32500	5200	49000
Retentate 150 kDa	50000	160000	8700	428000
Permeate 1 kDa	0	32000	4100	41000
Retentate 1 kDa	0	41000	4200	46500

	MB-C			**MB-D**		
	Main peaks (molecular weight range kDa)					
	150-35	35-15	15-5	520-270	270-45	45-12
Marinating brine	21000	51000	176000	33000	65000	51000
Bag PP 25 μm	19000	51000	174000	22000	63000	50000
Permeate 150 kDa	3500	32000	78000	0	0	7000
Retentate 150 kDa	13000	68000	500000	227000	227000	315000
Permeate 1 kDa	3500	22000	71000	0	0	6500
Retentate 1 kDa	< 1000	20000	58000	0	0	9700

separation (Drost *et al*. 2014).

The examined process waters contained proteins and PHP in the range from 13.76 to 25.73 g/dm^3 (Table 3), the differences between MB-M and MB-C were not significant. Low retention of proteins and PHP could result from short period of herring marination (7 days). For instance, Drost et al. (2014) reported similar concentration of proteins and PHP in brines which did not contain acetic acid, although created in equally short period of herring marination. However, Gringer *et al*. (2015) reported much higher concentration of proteins (41-57 mg/dm^3) in brines generated in the process of herring marination that last 190-200 days.

Each step of separation caused a decrease in the concentration of proteins and PHP (Table 3). The lowest reduction was observed in the stage with a PP bag of 25 μm, and the highest in the step using a membrane with the cut-off of 150 kDa. The total retention of proteins and PHP was relatively low and, depending on marinating brines put on test, ranged from 16% to 22%. To compare, Avula *et al*. (2009) acquired retention on the level of 86% while testing the level of proteins retention in industrial water with the usage of membrane with the cut-off of 10 kDa. Such a difference is probably connected with physiochemical composition of raw material from poultry farming and the lack of influence of acetic acid or sodium chloride on meat tissue, which are however present in the process of marinating fish. The level of proteins and PHP reduction acquired in our research was similar to the one acquired by Drost *et al*. (2014), who decreased the content of proteins and PHP in a permeate of about 28% by filtering the brine from the fish industry. It indicated similar composition and size of nitrogen compound in both kinds of processed waters from the fish industry. Szymczak and Kołakowski (2012) reported that during the marinating process a substantial amount of PHP is formed. That includes free amino acids,

Table 3 pH value, protein concentration and products of its hydrolysis (PHP) [g/dm^3], the level of retention [%] in marinating brines under study, solutions received as a result of separation at the particular level of scavenging (after PP sack of 25 μm and permeates after ceramic membranes with the cut-off of 150 and 1 kDa)

Type of marinating brine	Fraction	pH	Proteins and PHP g/dm^3	Retention [%]
MB-A	Marinating brine	4.15 ± 0.05^a	13.76 ± 0.17^a	
	Bag PP 25 μm	4.15 ± 0.05^a	13.01 ± 0.51	5.4
	Permeate 150 kDa	4.14 ± 0.04^a	11.42 ± 0.97	12.2
	Permeate 1 kDa	4.16 ± 0.04^a	10.75 ± 0.39	5.8
	Total retention of proteins and PHP [%]			**21.8**
MB-B	Marinating brine	4.33 ± 0.05^b	25.73 ± 0.58^b	
	Bag PP 25 μm	4.32 ± 0.05^b	24.12 ± 1.02	2.0
	Permeate 150 kDa	4.33 ± 0.04^b	21.10 ± 0.45	18.0
	Permeate 1 kDa	4.35 ± 0.04^b	20.40 ± 0.18	3.3
	Total retention of proteins and PHP [%]			**20.7**
MB-C	Marinating brine	4.44 ± 0.07^b	25.48 ± 1.57^b	
	Bag PP 25 μm	4.47 ± 0.05^b	24.16 ± 0.68	6.2
	Permeate 150 kDa	4.42 ± 0.06^b	21.38 ± 0.38	11.5
	Permeate 1 kDa	4.45 ± 0.04^b	19.98 ± 0.37	6.6
	Total retention of proteins and PHP [%]			**21.6**
MB-D	Marinating brine	4.73 ± 0.06^c	15.57 ± 0.16^c	
	Bag PP 25 μm	4.72 ± 0.05^c	15.25 ± 0.17	2.0
	Permeate 150 kDa	4.73 ± 0.04^c	14.36 ± 0.33	7.8
	Permeate 1 kDa	4.72 ± 0.04^c	13.11 ± 0.41	8.7
	Total retention of proteins and PHP [%]			**15.8**

^{a,b,c} Means without a common lowercase letter differ significantly ($P < 0.05$)

peptides and amino-compounds. Low proteins and PHP retention allows for conclusion that the molecular size of the compounds is probably smaller than the size of tested pores during examination of ceramic membranes.

The evaluated three-staged membrane system was characterised by diverse retention of compounds with the marked range of molecular weight (Table 2). The first stage of separation with the usage of PP bag with porosity of 25 μm usually caused low level retention, which did not exceed 10% for the majority of highlighted ranges of molecular weight. Higher retention was only proved for compounds in the weight range from 24 to 9 kDa (MB-A), compounds in the weight range of 100-20 kDa (MB-B) and compounds in the range of 520-270 (MB-D), 33, 25 and 33% respectively. The results are comparable to those acquired by Szaniawska *et al*. (2014) and Drost *et al*. (2014), who conducted the process of initial filtration of marinating brines in PP bag with the porosity of 1 μm and 25 μm respectively.

The highest retention was was reported for the separation stage using a membrane with the cut-off of 150 kDa (Table 2). Retentions above 70% were noticed for compounds in the range of

high molecular weight in MB-A, MB-B and MB-C. For the MB-D brinehowever, the retention level for compounds of weight ranged from 45 to 520 kDa. The retention for compounds of lower ranges of molecular weight ranged from 19 to 86% (MB-A and MB-D, respectively).

The UF processes are characterised by high level retention of high-molecular compounds, which includes i.e., proteins and the products of their hydrolysis (Kelly and Zydney 1995, Persson *et al*. 2003, De la Casa *et al*. 2007, Nędzarek *et al*. 2015a). For example, Tacharatanamanee *et al*. (2004) studying the process of protein fractioning, in surimi post-production water, proved that the usage of 100 and 300 kDa membrane substantially stopped the proteins of molecular weight ranging from 10 to 120 kDa. It was also found that UF fouling and polarisation layer, formed during the process, contributed to that results greatly. According to Nędzarek *et al*. (2015b), the fouling effect occurring while the process of the UF can contribute to the increase of compound level retention for molecular weight lower that the cut-off of the membranes used.

The third stage of separation conducted with the usage of membrane with the cut-off of 1 kDa proved full retention of compounds in the range of high molecular weight (MB-A and MB-C) and substantially low retention of the compounds in the range of low molecular weight (retention ranging from 1.5 to 16% for MB-A and MB-B respectively). We shall conclude that it is caused by the presence of compounds with relatively low molecular weight, what results from the fact that while fish marination in enzymatic processes, the products of protein hydrolysis with varied molecular weight are formed (Szymczak and Kołakowski 2012). For example, Jeon *et al*. (1999) while studying the process of UF hydrolysate from cod, using a membrane with the cut-off of 30, 10, 5 and 3 kDa, proved that mainly PHP with the molecular weight of 25, 13, 6, 3 and 1 kDa occurred in the hydrolysate. It was also proved that the usage of UF processes with the operation of tested membranes does not contribute to total elimination of those compounds from feed solution, but only reduces their content, what was also proved in our studies.

After a four-hour filtration through a membrane with the cut-off of 150 kDa, the following permeate was acquired from 50 kg of feed: 35 kg (MB-B), 36 kg (MB-D) and 37 kg (MB-A and MB-C. That contributes more than 70% of the feed. They included from 423 to 791 g of proteins and PHP (Table 4). The stage of ultrafiltration with the usage of a membrane with the cut-off of 1 kDa, after four hours generated permeate of 9 kg (MB-A, MB-C and MB-D) and 10 kg (MB-B), what constituted about 30% of the feed. In those parameters the proteins and PHP were ranging from 301-559 g (Table 4). In our research the proteins and PHP recovery was lower than the one acquired by Gringer *et al*. (2015). However, they filtrated brine with higher protein concentration, what could influence higher membrane clogging and, therefore, higher protein retention. The loss for proteins and PHP in the ultrafiltration process was low, for the UF 150 kDa maximum 5.2% (MB-B) and for the UF 1 kDa maximum 7.7% (MB-D) (Table 4). It allows for deduction that

Table 4 Weight balances of protein and PHP reported ing

Type of marinating brine	150 kDa					1 kDa				
	Inlet	Outlet		Loss		Inlet	Outlet		Loss	
		Permeate	Retentate	g	%		Permeate	Retentate	g	%
MB-A	651	423	214	14	2.1	423	301	101	20	4.7
MB-B	1262	739	457	66	5.2	739	510	206	23	3.1
MB-C	1208	791	397	20	1.6	791	559	183	49	6.2
MB-D	763	517	238	8	1.0	517	354	124	39	7.5

protein and PHP due to its low molecular weight and modest membrane-protein influence, which can be observed in case of acidic pH (compare De la Casa *et al*. 2007, Nędzarek *et al*. 2015), the degree of participation in the fouling creation was limited.

4. Conclusions

The process of marination contributes to the high ratio of nitrogen compounds characterised by low molecular weight, created as a result of protein hydrolysis, in the post-production marinating brines. When comparing the post-production brines, created in the process of marination of frozen-thawed and fresh herrings, a vast diversity in ranges of molecular weight can be noticed in the post-production brines created while marinating the frozen-thawing herring.

The three-staged system of pre-treating industrial marinating brines that was applied in our studies, contributed to reduction in the proteins and PHP content ranging from 16 to 22%, depending on the kind of raw material used to marinate. The highest retention of nitrogen compounds was gained using a membrane with the cut-off of 150 kDa. The three-staged treatment of marinating brines that was applied caused substantial reduction, or total elimination, of nitrogen compounds of high molecular weight and only reduced the content of nitrogen compounds with low molecular weight.

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