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Antioxidants, ACE I Inhibitory Peptides, and Physicochemical Composition, with a Special Focus on Trace Elements and Pollutants, of SPRING Spawning Atlantic Herring (*Clupea harengus*) Milt and Hydrolysates for Functional Food Applications

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Abstract: Norwegian spring spawning (NVG) herring milt is a raw material with high nutritional and functional values. However, its incorporation into food presents physicochemical and sensory challenges. Its high DNA content, the presence of TMA/TMAO and possibly heavy metal and/or environmental pollutants, and its bitter taste due to amino acids or peptides requires a careful approach to food development. Hydrolysis with food-grade enzymes enable an improvement in both the functional and sensory properties of the substrate and the increased stability of the raw materials and end products. HLPC, GC-MS, and in vitro protocols were used for the characterisation of manually extracted material (sample code: HMC) and milt from a fish-filleting line from early spring/late autumn catches. Three different food-grade protein hydrolysates were prepared from these raw materials (sample codes: H1, H2, and H3) as a means to estimate their functional food development potential. Combinations of three commercial enzymatic preparations were applied, targeting specific sensory properties. Parameters related to consumer safety (e.g., the presence of heavy metals and TMA/TMAO); beneficial health effects, such as antioxidant or antihypertensive bioactivities (measured using in vitro TAC, ORAC, DPPH, and ACE I inhibitory activity assays); the presence of beneficial fatty acids and micronutrients; and the protein quality were studied. On the basis of their total amino acid compositions, freeze-dried herring milt and hydrolysates could provide high-quality protein with most of the essential amino acids and taurine. Powdered milt has a particularly high fatty acid profile of bioavailable omega-3 fatty acids (2024.06 mg/100 g docosahexaenoic acid (DHA; 22:6n-3) and 884 mg/100 g eicosapentaenoic acid (EPA; 20:5n-3)). The experimentally measured levels of arsenic (3.9 ± 1.2 mg/kg) and cadmium (0.15 ± 0.05 mg/kg) were higher than the levels of the other two heavy metals (mercury and lead). The bioactivity is concentration-dependent. Overall, this work presents complementary information for the future utilisation of *C. harengus* powdered milt (possibly obtained directly from a fish-filleting line) and some of its protein hydrolysates as food ingredients.

Keywords: herring *Clupea harengus*; milt; hydrolysates; proximate composition; micronutrients; antioxidants; surface hydrophobicity; heavy metals

Key Contribution: Our work has investigated the food safety and quality of NVG Atlantic herring milt and three different types of food-grade enzymatic hydrolysates from both manually removed milt and milt obtained from a filleting line. The bioactivity and sensorial and nutrient qualities are discussed in view of the possible use of this raw material in functional food products.

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1. Introduction

Norwegian spring spawning (NVG) herring stocks belong to the highly migratory *Clupea harengus* species distributed throughout large parts of the Northeast Atlantic Ocean. Fisheries of these stocks are closely managed in Norway, with two approved fishing seasons per year (spring and autumn). The high nutritional quality of herring fillets is well known [1–3]. Herring is usually filleted prior to marketing, and three types of by-products predominate in herring processing lines. In addition to heads, bones, viscera, and belly flaps, liver, roe, and milt account for up to 25% of the total fish weight during the spawning season [4].

In Norway, which is one of the largest global fish and fish roe producers, milt, roe, and liver predominate in the production of feed for aquaculture and domestic animals [5,6]. In the EU, milt is still used mostly for the industrial manufacturing of DNA/protamine sulphate and the anti-HIV drug AZT for pharmaceutical purposes [7,8]. The use of herring (whole fish) for low-cost, high-quality nutraceuticals has recently been reviewed [9]. It is suggested that, through dietary intake, it may provide several potential health benefits, mainly due to its oil composition (EPA and DHA) but also due to its high mineral content (ash, including different amounts of Ca, Mg, Na, K, Fe, Zn, Cu, Mn, Se, F, and iodine). The selenium content in whole Atlantic herring is reported to be low [3], but this should be studied further as it is important during the production of fish concentrates due to its increased capacity to palliate, in organic form, the toxic effects of some heavy metals [10].

Roe from several fish species is consumed as a gourmet food in the form of salted or processed seafood products (e.g., caviar, bottarga, and taramasalata), while milt and liver are consumed to a much lesser extent [11,12]. Herring milt is traditionally consumed in the UK on toast as part of the British breakfast, and it is a niche product in several other specific country markets (Russia, Italy (together with tuna milt), the U.S.A., and Japan) [13,14]. One recent study evaluated consumer acceptance among teenagers in Ireland of newly developed herring (*Clupea harengus*) milt pâtés [14]. This study identified the sensory properties of milt pâtés (bitter taste due to high DNA content or strong "fishy" odour due to the presence of trimethylamine n-oxide (TMAO)/trimethylamine (TMA)) as the main challenges to good consumer acceptance, although the population studied generally recognised several dietary health benefits of herring milt pâté. Recently, some novel technologies for the removal of the most potent odour-active compounds from herring milt and its derived products (TMA/TMAO and dimethylamine (DMA)) have been suggested, such as electrodialysis, ion-exchange membrane fouling, and water dissociation [15].

Milt has a variable composition, depending on the fish species, nutrition/feed, season/maturation stage, rearing conditions, and/or stress, age, weight, and length, which is physiologically intended to provide the required chemical background for the survival and optimal performance of reproductive cells. The main components of milt—membrane, seminal fluid, and sperm cells—have a high content of (1) lipids (membrane), such as cholesterol and phospholipids, with a prevalence of polyunsaturated fatty acids (more than 10% of the total fatty acid profile in some species); (2) proteins (membrane, cytoskeleton, and nucleus) with different enzymatic, replication-supporting, motility, and structural functions; (3) nucleotide/DNA material, which is required for reproduction; (4) minerals (mostly K and Ca but also Mg, Na, Cl, and Se); and (5) vitamins (B12, C, and D) and monosaccharides, which maintain motility function [16]. Evidence of the accumulation of heavy metals, drug residues, or other environmental pollutants in fish roe and milt is growing, in parallel with their accumulation in aquatic environments worldwide; above certain levels, this also leads to higher fish mortality at early life stages [17,18]. Most available bibliographic reports focusing on the uses of the by-products of fish filleting, in general, have studied the levels of contaminants in the viscera as a whole fraction or in the liver. Not enough attention has been given to the quantification (variations by geographic zones and seasons) of the trace elements and pollutants in herring (*C. harengus*) milt.

A major challenge of using milt in food would be its standardised extraction and quality preservation for the development of targeted products for human consumption, which

would be hampered mostly by the abundant amounts of blood, water, and endogenous enzymes present in the by-products of fish filleting. Several different food-grade enzymatic hydrolysates have already been suggested as a means of ensuring the standardised production, improved quality, and possible incorporation of the resulting powdered milt concentrate for the enrichment of food matrices or nutraceuticals [6,18]. Food-grade enzymatic hydrolysis has been well established as a method for generating bioactive compounds (predominantly peptides, but also for the separation of proteins and oils). Several herring milt hydrolysates (HMHs) have been reported as having antioxidant, anti-inflammatory, and antidiabetic effects, both in vitro and in animal models (mice) [19–22]. In a previous study, the pilot-scale feasibility of the production of one type of food-grade hydrolysate from herring milt in mobile units was tested [3]. HMH's specific suitability for dietary inclusion for consumers with diabetes or metabolic syndrome has been suggested [21,23]. Furthermore, the higher digestibility and bioavailability of amino acids and peptides in protein hydrolysates are well documented [24–26].

However, the lack of a consumer-driven market, sensory challenges in the development of optimised food products, and the lack of established value chains for these are considered the main causes of the underutilisation of milt for human consumption. According to a report from the Norwegian Seafood Council for the first half of 2023, NVG herring landings in Norway have increased by 10,000 tonnes (for a total of 48,000 tonnes), or by 6%, over the same period in 2022 [27]. The same publication reports that, due to the small size of the herring, the preferential prices for fish oil and fish flour (meal), and the significant decline in the production and export of herring roe, almost half of all NVG herring landed has been used for oil and flour, with a very low product output with a higher added value.

In the present study, we have characterised the functional properties and proximate composition of initial NVG herring milt (from February and November catches), as well as several different enzymatic hydrolysates from this milt, as a means to estimate its functional, nutritional, and sensory qualities. The hydrolysates were prepared with combinations of three commercial food-grade enzymatic preparations, targeting specific sensory properties (less bitterness and less fishy smell), the effect of which on the proximate composition of NVG herring milt has not previously been reported. We have specifically analysed parameters of the hydrolysates related to consumer safety (e.g., the presence of heavy metals, TMA/TMAO), the presence of health-beneficial effects such as antioxidant and antihypertensive bioactivities (in vitro), beneficial fatty acids, micronutrients, and protein quality, as well as hydrophobicity, in order to estimate their potential for dietary inclusion, targeting specific segments of the population.

2. Materials and Methods

2.1. Raw Material

Freshly frozen whole Atlantic herring (*C. harengus*) from the spring (February) 2021 season (20 kg of fish per box; about 70 individual fish), caught in FAO zone 27, were provided by Fosnavåg Pelagic AS (Møre og Romsdal, Norway). These were stored at −30 °C in Longvagruppen's commercial freezing chamber at Gangstøvika, Ålesund, Norway, prior to milt extraction. Each box of 70 fish was semi-thawed and processed within 24 h. For protein concentrate production, the milt was removed, pooled, and weighed, then homogenised prior to hydrolysis. Two repetitions of each hydrolysis process were carried out to confirm the representativity of the results.

For comparison purposes, two batches of milt of approximately 1 kg each were obtained directly, by manual picking, from the by-product belt after filleting of fish from autumn catches at Fosnavåg Pelagic's factory, on 26 November 2021. Each batch was collected from the total by-product sample moving on the belt for a 20-min period. The unhydrolysed sample, used as a control for proximate composition analyses, was produced by pooling hand-extracted milt from the spring season, which was followed by direct lyophilisation in

a Labconco FreeZone 12-litre freeze-drier (Labconco, Kansas City, MO, USA) and posterior grinding of the dried powder in a coffee grinder.

2.2. Preparation of Enzymatic Hydrolysates

Each batch of milt was homogenised with distilled water at a 1:1 ratio with the use of CombiMax 600 blender equipment and hydrolysed in a Julabo Heidolph reactor system (Julabo, Seelbach, Germany). This system included two parallel 4 L working-volume glass reactors with Hei-TORQUE Precision 400 stirrers and was heated by a Julabo B33 water bath (Julabo, Germany). The homogenates were pre-heated to 50 °C; then, different commercial proteases were added for a final volume of 0.2%. Specifically, TailorFood Endocut-02L® (trademark of Tailorzyme ApS, Herlev, Denmark; used separately for preparation of hydrolysate 1 (H1)) and a cocktail of TailorFood Exocut-TR L® (80%) and Endocut-05L® (20%) for hydrolysate 2 (H2) or Endocut 07L (20%) for hydrolysate 3 (H3) were used for the experiments. These enzymes correspond to aminopeptidase from *Trichoderma reseei*, EC 3.4.11.15 (Exocut-TR L®; Tailorzyme ApS, Herlev, Denmark); metallo-endo-proteases from *Bacillus amyloliquefaciens*, EC no. 3.4.24.28 (Endocut-05L®; Tailorzyme ApS, Herlev, Denmark); and subtilisin serine endo-protease from *B. licheniformis*, EC 3.4.21.62 (Endocut-07L®; Tailorzyme ApS, Herlev, Denmark). No additional pH adjustment was made. Agitation at 120 rpm was applied throughout the whole hydrolysis process. After 120 min, the samples were treated for 15 min at 90 °C for enzyme inactivation and all material was centrifuged at 3000× *g* for 15 min at room temperature. Work continued with the soluble fraction after the centrifugation step. The protein in the supernatant fractions was dried in a Labconco FreeZone 12-Litre freeze-dryer (Labconco, Kansas City, MO, USA) prior to further characterisation. The pH was between 5 and 6 in all samples prior to freeze-drying. Samples produced from the manually extracted milt were coded as H1 and H2, while sample H3 was produced from the milt batch processed by machine fish filleting.

2.3. Physicochemical Characterisation

Moisture, ash content, and total fat were determined at Møreforsking's laboratory using the methods of AOAC 2000 [28]. The yield of fat, ash (% *v*/*w*), and protein recuperation (g/100 g or mL) per fraction was calculated. All measurements were performed in triplicate.

The fatty acid profile of all herring milt samples was analysed in accordance with the methods LM001.R02 and LM033.R00, using GC-MS, at the Institute of Aquaculture of the University of Stirling, UK, as part of its nutritional analysis services. The results were reported as a percentage of total fatty acids (FAs) and mg FA/100 g of total lipid from fish meal.

Micronutrients (metals) and heavy metals were analysed by Eurofins Food & Feed Testing, Norway, according to SS-EN ISO 17294-2-E29 [29], SS-EN-16277:2012 [30], and EN 15111 [31]. Trimethylamine/trimethylamine oxide (TMA/TMAO) was also analysed by Eurofins Norway, using the titrimetric determination protocol, based on Conway's method.

2.4. Total Amino Acid and Peptide Molecular Weight Profiling

Total amino acid profiling of the soluble *C. harengus* milt hydrolysate fractions was carried out by acid hydrolysis and UPLC (Waters, Milfiord, MA, USA), using the standard accredited method ISO 13903:2005/EU 152/2009 (F) [32]. As the method includes acid hydrolysis, Tryptophan is destroyed during the pretreatment. Cysteine and Methionine were present in their oxidated forms after the acid hydrolysis and were therefore only partially quantified in the total amino acid profile. The free amino acid profile, however, was carried out in the soluble fraction of the samples, by way of the same HPLC method but without acid hydrolysis. Therefore, the tryptophan was not destroyed in these analyses and could be quantified.

The assessment of the protein quality was carried out on the basis of their amino acid score (AAS) and essential amino acid index (EAAI). For this purpose, the following formulae were used:

$$\text{AAS (\%)} = [\text{mg AA in 1 g of tested dry sample/mg AA in 1 g reference protein}] \times 100 \quad (1)$$

$$\text{EAAI (\%)} = n\hat{}\ \log \text{EAA} \quad (2)$$

where log EAA = [1/*n*] [log (100 a1/a1R) + ... + log (100 an/*anR*)]; a is mg of amino acid in 1 g of tested dry sample; *aR* is mg of amino acid in 1 g of reference protein; and *n* is the number of amino acids considered for the calculation. AA and EAA stand for amino acid residue and essential amino acids in Equations (1) and (2), respectively. The reference protein used was the amino acid pattern defined by the World Health Organization and the Food and Agriculture Organization (FAO/WHO/UNU) [33]. The AAS of protein was considered the lowest AAS value within the detected essential amino acids.

The analysis of the peptide profile of the hydrolysates was carried out at Møreforsking's lab. Freeze-dried herring milt and hydrolysed samples were diluted with Milli-Q (MQ) water to a concentration of 10 mg/mL. Then, 100 µL of each diluted sample was further diluted with 900 µL of 10% acetonitrile in MQ water in an HPLC vial. Analysis was performed on an AQUITY UPLC H-Class PLUS System (Waters, Milford, MA, USA) with an AQUITY BEH125 SEC 1.7 u 4.6 mm × 150 mm column (Waters, Milford, MA, USA) and an AQUITY UPLC PDA Detector (Waters, Milford, MA, USA) set to 220 nm. Runs were isocratic, and a 100 mM phosphate buffer (pH 6.8) was used as the mobile phase with a 0.4 mL/minute flow rate, an injection volume of 2 µL, and a total run time of 10 min. The column temperature was set to 30 °C for analysis. Cytochrome C (12327 Da), aprotinin (6512 Da), insulin A (2531 Da), Leu-enkephalin (555.6 Da), Val-Tyr-Val (379.5 Da), and Gly-Tyr (238.2 Da) were used as standards. All were purchased from Merck, Germany. Chromatograms were manually integrated and separated into intervals of <0.2, 0.2–0.5, 0.5–1, 1–2, 2–5, and >5 kDa, expressed as percentages of the total area. All samples were analysed in triplicate.

2.5. DNA Quantification

Total DNA was extracted from the herring milt powdered samples after resuspension at 10 mg/mL in 75 mM sodium phosphate buffer pH 7.4 and agitation for 3 h at room temperature on a Kika Labortechnik KS250 basic shaker (ThermoFisher Scientific, Waltham, MA, USA), at 200 rpm speed, followed by a centrifugation step (15 min at 12,000× *g* at room temperature). Extraction was tested on both the pellets and the supernatants by use of the DNeasy Blood & Tissue kit's (Quiagen, Hamburg, Germany, product ref. 69504) spin column protocol reagents, according to the producer's instructions. Quantification was performed in the Invitrogen Qubit4 Fluorimeter (Thermo Fisher Scientific, Waltham, MA, USA), using the Qubit ssDNA quantification assay protocol (product ref. Q10212). Measurements were performed in three replicates for each sample, and dilutions were prepared in the AE buffer from the DNeasy Blood & Tissue kit (Quiagen, Hamburg, Germany), so as to obtain a statistically viable regression line from the Qubit4 Fluorimeter. Results were expressed in a ng/mg sample, based on standard line concentrations.

2.6. Bioactivity Characterisation

2.6.1. Antioxidative Capacity

The total non-enzymatic antioxidant capacity (TAC) was measured through the application of an assay kit from Sigma Aldrich (catalogue number MAK187), as per the producer's instructions. The kit allowed for simultaneous spectrophotometric quantification of both small molecules and protein antioxidants and the separate calculation of the corresponding parts of each of these (by application of a protein masking reagent).

The HMC samples were resuspended at 10 mg/mL in a 75 mM sodium phosphate buffer pH 7.4 (in duplicate) and then agitation was applied for 1 h at room temperature on a Kika Labortechnik KS250 basic shaker (ThermoFisher Scientific, Waltham, MA, USA) at 200 rpm, followed by a centrifugation step (15 min at 8000× *g* at room temperature). Work was continued with the soluble part (supernatant). MilliQ-grade water (Millipore water filtration equipment from Merck, Darmstadt, Germany) was used for the dilutions of these to 5 mg/mL, 1 mg/mL, and 0.1 mg/mL. The absorbance was measured at 570 nm in a Synergy HTX S1LFA plate reader (Agilent Technologies, Santa Clara, CA, USA) (similar to measurements for the DPPH method described below). Results were obtained in nM Trolox equivalents (TE), and measurements were performed within the range of 5 standard concentrations, namely, 4, 8, 12, 16, and 20 nM, as included in the kit.

The ORAC-antioxidative activity measurement method was used for the analysis of the herring milt protein concentrates and was carried out in black 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) in a BioTek Synergy HT plate reader (Agilent Technologies, Santa Clara, CA, USA), according to the protocol established by the equipment manufacturer [34]. The concentrated milt protein samples were resuspended in a 75 mM sodium phosphate buffer pH 7.4 at 100 mg/mL final concentration, similar to the dissolving procedure in the TAC protocol, with 2× and 10× dilutions prepared for testing from these in the same buffer. As controls, Trolox concentrations of 100, 50, 25, 12.5, and 6.25 µM were applied. Plates were incubated for 30 min at 37 °C in the plate reader, then 2.2′-Azobis (2-aminopropane) dihydrochloride (AAPH) was added at a 19 mM final concentration and fluorescence was measured at 485 nm excitation and 528 nm emission for 1 h. The results were calculated in µM TE or a minimum inhibition concentration of IC50.

For the DPPH assay [35], all samples were dissolved in three independent replicates each, at 100 mg/mL in a 0.05 M Na phosphate buffer (NaPB) at pH8, with agitation applied for 1 h at room temperature on a Kika Labortechnik KS250 basic shaker (ThermoFisher Scientific, Waltham, MA, USA) and at 200 rpm speed, followed by a centrifugation step (15 min at 8000× *g* at room temperature). 2.2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich D9132, Darmstadt, Germany) was dissolved at 0.15 mM in methanol. Trolox was chosen as standard and prepared at 100 mM in 50 mM NaPB and further diluted with the same buffer to 50, 25, 12.5, 6.25, and 0 µM for the standard curve. The protein concentrates were assessed at two-fold and ten-fold dilutions (the same as the concentrations used for the ORAC assay) in the assay buffer. A volume of 20 µL of all samples was combined with 180 µL of the 0.15 mM DPPH working solution and incubated for 30 min in the dark at room temperature, and absorbance was measured at 540 nm in a Synergy HTX S1LFA plate reader (Agilent Technologies, Santa Clara, CA, USA). The activity of the samples was calculated as µM TE, based on the standard curve, and plotted against the sample concentration [mg/mL].

2.6.2. ACE I Inhibitory Activity

For ACE I inhibitory activity measurements, the peptides were dissolved as described for the ORAC assay. The herring milt and hydrolysates were assessed at 0.1, 1, 5, and 10 mg/mL in three independent replicates. The Sigma Aldrich fluorometric angiotensin I-converting enzyme (ACE) activity assay kit (Merck, Darmstadt, Germany; Product CS0002) was used for the measurements, in accordance with the manufacturer's instructions. In 96-well plates, 50 µL of the samples and their respective buffers as blank controls were compared with 50 µL of standard solution of angiotensin I-converting enzyme (in the assay buffer) at dilutions of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.8 nM, and then the reaction was initiated by the addition of 50 µL of standard substrate solution to each well. ACE I-positive control was used in the kit to verify the functionality of the assay. Lisinopril at a 50 µM final concentration (Sigma Merck Cat. No. PHR 1143) or Captopril (Sigma Merck Cat. No C4042-5G) was used as a control for ACE I inhibition [36]. Fluorescence was measured every minute (at an excitation of 320 nm and an emission of 405 nm) at 37 °C for 5 min in a Synergy HTX S1LFA plate reader (Agilent Technologies, Santa Clara, CA, USA). The kit

manufacturer supplied a standard Excel sheet for calculation of the results from the assay. Linear regression curves were obtained for the decrease in absorbance over time for every sample and the percentage of ACE inhibition was calculated as follows:

[mU] ACE activity = (slope of the sample linear regression curve/slope of the standard linear regression curve)

One unit of ACE is defined as the amount of enzyme that releases 1 nmol of fluorescent product from the substrate, in 1 min, under the assay conditions, at 37 °C. The results were presented as lisinopril equivalents [μM] plotted against the sample concentration.

2.7. Techno-Functional Analysis

Surface hydrophobicity measurements were based on the ANS fluorescence method as per Felix et al. (2017) [37] with modifications. Namely, freeze-dried herring milt protein samples and ANS (1-anilino-8-naphtalene-sulfonate) were dissolved in a 75 mM sodium phosphate pH 7 buffer (ANS at 8 mM final concentration) prior to the analysis. Adequate dilutions of the protein samples (in the range 5 mg/mL–80 mg/mL) were prepared. The samples were analysed in black 96-well plates, following the addition of 4 μL of 8 mM ANS per 200 μL sample per well and incubation for 45 min at room temperature (25 °C) in the dark. Fluorescence was measured in a BioTek Synergy HT plate reader (Agilent Technologies, Santa Clara, CA, USA) at 365 nm excitation/484 nm emission. As sample blanks, the protein dissolutions without added ANS were used, and a reagent blank was included for the 75 mM sodium phosphate pH 7 buffer. The hydrophobicity (initial slope So) was calculated as a function of the fluorescence intensity vs. the protein concentration by linear regression analysis (Excel for Microsoft 365 MSO). Data from two independent experiments are shown.

2.8. Statistical Analysis

Statistical analyses were performed in GraphPad Prism Software v.9.5.0 (Dotmatics, Boston, MA, USA), as well as by using the Excel Stat data analysis tools in Microsoft Excel, unless specific Excel calculation sheets were provided for result analysis (e.g., for anti-ACE I activity, TAC, and ORAC). Results are expressed as mean values ± standard deviation if nothing else is stated. Differences were tested with ordinary one-way ANOVA, followed by an F-test for multiple comparisons. Statistical differences with a confidence level of $p \leq 0.05$ have been considered as significant.

3. Results and Discussion

3.1. Proximate Composition of NVG Herring Milt and Derived Concentrates

3.1.1. General Physicochemical Characterisation

The proximate composition of the herring milt and derived hydrolysates showed that the initial herring milt had relatively high ash (ranging between 12% and 22%) and total fat content (between 2% and 6%; Table 1), in comparison to the dietary intake recommendations of the EFSA. The values presented for the key nutritional parameters are for 100 g of dried product, which can be used only as a potential food ingredient and is not to be consumed directly without previous dilution or inclusion in a food matrix. In case of such inclusion, the quantity of ingredient to be used should be carefully considered so as to palliate the high mineral and salt content, or it should be submitted to further filtration or other purification treatment prior to use. The nutrient values in Table 1 for the two ingredients derived from the manually extracted milt (H1 and H2) and the milt from machine filleting (H3) are comparable (no significant differences are observed) and within the generally representative levels for fish and seafood [38]. The moisture and total ash values are close to those in previously reported data for other herring milt hydrolysates, prepared by Alcalase or Protamex: 6.6% moisture and 12.7%–13.8% ash [22]. For these hydrolysates, however, the total fat content was higher than in our case, ranging between 10.8% and 11.2% lipids. This could be a challenge during the storage of dry products, due to fat and protein oxidation.

Table 1. Basic nutritional composition of spring NVG herring milt and derived enzymatic hydrolysate ingredients compared to EFSA dietary reference values [39]. Results are expressed as mean ± SD from $n = 3$ replicates for each analysis, except where a percentage is used for value presentation.

Nutrient	Herring Milt (HM)	Hydrolysate H1	Hydrolysate H2	Hydrolysate H3	¤/¤¤Population Reference Intake (PRI)
Water content [%]	6.30 ± 0.03	10.10 ± 0.03	9.5 ± 0.03	9.9 ± 0.03	NA (adequate intake (AI) = 2 L/day)
True protein [ΣTAA] [mg/kg]	102.04 ± 2.04	63.51 ± 0.03	65.03 ± 0.01	95.45 ± 0.45	NA (AI) (AR § of 0.83 g/kg bw per day)
Ash [%]	21.78 ± 1.24	12.53 ± 0.74	15.35 ± 0.89	18.94 ± 0.57	NA
Fat [%] *	6.10 ± 0.48	2.20 ± 0.40	2.20 ± 0.09	3.10 ± 0.05	NA
DNA content of the soluble fraction [g/100 g sample] #	8.23 ± 1	21.60 ± 1	7.63 ± 1	11.00 ± 1	NA
Minerals:	**[mg/kg]**	**[mg/kg]**	**[mg/kg]**	**[mg/kg]**	
Copper (Cu)	3.2 ± 0.6	3.3 ± 0.7	3.1 ± 0.6	3.0 ± 0.6	NA (AI 1.3 mg (female) −1.6 mg (male)/day; UL = 5 mg/day) ##
Zinc (Zn)	24 ± 5	20 ± 4	43 ± 9	42 ± 8	7.5–12.7 mg/day (UL = 25 mg/day)
Iron (Fe)	53 ± 10	42 ± 8	55 ± 11	39 ± 8	11 mg/day
Calcium (Ca)	280 ± 56	540 ± 108	360 ± 72	280 ± 56	1000 mg/day (UL = 2500 mg/day)
Magnesium (Mg)	1300 ± 260	1300 ± 260	1300 ± 260	980 ± 196	NA (AI = 300–350 mg/day female/male)
Manganese (Mn)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	NA (AI = 3 mg/day)
Selenium (Se)	2.5 ± 0.5	2.0 ± 0.4	1.8 ± 0.4	2.3 ± 0.5	NA (AI = 70 µg/day; UL = 300 µg/day)
Total phosphorus (P)	31,000 ± 6200	25,000 ± 5000	27,000 ± 5400	25,000 ± 5000	NA (AI = 550 mg/day)
Iodine (I)	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	NA (AI = 150 µg/day; UL = 600 µg/day)
Sulphur (S)	7400 ± 1480	6700 ± 1340	6200 ± 1240	7700 ± 1540	NA
Potassium (K)	7000 ± 1400	6600 ± 1320	7400 ± 1480	6400 ± 1280	NA (AI = 3500 mg/day)
Sodium (Na)	8700 ± 1740	9900 ± 1980	9200 ± 1840	5800 ± 1160	Safe and adequate intake 2 g/day

* Total fat values in % are based on the sum of all fatty acids obtained during the fatty acid profiling analyses. # Values measured with the Qubit ssDNA assay kit in the samples resuspended at 10 mg/mL. ## Tolerable upper intake level (UL) is the maximum chronic daily intake of a nutrient (from all sources) judged to be unlikely to pose a risk of adverse health effects for humans. ¤ Nutrient intake likely to meet the needs of almost all healthy people in a population. ¤¤ Reference values for healthy adult population ≥ 18 years old, of both genders. § Average requirements (ARs) should be multiplied by the reference body weight to calculate values in g/day.

Thus, an estimated daily consumption of 10 g of herring milt ingredients, included in a food product, could provide between one-third and up to one-half of the recommended dietary intake of copper, zinc, magnesium, iodine, and calcium, minerals with established health claims that are essential for various metabolic functions in the human body (Regulation (EU) No. 1047/2012 amending Regulation (EC) No 1924/2006 with regard to the list of nutrition claims; EU Register on Nutrition and Health Claims: https://ec.europa.eu/food/food-feed-portal/backend/claims/files/euregister.pdf; accessed for the last time on 28 May 2024). The total selenium content in the samples, however, was high, and should be further specified for the ratio of organic (i.e., selenoproteins, Se–Cys, Se–Met, and others) to inorganic forms of the mineral. These data would support an adequate risk–benefit assessment and determination of the recommended maximum intake amounts of any of the milt ingredients. The general intake of organic

selenium through the diet is low in many European countries [40,41], and it performs vital functions in the activities of key metabolic enzymes (for the maintenance of fertility, immunity, thyroid hormone regulation, etc.), while inorganic selenium in excess can be toxic and can have cumulative effects [42,43].

The total DNA amount was measured as an indicator for the nucleotide content that would be delivered through the consumption of the ingredients or derived foods. The total DNA concentrations measured by way of different biochemical (chromatography, spectroscopy, mass spectrometry, NMR, spectrophotometry, fluorimetry) and molecular approaches (qPCR) in highly processed foods can vary depending on the extraction methodology and final level of achieved DNA purity [44]. The DNA extraction procedure we applied to the herring milt and hydrolysates has been adapted to animal tissue samples, not food samples, but can allow for higher salt tolerance and reduce the background signal from salts in the samples [45]. Also, the Qubit fluorometric quantification protocol enables specific quantification of single-stranded DNA (ssDNA), which is predominant in the gonads. The values we have measured for the hydrolysates in comparison to the initial herring milt are most probably slightly overestimated due to the lower level of purity/eventual contamination with proteins. The measured total DNA levels are comparable to previously reported quantities for other herring milt hydrolysates (7%–27.3%) [20]. Nucleotides are conditionally essential nutrients, depending on the developmental stage or health condition of the human body (e.g., infants, gastrointestinal, immune disease, trauma from burns, sportsmen) [46]. In newborns and infants (up to 3 years of age), dietary nucleotides are semi-essential nutrients, because de novo synthesis does not satisfy the growing body's requirements. Due to this, human milk contains large amounts of nucleotides (10–70 mg/100 mL) [47]. According to current legislation, infant formulas need and can be enriched with nucleotides up to a maximum level of 5 mg/100 kcal [48]. In healthy adults, however, the dietary ingestion of more than 2 g/day of nucleotides would result in a temporary acute increase in circulating uric acid levels, so a limit of 2 g/day has been set by FAO/WHO/UNICEF, for example, for the dietary nucleotide consumption of single-cell protein [49]. Our data could allow for a general estimation of the beneficial effects of herring milt ingredients if they are to be used for human nutrition, e.g., as regulatory factors in the human microbiota in adults [50], to have a positive effect on suppressed immune function, for baby food development, etc.

On the basis of their total amino acid composition, as obtained by HPLC, the powdered herring milt and hydrolysates can provide most essential amino acids and taurine (Table S1). It is important to underscore one of the weaknesses of our study: that tryptophan could not be quantified in the total amino acid profile due to methodological limitations (the very strong acid pretreatment of the samples required for full solubilisation). The estimated essential amino acid content was therefore calculated without this amino acid. Tryptophan has been quantified only in the free amino acid profile for our samples. In general, the analysed samples presented similar total amino acid profiles, although the amounts of some of the amino acids were significantly different in the samples ($p < 0.05$). The sum of total amino acids (ΣTAA) in each sample is an indicator of the true protein content of the herring milt and is thus the one referenced in Table 1. The ΣTAA for the four samples ranged between 63 and 102 g/kg. The experimentally established ratios of essential to nonessential amino acids (0.45 in the initial herring milt to 0.66 in H1, 0.42 in H2, and 0.49 in H3) are slightly lower but still comparable to those previously reported for a mix of by-products from herring *C. harengus* (heads, bones, viscera, belly flap, tail): 0.69 in viscera and belly flaps from the same season's raw material [1], various cultured seaweed species (0.58 to 0.69) [51], or protein hydrolysates from Baltic herring (*C. harengus membras*) (0.67) and roach (*Rutilus rutilus*) (0.65 to 0.70, depending on the enzymes used) [52]. The most abundant essential amino acids (EAAs) in the samples were leucine, phenylalanine, and valine, while histidine (in HM, H1, and H2) and lysine (in H3) were the limiting amino acids in the absence of values for tryptophane. Nonessential amino acids (NEAAs) were predominant in the herring milt and occurred in higher quantities than in the EAAs.

Among the NEAAs, the arginine content was especially high, followed by that of glutamic acid and glycine. Therefore, the lysine/arginine ratios were quite low and ranged between 0.08 and 0.18 (Table S1). A high arginine content was also reported in Baltic herring hydrolysates [51]. Table S2 presents the results from the estimation of the protein quality on the basis of the EAAI values, which ranged from 83.9% to 112.1% (the difference from 100%, where observed, arises from the inclusion of the ΣSTD in the individual amino acid values, calculated from peak areas by UPLC), thus supporting the possible consideration of high-quality protein in the initial herring milt and hydrolysates H1 and H3. EAAI values greater than 90% may align with high-quality proteins, while proteins with EAAI values between 70% and 89% may be considered of moderate quality [51], as in the case of sample H2.

The total fat content of the herring milt samples (Table 1) was similar to that of other marine species. The fatty acid (FA) profile acquired by way of gas chromatography is presented in Table 2. The unhydrolysed herring milt (freeze-dried raw material, sample HMC) and hydrolysate H3 contained significantly more unsaturated fatty acids (3.28 g/100 g total unsaturated fatty acids against 1.59 g/100 g total saturated fatty acids in the milt; 1.71 g/100 g total unsaturated against 1.42 g/100 g total saturated fatty acids in H3), while hydrolysates H1 and H2 contained predominantly saturated fatty acids (1.18 g/100 g and 1.19 g/100 g total saturated fatty acids against 0.72 g/100 g and 1.02 g/100 g total unsaturated fatty acids in H1 and H2, correspondingly), showing the influence of the type of enzymes used on the fatty acid fraction. The lipid profile of the freeze-dried herring milt was thus higher in polyunsaturated fatty acids (PUFAs) than monounsaturated fatty acids (MUFAs), while this ratio was inverted in all the derived hydrolysates. The profile of milt is particularly high in bioavailable omega-3 fatty acids (2024.06 mg/100 g docosahexaenoic acid (DHA; 22:6n-3) and 884 mg/100 g eicosapentaenoic acid (EPA; 20:5n-3)), with health-beneficial properties for the human diet comparable to those of fresh fish roe from, for example, Alaska pollock, cod, pike, trout, or herring [53,54]. Of the n-6 PUFAs, linolenic acid (18:2 n6), which is of particular interest from a nutritional point of view, was prevalent in all the samples and yielded about 1% of the total FAs in the freeze-dried material (1.12% in H, 0.78% in H1, 0.96% in H2, and 1.08% in H3). However, these values are lower than those reported for cod, capelin, lumpfish, or Alaskan Pollok roe [53]. The ratios of n3/n6 PUFAs (ranging from about 19 in the HM down to 4.5 in hydrolysate H2) are higher than previously reported values for capelin, salmon, cod, and pike roe, but lower than those for lumpfish roe. It is well known that treatment with proteolytic enzymes affects the lipid content of raw materials by generally reducing it and promoting a higher protein concentration in the final product.

Table 2. Experimentally determined fatty acid composition of total lipids in herring milt and the derived enzymatic hydrolysates (n = 3).

Fatty Acid (FA)	Unhydrolysed Herring Milt (HMC) [mg/100 g ds]	Sample H1 [mg/100 g ds]	Sample H2 [mg/100 g ds]	Sample H3 [mg/100 g ds]
Saturated				
14:0	56.32	39.86	50.41	108.85
15:0	33.39	25.78	28.38	39.42
16:0	1347.44	994.87	998.43	1124.38
18:0	154.35	116.72	115.62	143.49
20:0	<LOQ *	<LOQ	<LOQ	<LOQ
22:0	<LOQ	<LOQ	<LOQ	6.71
24:0	<LOQ	<LOQ	<LOQ	<LOQ
Total Σ saturated FA	**1591.49**	**1177.22**	**1192.84**	**1422.85**

Table 2. *Cont.*

Fatty Acid (FA)	Unhydrolysed Herring Milt (HMC) [mg/100 g ds]	Sample H1 [mg/100 g ds]	Sample H2 [mg/100 g ds]	Sample H3 [mg/100 g ds]
Monounsaturated (MU) FA				
16:1*n*-9	12.53	7.23	7.55	11.49
16:1*n*-7	65.94	42.73	42.32	79.39
18:1*n*-9	441.89	269.41	261.09	425.32
18:1*n*-7	511.88	367.41	353.58	358.38
20:1*n*-11	26.06	17.90	17.01	31.01
20:1*n*-9	103.38	62.80	64.01	206.89
20:1*n*-7	<LOQ	<LOQ	<LOQ	7.35
22:1*n*-11	46.46	27.84	37.04	191.73
22:1*n*-9	9.40	8.48	10.33	25.64
24:1*n*-9	23.69	19.63	19.82	33.68
Total Σ MUFA	**1241.22**	**823.43**	**812.75**	**1370.88**
Polyunsaturated (PU) FA				
18:2*n*-6	68.24	16.79	21.16	33.91
18:3*n*-6	12.38	<LOQ	<LOQ	<LOQ
20:2*n*-6	15.05	5.95	8.46	14.70
20:3*n*-6	<LOQ	<LOQ	<LOQ	<LOQ
20:4*n*-6	52.49	<LOQ	7.55	12.41
22:4*n*-6	<LOQ	<LOQ	<LOQ	<LOQ
22:5*n*-6	16.20	<LOQ	<LOQ	<LOQ
Total Σ *n*-6 PUFA	**164.36**	**22.73**	**37.17**	**61.02**
18:3*n*-3	28.73	<LOQ	5.98	14.61
18:4*n*-3	20.17	<LOQ	<LOQ	8.64
20:3*n*-3	7.34	<LOQ	<LOQ	8.18
20:4*n*-3	41.95	<LOQ	6.75	13.05
20:5*n*-3	884.00	47.94	58.53	79.95
21:5*n*-3	12.91	<LOQ	<LOQ	<LOQ
22:5*n*-3	98.80	6.08	6.69	11.26
22:6*n*-3	2024.06	71.99	89.08	142.57
Total Σ *n*-3 PUFA	**3117.96**	**126.01**	**167.03**	**278.25**
16:2	<LOQ	<LOQ	<LOQ	<LOQ
16:3	<LOQ	<LOQ	<LOQ	<LOQ
16:4	<LOQ	<LOQ	<LOQ	<LOQ
Total Σ PUFA	**3282.32**	**148.74**	**204.20**	**339.26**
Ratio saturated/unsaturated	**0.35**	**1.21**	**1.17**	**0.83**
Ratio *n*-3/*n*-6	**18.97**	**5.54**	**4.49**	**4.56**
TOTAL [Σ FA]	**6115.03**	**2149.39**	**2209.79**	**3132.99**

* LOQ = limit of quantification of the fatty acid analysis (0.06 mg/100 g).

3.1.2. Peptide Profile by Size Exclusion Chromatography

The peptide profile of the herring milt hydrolysates (Table 3, Figure S1) was determined in view of the functional (bioactivity and solubility) estimations of the protein fraction, as well as the possible presence of undigested high-molecular-weight proteins, which could have an allergenic effect in the case of human consumption. Of specific interest were the small peptides (below 1 kDa), due to the previously reported interesting techno-functional characteristics and health benefits of the consumption of such peptides

in fish, other herring milt, and roe hydrolysates [12,18,20,21,55]. Significantly different values for the peptide molecular weight distribution were determined only for the control sample HMC in comparison to the hydrolysates ($p < 0.05$). The observed differences among the percentages of each fraction in the hydrolysed samples H1 to H3 (the fraction below 1 kDa being of main interest for this study) did not show a statistical difference. The size distribution of the peptides in the present study is comparable to that of some previously reported hydrolysates; however, it is highly dependent on the type of protease, treatment duration, etc. Atlantic cod (*Gadus morhua*) hydrolysates and herring roe hydrolysates have shown health-beneficial effects, in vitro and in vivo, in adults with irritable bowel syndrome (IBS), with 75% to 78.78% of the peptides in the profile having molecular weights below 1 kDa [56,57]. The second most abundant fraction in the samples was that of peptides with sizes between 1 and 5 kDa (a total of 54%, 41%, and 31% for H1, H2, and H3, respectively). By comparison, the cod hydrolysate prepared with Protamex® (Novozymes AS) and tested for health benefits comprised about 20% peptides with a 2 kDa molecular weight [57]. The potential allergenicity of the protein hydrolysates would require further testing prior to any incorporation into products for human consumption or any definition of a recommended daily intake.

Table 3. Peptide profile by size exclusion chromatography on UPLC of the NVG herring milt hydrolysates ($n = 9$).

MW Distribution [kDa]	H1 [%]	H2 [%]	H3 [%]
<0.200	8.07 ± 0. 28	13.04 ± 0.06	15.00 ± 0.18
0.2–0.5	12.02 ± 0.31	10.37 ± 0.04	9.07 ± 0.34
0.5–1.0	37.27 ± 1.04	38.61 ± 0.43	35.41 ± 2.29
1.0–2.0	29.01 ± 0.60	26.20 ± 0.07	20.39 ± 0.61
2.0–5.0	9.96 ± 0.11	7.73 ± 0.04	6.28 ± 0.20
5.0–10.0	1.82 ± 0.05	1.8 ± 0.02	0.34 ± 0.16
10.0–15.0	1.43 ± 0.26	1.54 ± 0.04	3.05 ± 0.45
15–20	0.28 ± 0.06	0.40 ± 0.12	3.20 ± 1.62
20–50	0.10 ± 0.12	0.26 ± 0.07	1.27 ± 0.08
50–100	0.03 ± 0.01	0.08 ± 0.02	4.30 ± 0.58
100–200	0.01 ± 0.01	0.00 ± 0.01	1.72 ± 1.19
TOTAL % fraction above 1 kDa	42.66 ± 1.22	38.00 ± 0.39	40.53 ± 4.89
TOTAL % fraction below 1 kDa	57.36 ± 1.63	62.02 ± 0.53	59.48 ± 2.81

The LOD of the method is 0.02.

3.1.3. Presence of TMA/TMAO and Heavy Metals

Many fish contain significant amounts of TMAO that can be reduced by spoilage bacteria to TMA or dimethylamine (DMA), biogenic amines with a distinctive stale and unpleasant "fishy" flavour [58]. These compounds are water-soluble and can pass into the soluble protein fractions during processing. Blood-circulating levels of TMAO are a contributing factor in cardiovascular disease (CVD), leading to increased toxicity in patients with chronic kidney disease, and can also be generated by some human gut microbiota species [59,60]. Regarding TMAO, its maximum limits have not been established yet by EU legislation, while the total volatile basic nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N) are considered to be freshness indicators with set limits in unprocessed fishery and seafood products from certain aquatic species (Implementing Regulation EU 2019/627 [61]). It is important to control the TMA/TMAO dietary intake [62]. Therefore, we have analysed both compounds to estimate the total amount of TMA/TMAO in the initial herring milt

and prepared hydrolysates (Table 4). The values for the initial herring milt were the highest (302 mg/100 g); however, there was no significant difference between these and the values for hydrolysates H1 and H3. Only the total TMA and TMAO value in hydrolysate H2 was significantly lower (179 mg/100 g; $p < 0.05$). This may be due to the established food-grade proteolytic enzyme combination used and related hydrolysis conditions (pH, ionic strength, final amino acid composition, etc.). The TMA/TMAO values in the herring milt and hydrolysates that we obtained are comparable with these values in mackerel backbone hydrolysate (179 mg/100 g dry product), salmon viscera hydrolysate (250 mg/100 g dry product) [58], and those from previous reports on herring milt and hydrolysates [3,8].

Table 4. Proximate composition in terms of heavy metals and TMA/TMAO of the autumn NVG herring milt and hydrolysates (results are expressed as mean ± SD from $n = 3$ replicates for each analysis).

Sample	Mercury (Hg) (Total Content) [mg/kg]	Arsenic (As) (Total Content) [mg/kg]	Lead (Pb) (Total Content) [mg/kg]	Cadmium (Cd) (Total Content) [mg/kg]	TOTAL (TMA + TMAO) as Freshness Indicators [mg/100 g] §
HMC	0.047 ± 0.02 [a]	3.9 ± 1.2 [a]	<0.020	0.15 ± 0.05 [a]	302 [a]
H1	0.042 ± 0.02 [a]	3.1 ± 0.9 [a]	<0.020	0.11 ± 0.04 [a]	233 [a]
H2	0.025 ± 0.01 [b]	3.9 ± 1.2 [a]	<0.020	0.12 ± 0.04 [a]	179 [b]
H3	<0.020 *	3.8 ± 1.1 [a]	<0.020	0.07 ± 0.03 [b]	233 [a]
Maximum levels allowed in food ¥ [mg/kg]	0.30 (muscle meat of *C. harengus*)	0.020 (products to be placed on the market as powders)	0.30 (muscle meat of fish, or portion intended for consumption)	0.050 (muscle meat of fish, or portion intended for consumption)	60 mg of total volatile nitrogen/100 g of whole fishery product used directly for the preparation of fish (oil) for human consumption

* LOQ = limit of quantification of the analysis (0.020 mg/kg) [a] and [b]—significantly different value. ¥ As per the consolidated Commission Regulation (EU) 2023/915) [63]. § As per Implementing Regulation EU 2019/627 [61].

Table 4 also shows the results for the heavy metal content in the herring milt and hydrolysates compared to EU requirements for foodstuffs (EU regulation 2023/915). The heavy metal content depends on the proximity of fishing sites and heavy human industrial activities, the season, ocean currents, etc., which cause cumulative marine environment contamination. The level of mercury measured by us in the *C. harengus* milt is significantly lower than the legally set limits, while lead has not been detected. The experimentally measured levels of arsenic and cadmium are higher than the levels of the other two heavy metals. The text of the regulation EU 2023/915 does not refer specifically to fishery products with regard to arsenic content limits; however, it is well known that arsenic levels are high in fish and seafood, with a recently published example in marine fish of up to 10.26 mg/kg [64].

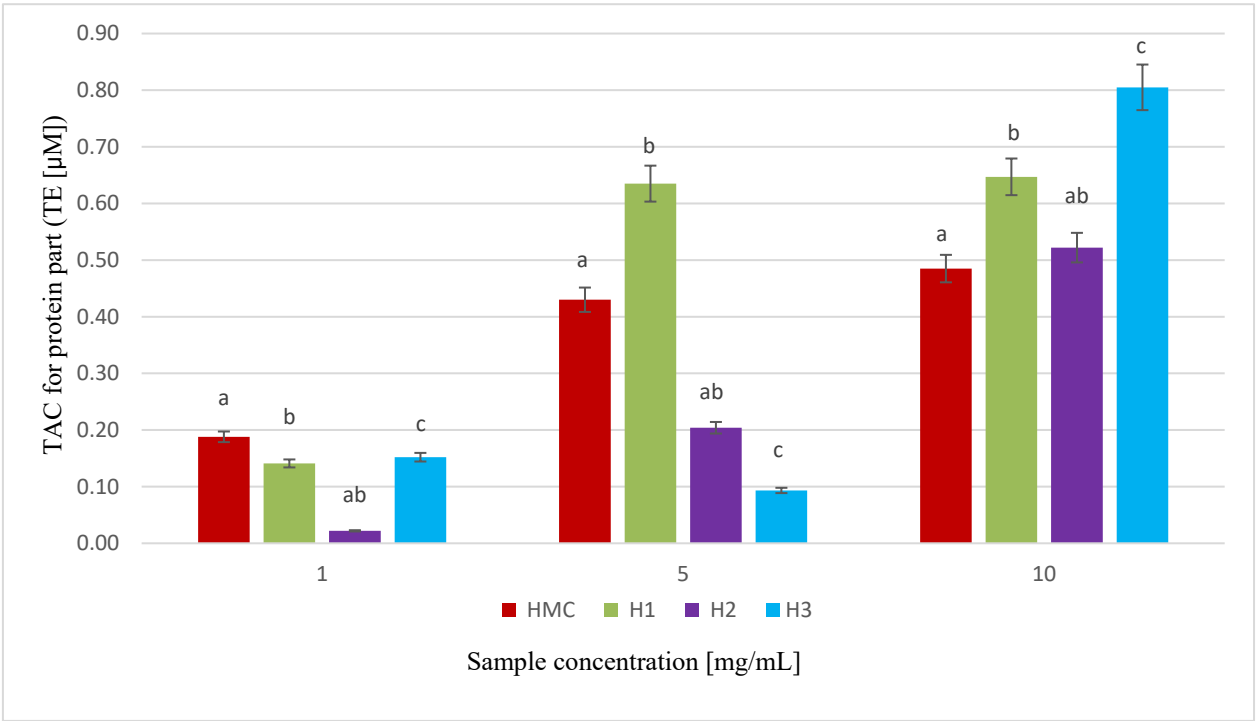
Regarding cadmium, reports of its accumulation in fish reproductive organs are abundant [65]. After entering the fish's body, most Cd ions form complexes with proteins (metallothionein) or, to a smaller extent, coordination compounds through ionic bonds with nucleosides, proteins, and other substances [66]. The legislative limit presented in Table 4 for Cd is for whole fish or sections of it intended for direct consumption and does not refer to "dried, diluted, processed and compound food", the category into which our samples will fall. For such food categories, the regulation EC 2023/915 refers to Article 3 of the same document, in which food business operators are required to provide and justify the specific concentrations of the contaminant. The Joint FAO/WHO Committee on Food Additives (JECFA) [67] and EFSA's CONTAM panel (based on a scientific opinion from 2011) have set a provisional tolerable weekly intake (TWI) value for cadmium of 2.5 µg/kg body weight (bw). Given all the known toxic effects of heavy metal accumulation in the human body, dietary exposure to heavy metals should be minimised as much as possible [68]. The method of determining the total heavy metal amounts in this study

was based on inductively coupled plasma mass spectrometry (ICP-MS) analysis, which does not distinguish between organic and inorganic forms of the metals. Thus, further studies should include metal form speciation in the samples, the seasonal variability of the levels, and a risk assessment of the potential contribution of heavy metal exposure through herring milt intake.

3.2. Bioactivity

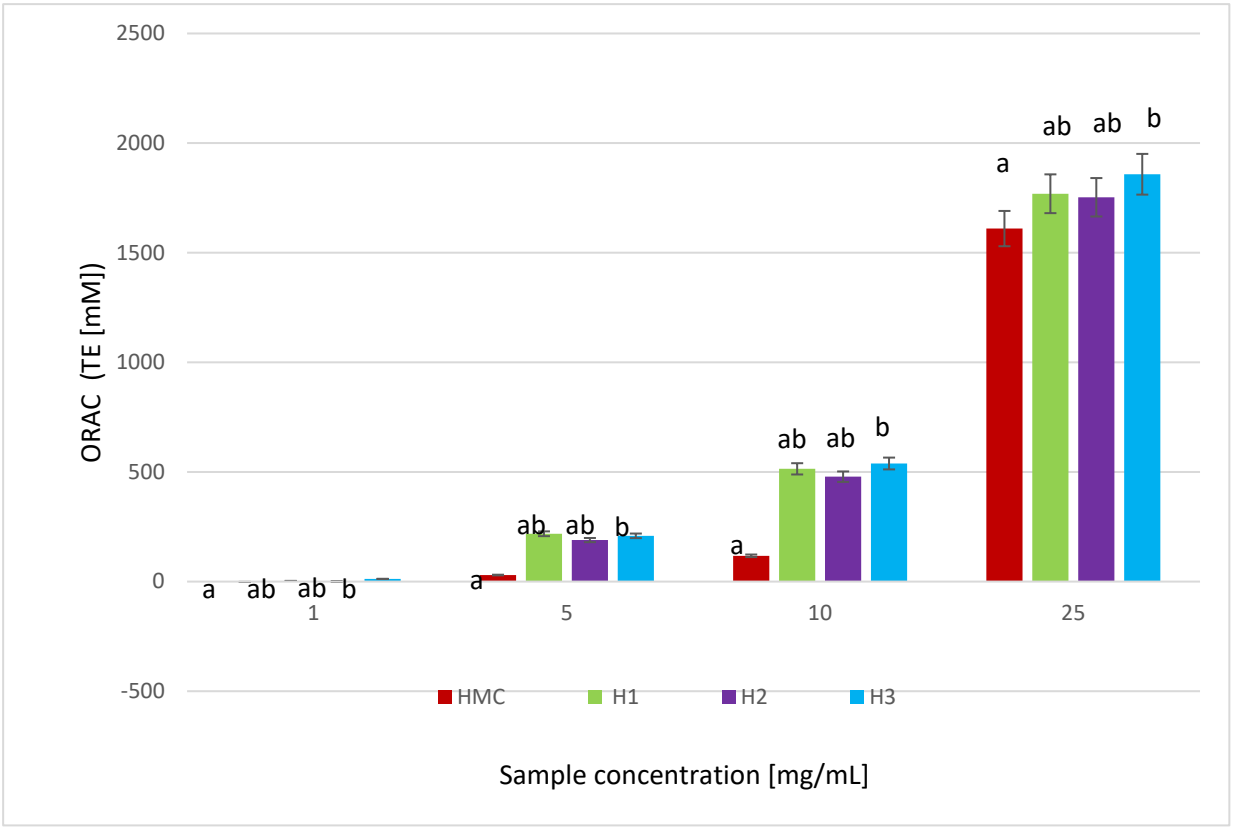
3.2.1. Antioxidant Capacity

The following three different in vitro analytical protocols were applied to perform the natural antioxidant capacity screening in the herring milt and hydrolysates: the total antioxidant capacity (TAC) kit, the ORAC protocol (widely used for the measurement of this parameter in biological samples and food products), and the DPPH assay protocol, usually adapted to samples with some organic and fat content. The results for the detected bioactivity in the samples from the three assay protocols are compared in Figure 1 and expressed in Trolox equivalents (TE). The bioactivity was dependent on the final sample concentration used for the testing and increased with increasing sample concentration ($p < 0.05$ for all assays). This is probably also due in part to the different solubility of the samples in the assay buffers at the chosen concentrations, and the related increase in bioactive compounds, independent of the use of a standardised solubilisation procedure. A certain level of large protein degradation was detected in the dried HMC sample during the peptide profiling analysis and, therefore, bioactivity was also analysed in this sample type.

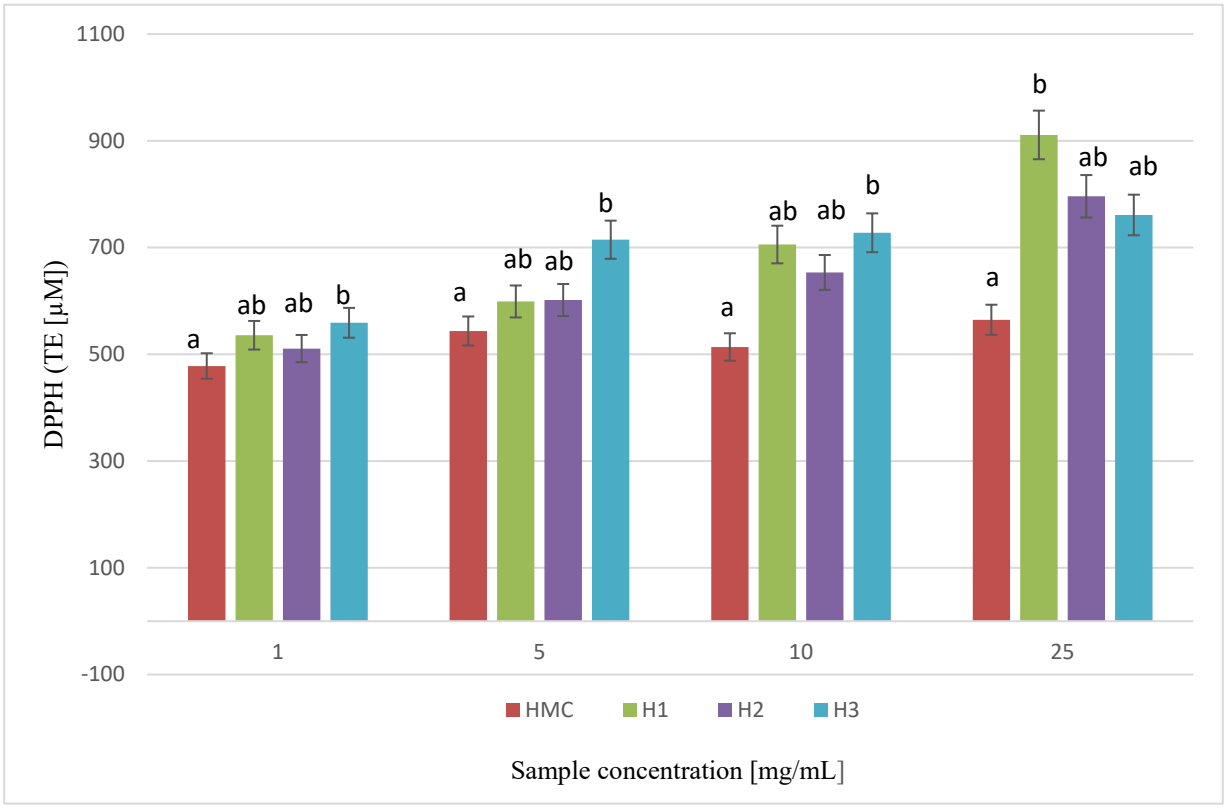


(**a**)

Figure 1. *Cont.*



(**b**)



(**c**)

Figure 1. Antioxidant capacity of herring milt powder and hydrolysates. (**a**) TAC values for the samples, with concentration dependence, as determined for the protein part of the samples; (**b**) comparison of the activity of the samples, as determined by the ORAC assay method; (**c**) comparison of the activity of the samples, as determined by the DPPH assay method. The bars indicate standard deviations (SDs). Significantly different values between sample types are marked with small letters ($p < 0.05$).

The total antioxidant capacity (TAC) method can discern the bioactivity contributed by the protein and small organic molecules in the samples by the addition of a protein-masking reagent when samples are resuspended in the corresponding hydrophilic buffering system at concentrations above 1 mg/mL (Figure 1a). Thus, we have established that the contribution of organic molecules in the samples to the total antioxidant activity could vary significantly (mean 72% for HMC, 26% for H1, 50% for H2, and 40% for H3). This is most probably due to the varying degrees of hydrolysis, the increased release of peptides with potential functional properties, and the presence of pigments (e.g., from blood or astaxanthin), metal ions (such as iron or other metals as stated in Table 1), or other small organic compounds in different concentrations. The hydrolysate H3 shows the highest concentration-dependent trend ($p < 0.05$) for the antioxidant activity associated with the protein part of the sample.

The ORAC method had a buffer for sample resuspension and dilution, in which the milt samples showed high solubility. The results obtained for the hydrolysates by the ORAC method were low if sample concentrations below 5 mg/mL were used for the testing (Figure 1b). The R^2 of the standard Trolox line achieved by this protocol was 0.98. The antioxidant activity detected at 25 mg/mL was quite similar among the samples, ranging from 1.6 M TE for the initial powdered herring milt, to 1.8 M TE for H1 and the same value (1.8 M TE) for H2, to 1.9 M TE for H3. Although the highest bioactivity was measured by this method from all three antioxidant protocols, the traditional ORAC protocol relies on the quantification of a normalised Area Under the Curve (net AUC) in fluorescence units and can result in ambiguous interpretations [69].

According to the DPPH method, the values measured for the TE were higher than those obtained with the TAC method and lower than those obtained with ORAC (Figure 1c; between 0.3 and 0.9 mM TE), with sample H1 showing the highest antioxidant value at 25 mg/mL (0.911 mM TE) and sample H3 exhibiting statistically different ($p < 0.05$) antioxidant values throughout the tested concentration range. Thus, lower concentrations of H3 could be used for inclusion in food, achieving a similar final radical scavenging capacity in the matrix, if needed. For sample H1, concentrations above 25 mg/mL brought to saturation in the detection with this method. This can be explained by the different observed solubilities of the samples in the systems containing organic solvents, even if they are polar (methanol).

In total, the measured TEs for the herring milt samples by DPPH and ORAC are within the range of previously reported values for Pacific hake [70], tilapia hydrolysates [71], and Alaska Pollack [72]. It is well known that enzymatic hydrolysis largely increases the measurable (and bioavailable) peptides with antioxidant capacity and, therefore, hydrolysis conditions (duration, number and combinations of enzymes, pH and temperature, and post-processing purification) largely affect these properties of the protein concentrates. The method of drying (spray-drying vs. freeze-drying or other method) may also be significant for the final antioxidant capacity of the samples [23,73].

3.2.2. ACE I Inhibitory Activity In Vitro

Angiotensin-converting enzyme (ACE) I inhibitors are widely used in the medical treatment of hypertension. The natural ACE I inhibitors, abundant in various food sources, are safer than medicines for blood pressure reduction and have no side effects. However, their bioavailability and respective minimal effective concentrations (MECs) should be confirmed prior to any suggested applications through nutraceutical or functional food consumption. Our results from the measured ACE I inhibitory activity in the powdered herring milt and hydrolysates are included in Figure 2. The highest level of activity was detected in sample H1 (equivalent to 0.375 µM lisinopril, an antihypertensive medication). The powdered herring milt also showed relatively high bioactivity (HMC, 0.150 µM lisinopril equivalents), comparable but slightly higher than that detected in sample H2 (0.110 µM lisinopril equivalents). By way of comparison, lisinopril can be prescribed in oral solution form at a concentration of 1 mg/mL, corresponding to a 2.5 mM concentration [74]. In vivo

supplementation in mice and rat obesity models with fractions of herring milt hydrolysates (after ultrafiltration) [21,22], or hydrolysates from herring rest raw materials [75], has already been proven to be efficient in reducing insulin tolerance and kidney damage and improving glucose regulation.

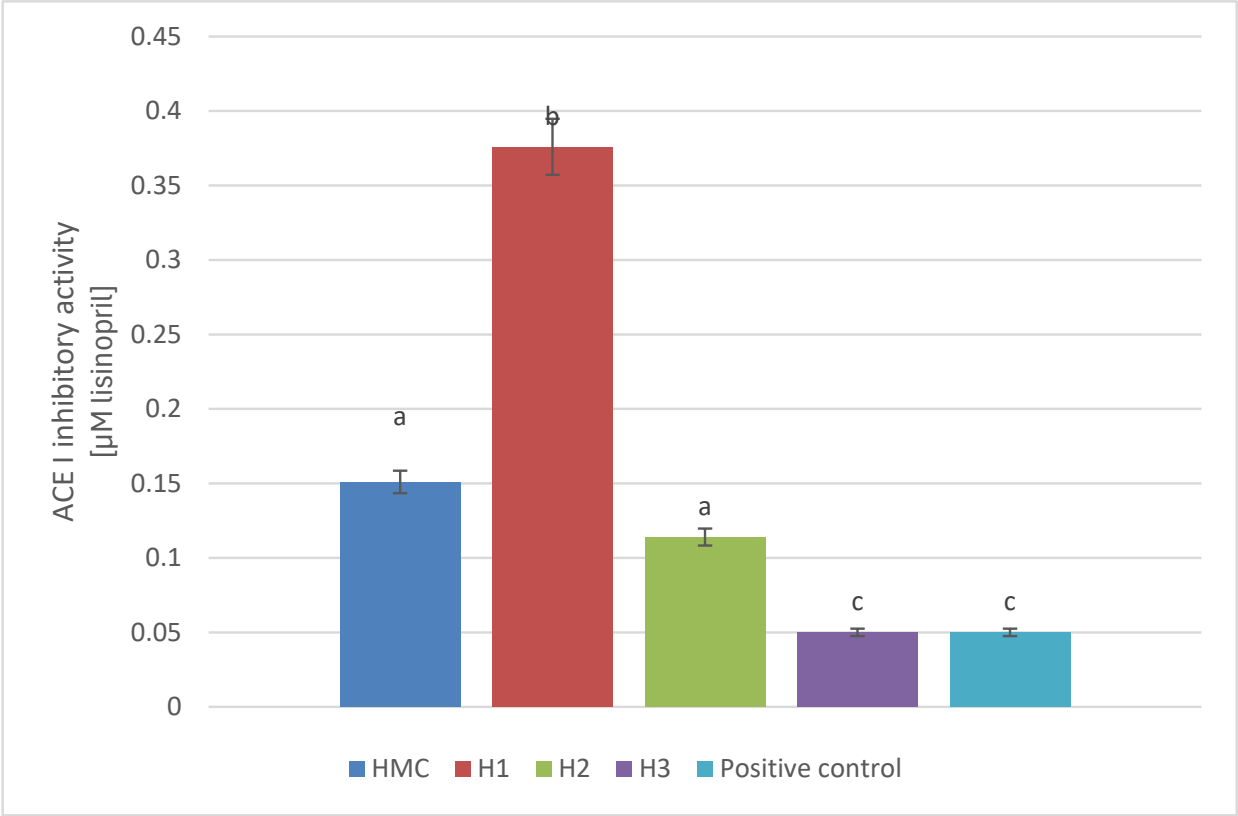


Figure 2. ACE I inhibitory activity of herring milt powder and enzymatic hydrolysates (HMC in dark blue, H1 in red, H2 in green, H3 in purple, and Lisinopril control in light blue). Bars indicate SD. Significantly different values are marked with small letters ($p < 0.05$).

3.3. Hydrophobicity

Although the hydrophobicity of a protein is highly dependent on the amino acid composition and can be estimated from it (e.g., as the values presented in Tables S1 and S3), the analytical measurement of this parameter by the ANS method can be considered a predictor of protein-exposed hydrophobicity in dissolutions with high ionic strength or emulsions [76]. Thus, the results for the protein-exposed hydrophobicity of the three hydrolysates, derived from NVG herring milt, are presented in Figure 3. The ANS hydrophobicity was determined in a hydrophilic system by using a 50 mM sodium phosphate buffer (pH 8). It is visible that the hydrophobicity is concentration-dependent, and that it is comparable with the three hydrolysate types at low concentrations (1–10 mg/mL) in the tested buffering system but increases significantly at sample concentrations of 25 and 50 mg/mL. This phenomenon is not due to the low initial solubility of the samples in the buffer since the insoluble parts were removed by centrifugation prior to measurement. Based on the highest content of hydrophobic amino acids (Table S3), the samples with the greatest hydrophobic characteristics would be the freeze-dried raw material (herring milt) and hydrolysate H3 (~38%), followed by hydrolysates H1 (29%) and H2 (24%). The protein-exposed hydrophobicity in the solution, at pH 8, of all the samples is the highest for the 50 mg/mL concentration and corresponds well with the behaviour expected on the basis of the total hydrophobic amino acid content. These properties are technologically relevant to any posterior inclusion of herring milt powder and protein concentrates in

liquid food matrices or emulsions for functional food development. It will be important to monitor possible complexation and insoluble particle formation should higher sample concentrations or temperature treatment be required for inclusion in specific food matrices. This may also be relevant in further encapsulation trials for bioactivity preservation.

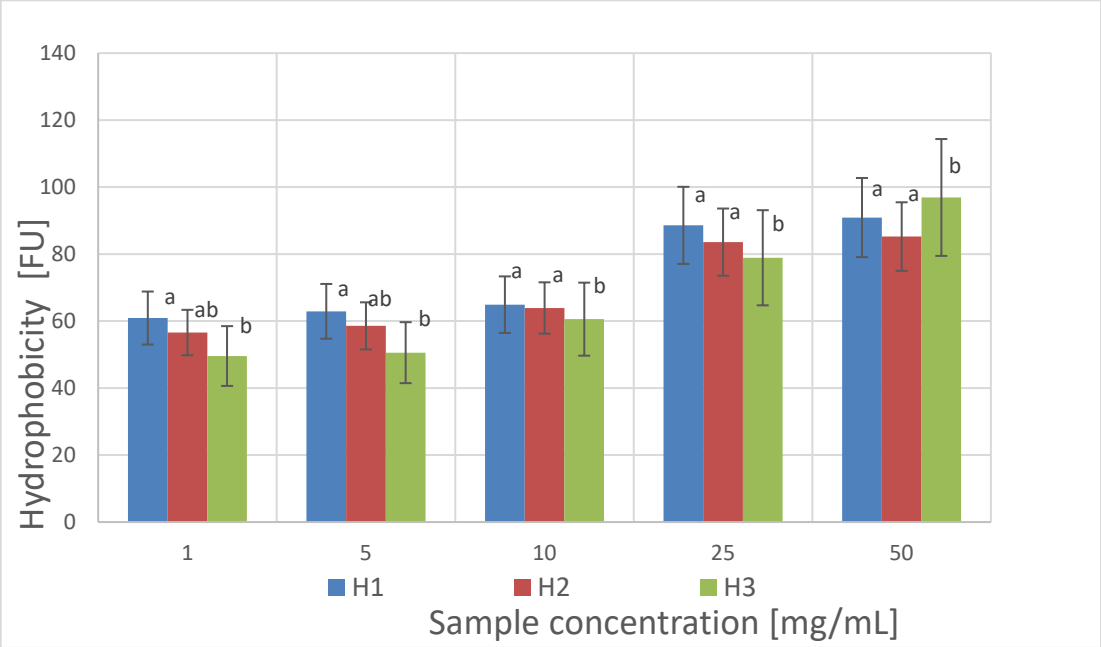


Figure 3. Concentration dependence of the protein-exposed hydrophobicity of herring milt hydrolysates (measurements per sample/concentration, $n = 3$). The bars indicate SD. Significantly different values are marked with letters ($p < 0.05$).

3.4. Estimation of Sensory Properties

Free amino acids derive from the hydrolysis of proteins and polypeptides under internal dipeptidase and aminopeptidase activity and are the main factors in taste perception (sweetness, umami, bitterness, and sourness), and are also precursors to volatile compounds such as alcohols, aldehydes, ketones, acids, and esters [1]. The experimentally defined composition of free amino acids in the studied herring milt samples is included in Supplementary Table S3 and is relatively high. The highest free amino acid content is thus found in sample H3 (7.2% in comparison to the total amino acid composition) and has an increasing trend from the freeze-dried raw material (HMC; 1.8% in comparison to TAAs) towards the protein concentrate H1 (approximately 2.3% of the TAAs), with H2 appearing similar to the HMC total FAA content (approximately 1.6% from TAAs). The composition of the free amino acids in the hydrolysates, however, varies quite significantly from that in the initial raw material. They are rich in amino acids such as Arginine (predominant in all the samples) and Alanine, followed by Glycine and Glutamic Acid, and have relatively high values of Leucine, Phenylalanine, Taurine, and Tyrosine. Argine, Phenylalanine, and Tyrosine, which are known to impart a bitter taste in synergy with nucleotides, are also taste-active [77]. Alanine, Glycine, and TMAO contribute to a sweet taste. The Glutamic Acid content, if considered separately, could contribute to a sour taste. However, the relatively high salt and nucleotide content in most of the samples (approximately 10% in the initial raw material and two of the protein concentrates) could interact with the glutamate and convey or synergistically enhance the umami taste [75,78]. Taurine, in combination with Glutamic Acid and nucleotides, or through metabolic conversions, can increase the umami taste of these products, and has, in parallel, important psychological impacts on consumers [75,77,79–81]. Other similar fish and seafood products have also been reported as having a predominant umami taste, with certain characteristics of bitterness [15,77].

Further testing with trained sensory panels and detailed analyses of volatile compounds could yield more precise knowledge about the sensory perceptions from the consumption of herring milt in general and the prepared hydrolysates specifically.

It can be seen that the enzyme combinations used by us for the preparation of samples H1, H2, and H3 have a clear effect on the free amino acid content. The enzymes were preselected for their known capacity to impart less bitterness and better sensorial attributes to the hydrolysates from a raw material with a high initial DNA content, as per advice from the enzyme producer (specifically, the combinations of the Exocut-TRL® and the Endocut-05L® and 07L® (samples H2 and H3)). The use of Endocut-02L® was tested as a "positive" control (sample H1) for achieving the highest degree of hydrolysis of the proteins in the initial herring milt. This sample was expected to have the highest levels of odorous compounds and bitterness. The unhydrolysed milt was also used in the proximate composition analyses by way of a comparison to judge the effects of the enzymes upon the nutritional and functional composition of the final products. Since it is well known that sensorially undesirable compounds such as TMA/TMAO and DMA are present in milt, the development of potential powdered ingredients for functional food could be a solution to modifying the negative sensory perception of many fresh food products, based on milt. The incorporation percentage of the samples to be selected, the pH, and the food matrix to be used for functional food development will alter the final sensory properties to a great extent.

4. Conclusions

This study describes the qualitative, nutritional, sensory, and bioactive characteristics and aptitude of NVG herring milt in powdered form and of three novel food-grade enzymatic hydrolysates for the development of functional foods or nutraceutical products. The tested enzymatic hydrolysis methodology, followed by drying, was selected because it is a simple and scalable approach. It does not involve the overprocessing of the raw material and extends its stability. The freeze-dried herring milt and hydrolysates could provide high-quality protein with most of the essential amino acids and taurine present. The peptide fraction below 1 kDa is predominant in all of the hydrolysates. The general mineral content of the four potential food ingredients can cover from one-third up to one-half of the recommended dietary intake of copper, zinc, magnesium, iodine, and calcium, minerals with established health claims for human nutrition. The fatty acid profile of powdered milt is particularly high in bioavailable omega-3 fatty acids, such as DHA and EPA, with health-beneficial properties for the human body. The level of mercury measured is significantly lower than the legally set limits, while lead has not been detected. The total arsenic and cadmium have been measured at levels above limits defined by food legislation. Thus, the amounts of milt products to be used as potential ingredients should be the subject of careful consideration. The determined functional properties of the four ingredients, such as the in vitro antioxidant and ACE-I inhibitory bioactivities, are of interest for the development of functional food products and nutraceuticals. Additionally, based on the estimation of the amino acid and biogenic amine content, it has been concluded that there are several umami-contributing components leading to a possible balance between the bitter taste with a sweet or salty taste. Further studies of the large-scale feasibility and the sensory properties of the developed herring milt ingredients and of food matrices including them should be carried out to confirm the sensory aptitude results.

The bioaccessibility of the two heavy metals detected (As and Cd) and some minerals (e.g., Se) in the samples and the antioxidant and ACE I inhibitory capacity should be further estimated following the application of the standardised simulated human digestion protocol, with the use of a Caco-2 cell culture model or 3D intestinal models. Only after assessing the exact uptake of the minerals, their organic/inorganic form distribution in the raw material, and the possible allergenicity of the herring milt samples would it be possible to suggest concrete functional food applications, such as for metabolic disease prevention or nutritional fortification. Overall, this work represents complementary information for

the future utilisation of *C. harengus* powdered milt (possibly removed directly from a fish-filleting line) and some of its protein hydrolysates as food ingredients.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fishes9110456/s1: Table S1: Total amino acid profile of the NVG herring milt and hydrolysates; Table S2: Amino acid score (AAS) and essential amino acid index (EAAI) of NVG herring (*C. harengus*) milt and hydrolysates; Table S3: Free amino acid profile of the NVG herring milt and hydrolysates by UPLC analysis (ds = dry sample); Figure S1: Peptide profile of herring milt and hydrolysates by electrophoretic analysis, by use of the BioRad Mini Protean system.

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