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Research article

A standardized, innovative method to characterize the structure of aquatic protein hydrolysates

Alexandre Leduc [a,b], Vincent Fournier [b], Joël Henry [a,*]

[a] Normandie University, UNICAEN, Sorbonne Universités, MNHN, UPMC Univ Paris 06, UA, CNRS, IRD, Biologie des Organismes et Ecosystèmes Aquatiques (BOREA), 14032, Caen, France
[b] DianaAqua, Symrise Group, Elven, France

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ABSTRACT

The performances of protein hydrolysates highly depend on their peptide composition (amount, size and diversity), which itself closely depends on raw material origin and the hydrolysis parameters of the manufacturing process. The current analyses that characterize protein hydrolysates provide information on the level of hydrolysis (degree of hydrolysis, DH). However, they need additional describers to better characterize peptide profiles and product standardization. To reach this objective, we developed a fast and standardized method to characterize the abundance and the diversity of low-molecular-weight peptides in protein hydrolysates. This method innovatively combines classical HPSEC and nLC-ESI-MS analytical tools to characterize any kind of hydrolysate, whether solid or liquid, in terms of peptide level and diversity, and then merge peptides into 2D diagrams to visualize comparisons between protein hydrolysates. The targeted applications of this new tool for characterizing complex protein hydrolysates are (i) verifying the standardization of the produced products across batches, and (ii) analyzing and understanding the consequences of the modifications of the hydrolysis process on the molecular profiles of the generated peptides. The sample standardization described in this study is therefore an essential prerequisite for the functional characterization of hydrolysates *in vitro*.

1. Introduction

Interest for protein hydrolysates has been growing in the last decades. Whatever their origin (plants, marine animals, meat, milk, etc.), protein hydrolysates have demonstrated both nutritional and functional benefits for human food and animal feed (Shahidi and Janak Kamil, 2001). Hydrolysis of raw materials produces peptides with biological activities (antimicrobial, antioxidant, hypotensive…) (Kim and Mendis, 2006). Protein hydrolysates of marine origin are the most represented hydrolysates on the food and feed markets. In aquaculture feeds, they are used for their palatability, nutrition and health performances (Cahu et al., 1999; Refstie et al., 2004; Choi et al., 2009; Gisbert et al., 2012; Khosravi et al., 2015a, b, 2017).

Enzymatic hydrolysis of proteins yields a mixture of free amino acids (AA), of di-, tri- and oligo-peptides, and increases the occurrence of polar groups and the solubility of hydrolysate compounds (Kristinsson and Rasco, 2000). In aquaculture feeds and other applications, the functional properties of protein hydrolysates directly result from their amino acid and peptide composition (Espe et al., 1999), as well as from peptide molecular weight (Liaset et al., 2000). The advantage of hydrolysis is that different profiles of peptide mixtures may be produced from a same raw material. These deviations of peptide profiles are highly dependent on the processing parameters (enzyme specifications, hydrolysis temperature and duration, raw material/water ratio, etc.), and could result in finished products with very highly diverse functional properties.

The hydrolysis level of proteins is usually characterized by the degree of hydrolysis (DH), *i.e.* the number of cleaved peptide bonds relatively to the number of initial peptide bonds (Mullally et al., 1995). The DH is determined at the end of hydrolysis. It does not provide any information on the size of the peptides cleaved during hydrolysis, so that it cannot be applied to fine-tune the parameters of the hydrolysis process. To further characterize protein hydrolysates, the peptide profile still remains the most relevant parameter. Several methods can be applied to analyze peptide profiles: sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Liceaga-Gesualdo and Li-Chan, 1999), high-performance size exclusion chromatography (HPSEC) (Guérard et al., 2001), or mass spectrometry (Robert et al., 2015). However, these methods utilized alone are not sufficient to fully characterize protein hydrolysates and

* Corresponding author.
E-mail address: joel.henry@unicaen.fr (J. Henry).

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should be coupled with one another to reach a better overview of protein hydrolysate specifications. Complex protein hydrolysates can indeed contain more than 1,000 peptides of different sizes (Robert et al., 2014, 2015).

Peptide extraction from protein hydrolysates or animal tissues is well documented, and many protocols deal with the purification of different mass ranges or specific peptides (see German et al., 2015 for a review). Nevertheless, it could be difficult to compare quantitative results when peptide amounts in the hydrolysates vary significantly.

This study presents a fast and standardized method to characterize complex protein hydrolysates. We used different products of various origins containing different protein concentrations to validate a method based on the use of two indexes taking low-molecular-weight (LMW) peptide abundance and diversity into account.

2. Materials and methods

2.1. Protein hydrolysate sampling

Raw materials of different origins and different hydrolysis processes were selected to compare a wide panel of protein hydrolysates. The finished products were provided by DianaAqua (Symrise group, Elven, France). The protein hydrolysates were produced as follows: two white shrimp (*Litopenaeus vannamei)* hydrolysates, produced by two different enzymatic treatments; one Nile tilapia (*Oreochromis niloticus*) hydrolysate; two salmon (*Salmo salmar*) hydrolysates produced from 2 different production sites and raw materials but using the same enzyme; two tuna (*Thunnus sp.*) hydrolysates produced with different hydrolysis times and from different raw materials; one Argentinean shortfin squid (*Illex argentinus*) hydrolysate; one cod (*Gadus morua*) hydrolysate; and two Antarctic krill (*Euphausia superba*) hydrolysates, with an additional post hydrolysis thermal treatment for one product. Protein hydrolysates were produced from the cephalothorax of white shrimp, from the frames of fish and squid from food processing plants, or from whole animals (krill).

2.2. Extraction of hydrolytic peptides and normalization of concentrations before analysis

The method is summarized in Figure 1. Peptides were extracted prior to mass spectrometry and liquid chromatography analyses. One gram of hydrolysate powder or 1 ml of liquid hydrolysate was homogenized in 10 ml of 0.1% HPLC-grade trifluoroacetic acid (TFA – Sigma-Aldrich, Saint-Louis, Missouri, USA) solution for 10 min. Samples were centrifuged 10 min at 20,000 × g. Whatman paper-filtered supernatants were diluted to 1 absorbance unit (AU) at a wavelength of 214 nm in an HPLC-grade 0.1% formic acid (FA – Sigma-Aldrich) solution to obtain normalized samples. A constant volume of 1 AU-diluted hydrolysate extract (50 ml) was concentrated on a Sep-Pak C18ec classic cartridge (Waters, Milford, Massachusetts, USA), eluted by acetonitrile (Sigma-Aldrich)/water/FA

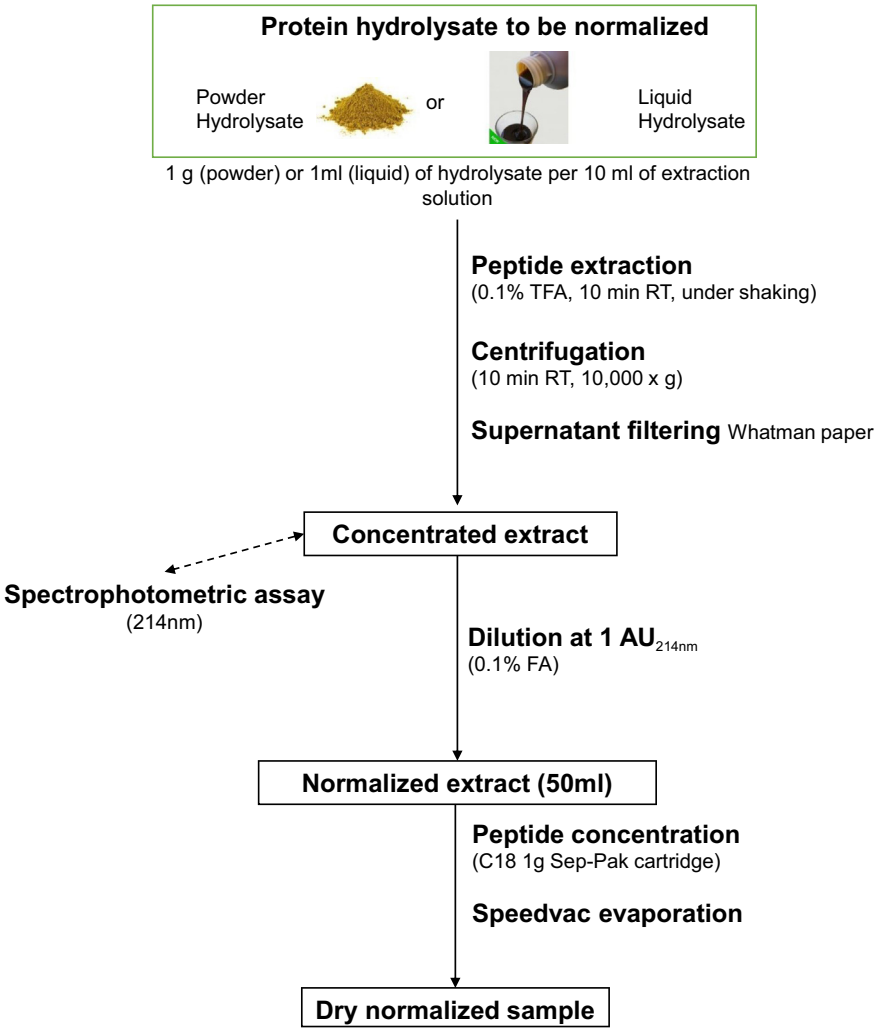

Figure 1. Method used to normalize protein hydrolysate samples before analysis. AU: absorbance unit; FA: formic acid; RT: room temperature; TFA: trifluoro-acetic acid.

(80/19.9/0.1, v/v/v), evaporated on a SpeedVac concentrator, and kept at 4 °C until mass spectrometry and HPSEC analyses. Thus, all standardized samples contained 50 ml at 1 AU of the same quantity of peptides regardless of their initial galenic form (powder, liquid, or concentrate).

2.3. Diversity of peptides in protein hydrolysates

Peptide extracts were first desalted and concentrated onto a μC18 Omix (Agilent) before analysis. The chromatography step was performed on a NanoElute (Bruker Daltonics) ultra-high-pressure nano flow chromatography system. Peptides were concentrated onto a C18 pepmap 100 (5 mm × 300 μm i.d.) precolumn (Thermo Scientific) and separated at 50 °C on a reversed phase Reprosil column (25 cm × 75 μm i.d.) packed with 1.6μm C18 coated porous silica beads (Ionopticks). Mobile phases consisted of 0.1% formic acid, 99.9% water (v/v) (A) and 0.1% formic acid in 99.9% ACN (v/v) (B). The nanoflow rate was set at 400 nl/min, and the gradient profile was as follows: from 2 to 15% B within 20 min, followed by an increase to 25% B within 10 min and further to 37% B within 5 min, followed by a washing step at 95% B and reequilibration.

Mass spectrometry analyses were carried out on a TIMS-TOF pro mass spectrometer (Bruker Daltonics) with a modified nano electrospray ion source (CaptiveSpray, Bruker Daltonics). The system was calibrated every week and mass precision was better than 1 ppm. A 1,400 capillary voltage was typically employed for ionizing. MS spectra were acquired in the positive mode in the 100 to 1,700 m/z mass range. In the experiments described here, the mass spectrometer was operated in PASEF mode without exclusion of single charged peptides. A number of 10 PASEF MS/MS scans was performed during 1.25 s from mass range 0–5.

2.4. Abundance of peptides in protein hydrolysates

High-performance steric exclusion chromatography (HPSEC) characterization was performed on a VARIAN system equipped with a UV (214-nm wavelength) detector (Agilent technologies, Santa Clara, CA, USA). Elution was carried out on a 300 × 8mm, 5μm ReproSil 50 SEC column (AIT, Houilles, France) with acetonitrile/water/TFA (10/89.9/0.1, v/v/v) at a flow rate of 0.7 ml/min. Each peptide extract was dissolved in 100 μl of HPSEC eluent, and 20 μl were injected. The HPSEC system was calibrated with three synthetic peptides: 949.59 Da (KKPLFGLF: 84.41% purity – Proteogenix, Schiltigheim, France), 1,000.13 Da (PRFQGNGKP: 98.89% purity – Genecust, Ellange, Luxembourg), and 1,058.71 Da (KKKKPLFGL: 91.25% purity – Proteogenix).

2.5. Determination of the peptide abundance index in protein hydrolysates

The peptide abundance index of LMW peptides in a protein hydrolysate represents the percentage of peptides with molecular weights below 1,000 Da. Based on the integration of the area below the curve extracted from chromatogram data, the abundance index was calculated as follows:

$$\text{Abundance (\%)} = 100 \text{ x [area of peptides with a MW} < 1{,}000 \text{ Da x total sample area}^{-1}] \quad (1)$$

2.6. Determination of the peptide diversity index in protein hydrolysates

The diversity index of LMW peptides in a protein hydrolysate represents the percentage of peptides with molecular weights below 1,000 Da. To determine this index, peptides of identical measured molecular masses (±0.0001 Da) were removed from analysis. Based on the peaklist data recorded from the nLC-ESI-MS analysis, the diversity index was calculated as follows:

$$\text{Diversity (\%)} = 100 \text{ x [number of peptides with a MW} < 1{,}000 \text{ Da x total number of measured peptides MW}^{-1}] \quad (2)$$

2.7. Statistical analyses

Extraction, normalization, HPSEC and nLC-ESI-MS analyses were performed in triplicate with all samples. Peptide abundance and diversity indexes were expressed as means ± standard deviations (SDs). Peptide abundance and diversity indexes were analyzed each by one-way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

3. Results and discussion

Because the protein hydrolysate samples were in different galenic forms (liquid, powder, concentrate), their peptide contents were determined beforehand to analyze LMW peptide abundance and diversity. Total peptide content was quantified in the protein hydrolysate extracts by measuring absorbance at a wavelength of 214 nm corresponding to the maximum absorbance of the peptide bond. This very common method bypasses the amino acid composition of hydrolysates, for although the Coomassie Blue assay is widely used for protein quantification, it cannot be used to estimate the peptide content of protein hydrolysates (Kilkowski and Gross, 1999). In fact, Coomassie blue only interacts with aromatic amino acids (F, H, W and Y). This assay should be therefore reserved for the determination of the total protein quantities, since they have more constant levels of aromatic AA unlike peptides which have very variable levels of aromatic AA.

The normalization step described here is a key point in the methodology of protein hydrolysate characterization. Protein hydrolysates produced from aquatic by-products have demonstrated functional (antibacterial, antioxidant, immunostimulant) properties *in vitro* linked to the presence of bioactive peptides that could have applications in animal and human health (Kim and Mendis, 2006). It is essential to carry out functional tests on protein hydrolysate samples of different origins and galenic forms (liquid or powder) in standardized conditions. The use of rigorous normalized sample replicates allows statistical treatment so as to compare their specifications and understand their functional performances during *in vitro* trials (Leduc et al., 2018).

The HPSEC chromatograms and nLC-ESI-MS peak list histogram corresponding to the shrimp hydrolysate with an advanced hydrolysis process are given in Figure 2 as an example. A 1,000 Da threshold is represented in both diagrams. We selected a threshold of 1,000 Da, *i.e.* peptides containing less than ~ 10 amino acids. They represent the most abundant peptide fraction in aquatic protein hydrolysates and the most efficient one to supply palatability, nutrition and health benefits in aquaculture feeds that regularly contain aquatic protein hydrolysates (Cahu et al., 1999; Refstie et al., 2004; Choi et al., 2009; Gisbert et al., 2012; Khosravi et al., 2015a, b; Khosravi et al., 2017), even if high contents of LMW peptides could cause the aquaculture feed to taste bitter (Adler-Nissen, 1984).

The liquid chromatography method analyzes the peptide profiles of a protein hydrolysate by separating peptides according to their molecular weight. This method has been used to characterize peptides and protein hydrolysates for 40 years (Guérard et al., 2001; Irvine, 2003). To complete this analysis, we measured peptide diversity using nLC-ESI-MS, a tandem liquid chromatography technique coupled with mass spectrometry. This technique is widely used for peptidomic analyses of food products (Picariello et al., 2012), but to our knowledge it had never been used to analyze complex protein hydrolysates. nLC-ESI-MS is very sensitive, it measures peptide masses down to the picomole level (Arnott et al., 1993).

Whole results are summarized in Table 1. Associated statistical comparisons of indexes are given in Table 2. Average MW was under

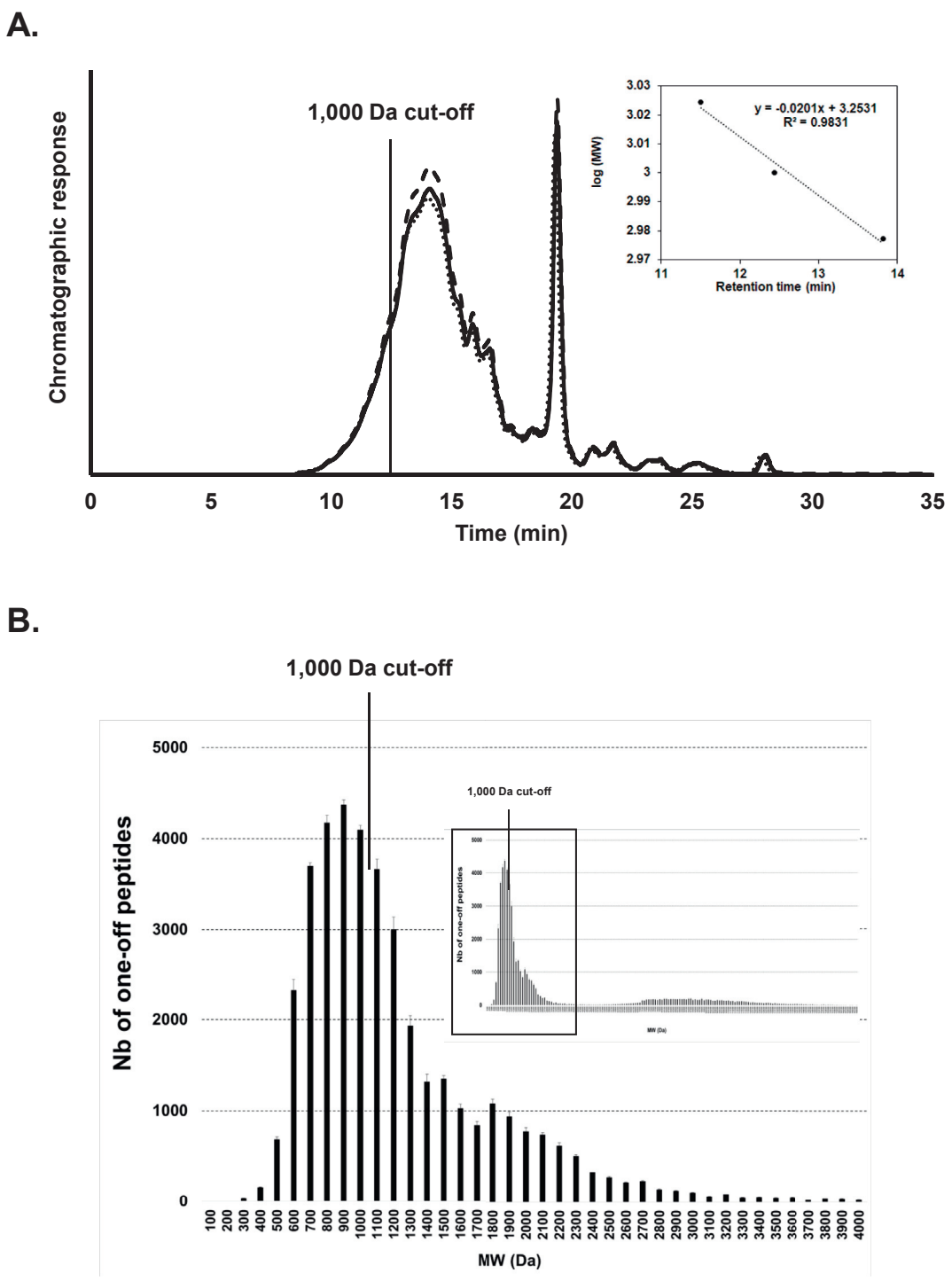

Figure 2. HPSEC and mass spectrometry profiles of high hydrolyzed shrimp by-product. A. HPSEC chromatograms and calibration curve (n = 3). B. nLC-ESI-MS peaklists (n = 3). Error bars correspond to the SDs.

1,000 Da for all samples, and ranged from 234.06 to 744.04 Da for Nile tilapia and tuna hydrolysates, respectively. The abundance indexes ranged from 47.60% to 90.20% between protein hydrolysates. The range of diversity indexes was narrower, from 33.12% to 47.55%. Variability among replicates was very low for all hydrolysates, both by HPSEC (CV <1%) and nLC-ESI-MS (CV <6%).

Both shrimp hydrolysates (low and high hydrolyzed) had very high LMW peptide abundance indexes, but a high hydrolysis process significantly increased the index. And despite the higher level of hydrolysis, peptide diversity remained stable. But even if the numerical value of the diversity index did not significantly vary, the high hydrolysis process reduced CV by 57%, *i.e* from 5.93% to 3.36% for low and high shrimp hydrolysis, respectively. This finding indicates that a stronger hydrolysis process allows for a better standardization of the diversity of LMW peptides.

The two tuna hydrolysates had significantly different abundance indexes that could be explained by (i) the different hydrolysis processes and (ii) the raw material origin. The first tuna hydrolysate was manufactured from tuna viscera, whereas the second one included cooked red meat that contained more collagen than viscera did. The structure and

Table 1. Abundance and diversity of peptides below 1,000 Da in hydrolysates.

Parameters	Raw material	Galenic Form	Average MW (Mean ± SD (CV))	LMW peptide abundance (Mean ± SD (CV))	One-off m/z	LMW peptide diversity (Mean ± SD (CV))
Hydrolysis process						
(low)	Shrimp	Powder	535.95 ± 20.9 Da (3.90%)	84.87 ± 0.39% (0.45%)	50114	40.93 ± 2.43% (5.93%)
(high)	Shrimp	Powder	614.92 ± 42.08 Da (6.84%)	90.20 ± 0.44% (0.48%)	48733	40.11 ± 1.35% (3.36%)
Production site						
(site 1)	Salmon	Liquid	377.76 ± 21.19 Da (5.61%)	75.86 ± 0.6% (0.79%)	67099	33.92 ± 0.38% (1.13%)
(site 2)	Salmon	Liquid	457.50 ± 39.72 Da (8.68%)	71.35 ± 0.6% (0.84%)	70487	32.63 ± 0.99% (3.04%)
Raw material						
(raw material 1)	Tuna	Liquid	529.91 ± 24.98 Da (4.71%)	87.02 ± 0.39% (0.45%)	54948	39.01 ± 1.43% (3.67%)
(raw material 2)	Tuna	Liquid	744.04 ± 88.04 Da (11.83%)	47.60 ± 0.39% (0.83%)	41131	33.12 ± 0.65% (1.96%)
Post hydrolysis thermal process						
(without)	Krill	Liquid	512.38 ± 19.03 Da (3.71%)	76.54 ± 0.55% (0.72%)	59524	37.38 ± 0.86% (2.30%)
(with)	Krill	Liquid	344.60 ± 19.85 Da (5.76%)	73.33 ± 0.24% (0.33%)	64005	39.00 ± 0.54% (1.39%)
Single analysis						
	Nile tilapia	Powder	234.06 ± 22.55 Da (9.63%)	68.43 ± 0.44% (0.64%)	58450	39.82 ± 0.26% (0.66%)
	Argentinean shortfin squid	Liquid	523.80 ± 13.88 Da (2.65%)	67.57 ± 0.39% (0.58%)	55479	36.76 ± 0.77% (2.08%)
	Cod	Powder	304.84 ± 12.54 Da (4.11%)	82.47 ± 0.40% (0.48%)	58302	47.55 ± 0.45% (0.95%)

The determination of the average molecular weight (MW) and abundance indexe was based on HPSEC analytical results (Equation 1). The determination of the one-off m/z and diversity indexe was based on nLC-ESI-MS analytical results (Equation 2). Values are means of 3 replicates ± standard deviations (SDs). Coefficients of variation (CV) are given for each sample. The abundance index of low molecular weight (LMW) peptides represents the amount of peptides with molecular weights below 1,000 Da in the protein hydrolysates, based on the integration of the area below the curve extracted from HPSEC chromatogram data. The diversity index of LMW peptides represents the diversity of peptides with molecular weights below 1,000 Da in the protein hydrolysates, based on nLC-ESI-MS peak list data. Identical molecular masses were removed from analysis to determine off-m/z and diversity indexes (±0.0001 Da).

Table 2. Statistical comparisons of peptide abundance and diversity indexes among protein hydrolysates.

Compared to →	Shrimp low hydrolysis	Shrimp high hydrolysis	Salmon site 1	Salmon site 2	Tuna raw_1	Tuna raw_2	Krill	Krill heated	Tilapia	Squid	Cod
Shrimp low hydrolysis	-	↓/NS	↑/↑	↑/↑	↓/NS	↑/↑	↑/↑	↑/NS	↑/NS	↑/↑	↑/↓
Shrimp high hydrolysis	↑/NS	-	↑/↑	↑/↑	↑/NS	↑/↑	↑/↑	↑/NS	↑/NS	↑/↑	↑/↓
Salmon site 1	↓/↓	↓/↓	-	↑/NS	↓/↓	↑/NS	NS/↓	↑/↓	↑/↓	↑/↓	↓/↓
Salmon site 2	↓/↓	↓/↓	↓/NS	-	↓/↓↓	↑/NS	↓/↓	↓/↓	↑/↓	↑/↓	↓/↓
Tuna raw 1	↑/NS	↓/NS	↑/↑	↑/↑	-	↑/↑	↑/NS	↑/NS	↑/NS	↑/↑	↑/↓
Tuna raw 2	↓/↓	↓/↓	↓/NS	↓/NS	↓/↓	-	↓/↓	↓/↓	↓/↓	↓/↓	↓/↓
Krill	↓/↓	↓/↓	NS/↑	↑/↑	↓/NS	↑/↑	-	↑/NS	↑/↓	↑/NS	↓/↓
Krill heated	↓/NS	↓/NS	↓/↑	↑/↑	↓/NS	↑/↑	↓/NS	-	↑/NS	↑/↑	↓/↓
Tilapia	↓/NS	↓/NS	↓/↑	↓/↑	↓/NS	↑/↑	↓/↑	↓/NS	-	↑/↑	↓/↓
Squid	↓/↓	↓/↓	↓/↑	↓/↑	↓/↓	↑/↑	↓/NS	↓/↓	↓/↓	-	↓/↓
Cod	↓/↑	↓/↑	↑/↑	↑/↑	↓/↑	↑/↑	↑/↑	↑/↑	↑/↑	↑/↑	-

(Peptide abundance index/Peptide diversity index); ↑: significant higher index; ↓: significant lower index; NS: non-significant differences (1-way anova; $P > 0.05$).

the polymeric nature of collagen and the denaturation of red meat proteins during the thermal treatment of the raw materials are two plausible explanations for the lower hydrolysis level in the second tuna hydrolysate, which resulted in a lower LMW peptide abundance and a higher average MW. To a lesser extent, the diversity index of the second tuna hydrolysate was also impacted and was lower than in the viscera hydrolysate.

Salmon hydrolysates were also produced from identical raw materials (viscera and frames) but collected from two different sites and processed in two different factories using the same hydrolysis process. The differences between the two hydrolysates were lower than those observed with tuna hydrolysates. Concerning the peptide diversity index, there was no significant difference between the two salmon hydrolysates ($P > 0.05$).

Krill hydrolysates had relatively similar profiles despite the additional thermal process applied to one of the hydrolysates. This provides evidence of the high thermal stability of LMW peptides.

The levels of peptide abundance were very similar in the other protein hydrolysates, processed from squid and two species of fish, cod from cold water and tilapia from warm water. However, peptide diversity was much higher in the cod protein hydrolysate.

Interestingly, among all fish protein hydrolysates, salmon hydrolysates had abundance indexes similar to the tilapia or cod hydrolysate indexes, but their diversity indexes ranged from 32.63% for salmon hydrolysate to 47.60% for cod hydrolysate. Cod hydrolysate presented the highest diversity index, even higher than that of the high hydrolyzed shrimp hydrolysate, considered as the most hydrolyzed product before this study.

These results show that most of the protein hydrolysates analyzed in this study had an abundance of LMW peptides (below 1,000 Da) of around 70%, but were well distributed along the "diversity" axis. Similarly, the products with the highest diversity indexes (high hydrolyzed shrimp and cod hydrolysates) had very different abundance indexes. This result emphasizes that an analysis only based on the abundance of LMW peptides or average MW would give a fragmentary view of the peptide composition of the protein hydrolysate. This is again evidence that both abundance and diversity analyses are required to characterize protein hydrolysates.

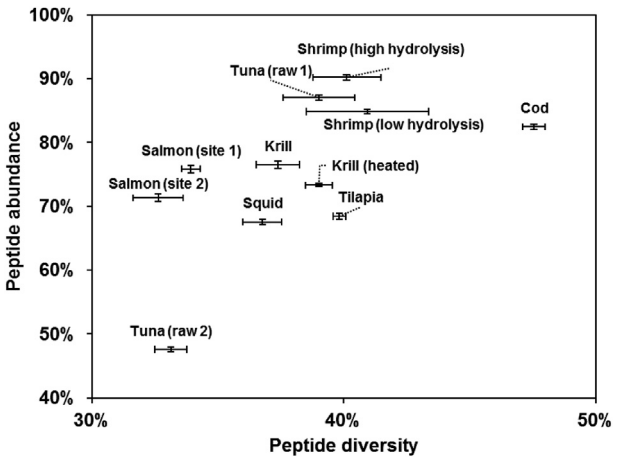

Figure 3. 2D Diagram of abundance and diversity indexes. Graphical representation of peptide abundance and diversity of peptides < 1,000 Da in protein hydrolysates. Error bars correspond to the SDs.

HPSEC and nLC-ESI-MS analysis results are presented in a graph of the peptide abundance and diversity of the protein hydrolysates (Figure 3). Such plotting of protein hydrolysates is really helpful to have a full picture of the peptide composition of protein hydrolysates and to determine the effect of raw material origin and processing on their final specifications.

Many years ago, the utilization of HPSEC to characterize protein hydrolysates was a great improvement compared to the measurement of the degree of hydrolysis (DH). While the DH provided information on the number of cleaved peptide bounds, HPSEC analysis showed the peptide profile produced during the hydrolysis process, therefore details about where/how the protein was cleaved. Now HPSEC is usually used and the new method implemented in this study gives new information on peptide diversity and shows that for very close peptide contents in two hydrolysates, diversity can vary a lot, and this may have consequences on the functional performances of the product. This study shows a very good repeatability of the analysis, with a low deviation between replicates. Therefore, it is obviously up to each user to define the number of replicates and the cut-off values to adapt the method and achieve their objectives.

The functional performance of a protein hydrolysate is closely correlated to its peptide profile and structure (Espe et al., 1999; Liaset et al., 2000). This is particularly true when the hydrolysis process is applied to produce bioactive peptides. Therefore, it is critical to control the performance and the standardization of the hydrolysis process and the specifications of the finished products to guarantee a high and consistent performance of protein hydrolysates. This fast and efficient method represents a clear improvement for quality control and a validation step for industrial hydrolysate production purposes.

The method developed in this study also offers protein hydrolysate manufacturers an efficient tool for adapting hydrolysis process parameters so as to reach specific targets in terms of peptide abundance and diversity. It could also be used to predict the zootechnical and functional performances of newly developed protein hydrolysates by comparing their position in the 2D diagram with known protein hydrolysates, *i.e.* shrimp, tilapia or krill hydrolysates tested in *in vivo* trials (Bui et al., 2014).

Our method significantly improves the deep characterization of complex protein hydrolysates. For the first time, it is possible to develop protein hydrolysates while better understanding the impact of processing modifications on their structural properties, in view of correlating their characteristics to their functional (*in vitro* and *in vivo*) properties to fine-tune hydrolysis and reach specific performances.

Declarations

Author contribution statement

Alexandre Leduc: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Vincent Fournier: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Joël Henry: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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