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Peptides in rainbow trout (*Oncorhynchus mykiss*) muscle subjected to ice storage and cooking

Caroline Bauchart [a,b], Christophe Chambon [a], Philippe Patureau Mirand [b], Isabelle Savary-Auzeloux [c], Didier Rémond [b], Martine Morzel [a,*]

[a] *Qualité des Produits Animaux, Institut National de la Recherche Agronomique, Centre de Theix, 63122 Saint-Genès Champanelle, France*
[b] *Unité Nutrition et Métabolisme Protéique, Institut National de la Recherche Agronomique, Centre de Theix, 63122 Saint-Genès Champanelle, France*
[c] *Unité de Recherches sur les Herbivores, Institut National de la Recherche Agronomique, Centre de Theix, 63122 Saint-Genès Champanelle, France*

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Abstract

Although proteolysis during *post mortem* storage is an important factor affecting fish texture, little is known about degradation end-products. This study was performed to investigate the occurrence of low molecular weight peptides (<5 kDa) *in post mortem* rainbow trout muscle, during ice storage, and to evaluate their stability during cooking. It combined quantitative (amino acid analysis) and qualitative approaches (mass spectrometry). The results showed that muscle of trout was poor in peptides. These were mainly anserine and glutathione. Their concentration was almost unaffected by the seven days of ice storage and vacuum cooking for 5 min at 70 °C. MS analysis revealed a limited but highly reproducible appearance of small peptides in trout muscle during the ice storage and after cooking. The compounds detected by MS analysis remain to be characterised.

Keywords: Trout; Muscle; Peptides; Proteolysis; *Post mortem*; Cooking

1. Introduction

Peptides are molecules which can be present in fish muscle *intra vitam* (e.g. anserine, glutathione) and/or could be produced by proteolysis during *post mortem* storage. Immediately after death, several biochemical and enzymatic changes are triggered in fish muscle, resulting in the gradual loss of fish freshness (Botta, 1994; Sikorski, Kolakowska, & Burt, 1990) and texture deterioration. Texture changes can be due to degradation of the extracellular matrix (Bremner, 1992; Sato et al., 1997) and weakening of the myofibrillar structure (Ladrat, Verrez-Bagnis, & Fleurence, 2003). Deterioration of the myofibrillar structure results from the action of endogenous proteolytic systems, namely the neutral calcium-activated calpains and lysosomal cathepsins. For instance, calpains may initiate the disintegration of the Z line by a titin cleavage (Astier, Labbe, Roustan, & Benyamin, 1991), which weakens the titin/α-actinin interaction and results in the release of intact α-actinin from Z lines (Papa, Alvarez, Verrez-Bagnis, Fleurence, & Benyamin, 1996). Similarly, cathepsins have been reported to induce muscle softening (Yamashita & Konagaya, 1991). Furthermore, cathepsins remain active at temperatures close to cooking temperature (Geist & Crawford, 1974). For all these reasons, proteolysis during *post mortem* storage is an important factor affecting fillet texture.

Despite the importance of proteolysis on texture and quality of fish muscle, little is known about products of proteolysis during *post mortem* storage and cooking, especially small peptides (<5 kDa). Such peptides are of interest because it is in this molecular weight range that most bioactive peptides can be found (Korhonen & Pihlanto, 2003). Bioactive peptides are dietary peptides with physiological properties, such as immunostimulating or antihypertensive activity (Kitts & Weiler, 2003; Korhonen & Pihlanto,

* Corresponding author. Tel.: +33 473 62 45 76; fax: +33 473 62 42 68.
E-mail address: morzel@clermont.inra.fr (M. Morzel).

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2003). These peptides could contribute to the nutritional value of fish.

In this context, the objective of this study was to investigate the occurrence of low molecular weight peptides (<5 kDa) *in post mortem* trout muscle, during ice storage, and to evaluate their stability during cooking. It combined quantitative (amino acid analysis) and qualitative approaches (mass spectrometry).

2. Materials and methods

2.1. Fish and experimental procedure

Six rainbow trout (*Oncorhynchus mykiss*) (mean weight 215 ± 18 g) were obtained from a local fish farm (Moulin de Pagnat, Saint-Saturnin, France). After four days of starvation, fish were killed by a blow on the head, eviscerated and immediately put in a plastic film on ice. Muscle samples were taken from the white dorsal muscle 30 min to 1 h 30 after death (**T0**), and after seven days of ice storage (**T7**). Part of the flesh collected at T7 was cooked under vacuum for 5 min at 70 °C in a water bath (**T7c**). After processing, all muscle samples were stored at −80 °C prior to use.

2.2. Peptide extraction

After thawing on ice, 2.5 g of muscle samples were homogenized in 12.5 ml of 3% perchloric acid (PCA) with 100 μl of 27 mM norleucine (internal standard), using an Ultra-Turrax homogenizer (15,000 rpm twice for 15 s separated by 15 s on ice). The homogenate was then centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was submitted to an ultra-filtration with a 5 kDa cut off (Vivaspin 15, VIVASCIENCE, Hanover, Germany) at 2,000 g for 2 h at 4 °C. Extract aliquots (500 μl) were stored at −20 °C prior to analysis.

2.3. Nitrogen content and amino acids analysis

Total nitrogen content was determined by the Kjeldahl method on muscle and extracts. For free amino acids (FAAs) analysis, 400 μl of extract were applied to 2 ml of AG-50 resin, 100–200 mesh, in the H^+ form (Bio-Rad Laboratories, Hercules, CA, USA), followed by deionised water wash. The amino acids were then eluted with 10 ml of 4 N NH_4OH. Eluate was evaporated overnight under vacuum and resuspended in 400 μl of 0.1 M lithium buffer (pH 2.2). For total amino acids (TAAs) analysis, 400 μl of extract were hydrolyzed in 6 N HCl at 110 °C for 24 h. Hydrolysate was evaporated and the dry residue was resuspended in 400 μl of 0.1 M lithium buffer (pH 2.2). Samples were stored at −20 °C prior to analysis. Amino acids were analyzed by ion-exchange chromatography on a HPLC System (BioTek Kontron, Rotkreuz, Switzerland), using postcolumn derivatisation with ninhydrin. The peptidic amino acids (PAAs) fraction was calculated by difference between TAAs and FAAs. Cysteine, methionine, and tyrosine, which are severely affected by acid hydrolysis, were not considered. Furthermore, during hydrolysis, asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively. The peptidic concentrations of aspartic acid and glutamic acid were therefore calculated as follows:

$$[\mathrm{Asp}]_{\mathrm{PAA}} = [\mathrm{Asp}]_{\mathrm{TAA}} - ([\mathrm{Asp}]_{\mathrm{FAA}} + [\mathrm{Asn}]_{\mathrm{FAA}})$$
$$[\mathrm{Glu}]_{\mathrm{PAA}} = [\mathrm{Glu}]_{\mathrm{TAA}} - ([\mathrm{Glu}]_{\mathrm{FAA}} + [\mathrm{Gln}]_{\mathrm{FAA}})$$

2.4. Glutathione and anserine determination

Total glutathione (oxidized and reduced forms) content was assayed using the enzymatic recycling procedure described by Robinson, Rounds, Hong, Jacobs, and Wilmore (1992). Anserine content was quantified by RP-HPLC, using precolumn derivatisation with *O*-phthaldehyde reagent (column: C18-HDO 250 × 4.6 mm, 5 μm; Uptisphere, Interchim), adapted from the method of Maynard, Boissonneault, Chow, and Bruckner (2001).

2.5. Matrix-assisted laser desorption ionization (MALDI) – time-of-flight (ToF) mass spectrometry (MS) analysis

Extracts (200 μl) were applied on C18 membrane (Vivapure MALDI-Prep, VIVASCIENCE, Hanover, Germany) according to the manufacturer's protocol, in order to remove PCA. Samples were eluted with 5 μl of 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). An aliquot (0.9 μl) of each eluate was spotted on the plate with an equal volume of a matrix solution (CHCA: α-cyano-4-hydroxycinnamic acid), at 10 mg/ml, prepared in 50% ACN/0.1% TFA. The mixture was allowed to dry before analysis. Positive ions ($M + H^+$) spectra were recorded in both the reflectron mode and linear mode of MALDI-ToF MS (Voyager DE-Pro, Per-Septive BioSystems, Framingham, MA, USA) using Voyager software for data collection and analysis. The MS was calibrated with a standard peptide solution (Proteomix, LaserBio Labs, Sophia-Antipolis, France). The signal *m*/*z* was measured. The conversion of *m*/*z* into Dalton is obtained by subtracting the mass of H^+ (1.008) from *m*/*z*. Compounds from 0 to 5000 Da were listed.

2.6. Nano-electrospray-ionisation-MS/MS (nano-ESI-MS/MS) analysis

The nano-ESI-MS/MS analysis was carried out on a mass spectrometer LCQ Ion Trap equipped with a nano-electrospray source (Thermofinnigan, San Jose, CA, USA). Extracts (200 μl) were applied on to C18 membrane (Vivapure MALDI-Prep, VIVASCIENCE, Hanover, Germany). Samples were eluted with 15 μl of 65% ACN/1% formic acid. The nanoelectrospray capillaries (Protana, Odense, Denmark) were loaded with 3 μl of resulting sample. Ionisation was performed with a liquid junction and a

non-coated capillary probe (New Objective, Cambridge, USA). Data acquisition was performed in a manual mode and the collision-induced dissociation (CID) of selected precursor ions was performed using 30% to 40% relative collision energy. The MS/MS data were searched against NCBI databases with the search engine SEQUEST (LCQ-Deca software package).

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Frozen muscle samples (0.1 g) were homogenized in 1 ml of extraction buffer (8 M urea, 5 mM Pefabloc®-SC, 4% w/v CHAPS in 40 mM Tris-base) in an Eppendorf tube containing a glass bead in a Retsch agitator for 1 h at 4 °C. The extracts were centrifuged at 10,000*g* for 1 h at 10 °C and the clear supernatant was collected. Protein concentration was determined by the method of Bradford (1976), SDS–PAGE was performed according to the method of Laemmli (1970), using an 11% polyacrylamide gel and 30 μg of protein were loaded per well.

2.8. Statistical analysis

A Student paired *t*-test was used to compare individual PAA concentration with zero. Only PAA concentrations significantly different from zero ($P < 0.05$) have been considered. The significance of the difference between fresh and stored muscle, and between uncooked and cooked muscle, was analysed using Student paired *t*-test.

3. Results and discussion

3.1. Fresh muscle

After slaughter, protein concentration ($N \times 6.25$) in white dorsal muscle represented 20.1% of muscle weight. This value is in agreement with the observations of Venugopal and Shahidi (1996), and Gokoglu, Yerlikaya, and Cengiz (2004) in raw rainbow trout. Nitrogen content in acid extract was about 2.6 mg of N/g of wet muscle, accounting for 8% of muscle total nitrogen. Nitrogen, measured from amino acids (FAAs + PAAs), accounted for 55% of total nitrogen in extracts, of which 62% is PAA nitrogen. Non-protein nitrogen compounds, present in fish muscle, such as creatine, creatinine, guanidine compounds, trimethylamine oxide, purines, and also not measured free and peptidic amino acids, could constitute the remaining nitrogen fraction.

In fresh muscle, free amino acid pattern (Table 1) was consistent with previously reported data for white muscle of rainbow trout (Kaushik & Luquet, 1979; Yokoyama & Nakazoe, 1991); glycine, alanine, taurine and histidine being, on a molar basis, the most abundant free amino acids. Few amino acids were evidenced as peptidic amino acids (Table 2). The two predominant peptidic amino acids in extracts, 1-methyl-histidine and β-alanine, correspond to the constitutive amino acids of anserine (β-Ala-1-methyl-His). The difference between 1-methyl-histidine and β-alanine concentrations was explained by a loss of β-alanine during hydrolysis, verified by an analysis of total amino acids in extract after addition of a known amount of anserine. A loss of β-alanine during acid hydrolysis of balenine (β-Ala-3-methyl-his) has also been evidenced by Aristoy, Soler, and Toldrá (2004). The presence of anserine was confirmed by a specific assay at a level matching the concentration of peptidic 1-methyl-histidine. Anserine concentration in the present study was in agreement with previously reported data for white muscle of rainbow trout (Abe, 1983; Abe, Bobson, Hoeger, & Parkhouse, 1985; Kaushik & Luquet, 1979). The third most abundant peptidic amino acid was glycine. Such a high concentration is difficult to explain with regard to the concentration of the other peptidic amino acids: glycine accounted for 89% of non-anserine PAAs. It suggests the presence of polyglycine peptides in the extract. Some of the peptidic glycine came from glutathione (γ-Glu–Cys–Gly), another endogenous peptide naturally found in muscles, but glutathione-derived glycine accounted for only 4% of peptidic glycine. Total glutathione concentration in the present study (Table 3) was twice that observed in muscle of rainbow trout by Passi et al. (2004). This difference of glutathione concentration could be due to differences in fish husbandry and oxidative status. The fourth most abundant peptidic amino acid in the fresh muscle was glutamic acid; its concentration was in agreement with the observed glutathione concentration.

Extracts were analysed by MALDI-ToF and detected compounds found in at least three animals out of six are listed in Table 4. The MS analysis of fresh muscle extracts revealed the presence of 22 compounds with a mass <4000 Da. These compounds were then analysed by nano-ESI-MS/MS. However, because of the scarcity of records on fish proteins in databases, no compound present in the extract was clearly identified. The internal standard (norleucine, 131 Da) was detected, but it was not possible to evidence the presence of anserine (240 Da) and glutathione (reduced form, 307.3 Da; oxidized form, 612.6 Da). These peptides were probably lost during the process of PCA removal, a step essential in sample preparation for a satisfactory crystallisation for MALDI-ToF analysis. It might lead to some compounds loss, and therefore the list was not exhaustive. Few works have provided identification of peptides in fish muscle. Kohama et al. (1988) have isolated and identified an octopeptide (952 Da), with antihypertensive property, in acid extract of tuna muscle. This peptide was not evidenced in the present study on trout muscle extract.

3.2. Effect of ice storage

Total nitrogen content of acid muscle extract was not affected by ice storage (data not shown). The pool of most free amino acids slightly increased during storage, probably

Table 1
Free amino acids (FAAs) concentration (μmol/g of wet muscle) in trout muscle extracts at T0 (fresh muscle), T7 (after seven days of ice storage) and T7c (cooked after seven days of ice storage)

FAAs	Treatments			Effects[a]	
	T0	T7	T7c	Storage	Cooking
Phosphoserine	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	NS	NS
Taurine	1.51 ± 0.25	6.20 ± 0.41	4.16 ± 0.37	***	**
Aspartic acid	ND	ND	ND	–	–
Threonine	0.48 ± 0.05	0.60 ± 0.03	0.56 ± 0.04	*	NS
Serine	0.71 ± 0.05	0.96 ± 0.07	0.82 ± 0.03	***	*
Asparagine	0.07 ± 0.04	0.05 ± 0.00	0.04 ± 0.01	NS	NS
Glutamic acid	0.02 ± 0.01	0.15 ± 0.03	0.11 ± 0.01	**	NS
Glutamine	0.06 ± 0.03	ND	ND	–	–
Glycine	25.6 ± 0.99	15.0 ± 0.86	20.5 ± 1.47	***	*
Alanine	3.04 ± 0.37	3.69 ± 0.43	3.15 ± 0.31	*	*
Citrulline	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	NS	NS
Valine	0.33 ± 0.02	0.50 ± 0.02	0.45 ± 0.00	***	**
Cystine	0.02 ± 0.00	ND	ND	–	–
Methionine	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	**	NS
Isoleucine	0.17 ± 0.01	0.26 ± 0.01	0.21 ± 0.01	**	NS
Leucine	0.26 ± 0.02	0.40 ± 0.02	0.34 ± 0.01	***	**
Tyrosine	0.04 ± 0.01	0.10 ± 0.01	0.07 ± 0.00	***	**
Phenylalanine	0.07 ± 0.01	0.12 ± 0.01	0.12 ± 0.00	***	NS
Histidine	1.46 ± 0.31	0.76 ± 0.14	1.10 ± 0.28	**	NS
Ornithine	0.02 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	**	*
Lysine	0.25 ± 0.04	0.39 ± 0.03	0.37 ± 0.03	**	NS
Arginine	0.07 ± 0.02	0.11 ± 0.01	0.10 ± 0.01	**	NS
Hydroxyproline	0.23 ± 0.03	0.19 ± 0.02	0.21 ± 0.02	NS	NS
Proline	0.18 ± 0.05	0.30 ± 0.05	0.28 ± 0.03	**	NS
β-Alanine	0.28 ± 0.09	1.87 ± 0.14	2.08 ± 0.16	***	NS
1-Methyl-histidine	0.08 ± 0.01	1.74 ± 0.12	1.82 ± 0.29	***	NS
Total	34.96 ± 1.59	33.24 ± 1.38	36.57 ± 1.96	NS	NS

Values are shown as means ± standard error ($n = 6$). ND, not detectable.
[a] NS, non-significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 2
Peptidic amino acids (PAAs) concentration (μmol/g of wet muscle) in trout muscle extracts at T0 (fresh muscle), T7 (after seven days of ice storage) and T7c (cooked after seven days of ice storage)

PAAs	Treatments			Effects[a]	
	T0	T7	T7c	Storage	Cooking
Phosphoserine	0.10 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	NS	NS
Aspartic acid	0.13 ± 0.02	0.18 ± 0.03	0.25 ± 0.02	NS	NS
Glutamic acid	0.42 ± 0.05	1.06 ± 0.06	0.98 ± 0.06	***	NS
Glycine	8.18 ± 0.79	7.16 ± 0.36	7.26 ± 0.37	NS	NS
Valine	ND	ND	0.03 ± 0.01	–	–
Leucine	0.02 ± 0.01	ND	0.04 ± 0.01	–	–
Histidine	0.26 ± 0.08	0.26 ± 0.04	0.20 ± 0.03	NS	NS
Lysine	0.12 ± 0.02	0.05 ± 0.02	0.08 ± 0.01	NS	NS
β-Alanine	9.71 ± 1.09	10.53 ± 0.86	9.05 ± 1.15	NS	NS
1-Methyl-histidine	14.7 ± 0.85	14.6 ± 1.24	13.2 ± 1.10	NS	NS
Total	33.60 ± 2.29	34.01 ± 2.10	31.28 ± 2.45	NS	NS

Values are shown as means ± standard error ($n = 6$). ND, not detectable.
[a] NS, non-significant.
*** $P < 0.001$.

as a result of muscle autolysis (Table 1). For an unknown reason, taurine concentration was fourfold greater after seven days of ice storage. Conversely, a large decrease in glycine and histidine was observed. Such a decrease in glycine concentration was previously reported by Ruiz-Capillas and Moral (2003) in white muscle of tuna stored in

Table 3
Total glutathione and anserine concentrations (μmol/g of wet muscle) in trout muscle extracts at T0 (fresh muscle), T7 (after seven days of ice storage) and T7c (cooked after seven days of ice storage)

	Treatments			Effects[a]	
	T0	T7	T7c	Storage	Cooking
Glutathione	0.31 ± 0.02	0.34 ± 0.01	0.30 ± 0.02	NS	*
Anserine	15.3 ± 0.84	15.0 ± 0.76	13.0 ± 0.59	NS	*

Values are shown as means ± standard error ($n = 6$).
[a] NS, non-significant.
* $P < 0.05$.

Table 4
MS analysis by MALDI-ToF of trout muscle extracts at T0 (fresh muscle), T7 (after seven days of ice storage) and T7c (cooked after seven days of ice storage)

T0	T7	T7c
132	132	303
135	378	972
162	457	1101
457	843	1230
972	972	1544
989	989	1932
1101	1056	2588
1208	1101	3385
1230	1230	3644
1304	1360	3731
1666	1666	3798
1697	2801	3885
1714	3201	4295
1759	3259	4733
1783	3731	4804
1794	3885	4820
2011		
2570		
2596		
2743		
3731		
3885		

Mass of detected compounds (Da) found in at least three of the six animals. Da, $m/z - 1.008$.

ice. Ice storage had no effect on glutathione concentration in acid extract (Table 3). Although it was not possible to detect a significant decrease in anserine (Table 3) or in its peptidic components (Table 2) during ice storage, concentrations of free β-alanine and 1-methylhistidine in extracts slightly increased (Table 1). Similar observations were reported by Ruiz-Capillas and Moral (2001) in hake (*Merluccius merluccius* L.) muscle after five days of ice storage. In their study, the progressive loss of anserine during storage (up to 35 days) was attributed to anserinase activity, and the authors proposed that anserine and its degradation compounds could be used as quality control indices for chilled hake.

A long time of storage (seven days) was chosen so as to favour peptide occurrence. Indeed, a sharp increase in TCA-soluble peptides was observed in seabass muscle after six days at 4 °C (Masniyom, Benjakul, & Vissanguan, 2004). In our study, no large increase in peptidic amino acid concentrations was evidenced after seven days of ice storage (Table 2). Only peptidic glutamic acid was significantly increased. In beef and pork muscles, Claeys, De Smet, Balcaen, Raes, and Demeyer (2004) observed an increase in peptide concentrations in the 3-17 kDa molecular weight range during 14 days of *post mortem* ageing. In lamb muscle, an increase of small peptides (<2.4 kDa) was also observed during 21 days of meat ageing (Sylvestre, Feidt, & Brun-Bellut, 2001). The difference between our results and those in seabass and mammalian muscles may be explained by the muscle composition and the technological parameters (time and temperature of storage) specific to the various species.

The sensitivity of MS analysis allowed distinction of some differences of patterns between T0 and T7, mainly a disappearance of compounds between 1700 and 2800 Da and the appearance of compounds between 2800 and 3700 Da (Table 4). Regarding peptidic composition of fresh and stored muscle, it is noteworthy that nine of the compounds detected by MS in the fresh muscle extract were still present after seven days of ice storage. The disappearance of smaller compounds argues in favour of moderate peptide degradation, in line with the slight increase in free amino acid concentration in extracts. The appearance of larger compounds suggests that *post mortem* proteolysis occurred but was limited and did not produce high amounts of low molecular weight peptides (<5 kDa) in trout muscle during ice storage. The stability of the electrophoretic protein pattern (Fig. 1) confirmed that little proteolysis occurred in trout muscle, in accordance with the

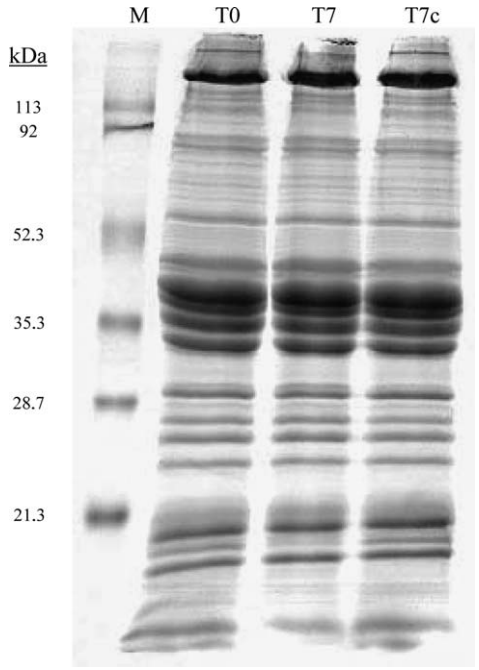

Fig. 1. SDS–PAGE gel electrophoresis of total proteins extracted from trout muscle at T0 (fresh muscle), T7 (after seven days of storage) and T7c (cooked after seven days of storage). M, molecular weight markers.

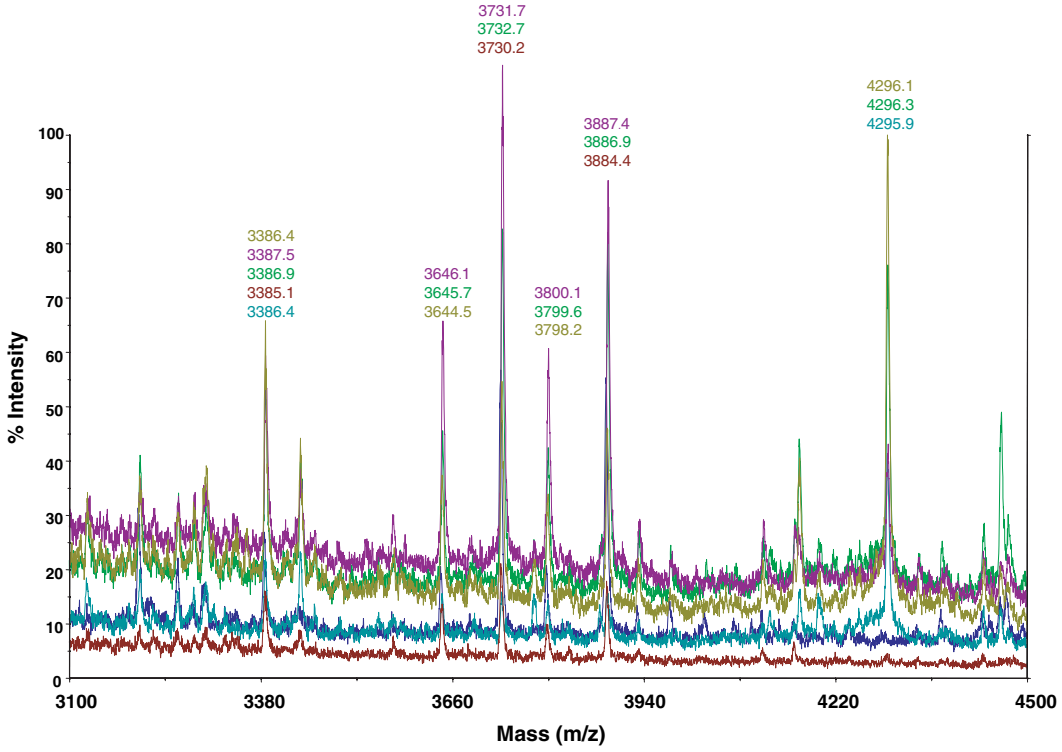

Fig. 2. MS/MALDI-ToF analysis spectra of cooked muscle extracts of six animals (example of a zone between 3100 and 4500 *m/z*).

result observed in rainbow trout muscle after 14 days *of post mortem* storage at 0 °C (Tsuchiya, Kita, & Seki, 1992). This finding is somewhat similar to those found in sea bass (Verrez-Bagnis, Ladrat, Morzel, Noel, & Fleurence, 2001) and cod muscle (Kjærsgård & Jessen, 2003) where authors described little protein degradation.

3.3. Effect of cooking

Cooking had little impact on free amino acid concentration in muscle extract. The bulk of free amino acids tended to decrease, but glycine and histidine concentrations significantly increased (Table 1). Anserine and glutathione concentrations were lower after cooking (about 12% of decrease) (Table 3). As for free amino acids, this variation could probably be explained by a loss in the cooking juice, since anserine was shown to be stable during heat treatments (Aristoy et al., 2004). During cooking, no significant hydrolysis of trout muscle proteins seemed to occur, based on electrophoresis results (Fig. 1) and peptidic amino acid concentration (Table 2). However, MS analysis evidenced the appearance of compounds with a mass above 4000 Da. These new compounds could be peptides that came from hydrolysed muscle proteins. Five compounds initially found in the fresh muscle were still present after cooking. Molecular weights of these compounds were 972, 1101, 1230, 3731 and 3885 Da. Moreover, it should be noted that the highly reproducible peaks pattern (Fig. 2) indicates that sites of proteolysis (whether of an enzymatic or chemical nature) are highly specific.

4. Conclusion

Fresh muscle of rainbow trout contained two major endogenous peptides, anserine and glutathione, the concentrations of which were almost unaffected by the seven days of ice storage and the cooking conditions applied in the present work. These processes generated low molecular weight peptides (<5 kDa), but in very low concentrations. Furthermore, protein electrophoretic patterns were very stable. Together, these results suggest that *post mortem* proteolysis was present but limited and produced few low molecular weight peptides. Overall, mass spectra of acid extracts were extremely reproducible between animals. The present study evidenced compounds that were initially present in trout muscle and were stable during storage and cooking, and other compounds that appeared (protein degradation) or disappeared (peptidolysis) throughout fish processing. The next step will be to identify these molecules.

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