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NMR approach for monitoring post-mortem changes in Atlantic salmon fillets stored at 0 and 4 °C

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ABSTRACT

High resolution NMR technique has been used to monitor post-mortem changes in salmon (*Salmo salar*) fillets upon storage at 4 and 0 °C. Thirty-one different fish metabolites influencing freshness and taste properties have been unequivocally assigned by NMR using either available standard compounds or *ad hoc* acquired 2D ^{1}H–^{1}H TOCSY and ^{1}H–^{13}C HSQC spectra. The monitored fish metabolites include amino acids, dipeptides, sugars, vitamins, biogenic amines, as well as different products of the ATP degradation. The detection and monitoring of biogenic amines by NMR, upon fish storage, is information of interest for consumers, since some of these compounds are toxic. The data from this study shows that NMR spectroscopy also provides the amount of all metabolites necessary for the calculation of the *K*-index used to express fish freshness. A good correlation was found between the *K*-index increase and the formation of the undesired biogenic amines. The metabolite concentrations and the *K*-index found in this work were compared and found coherent with literature data. The performed study reveals the strengths and the suitability of the NMR approach to monitor different biochemical processes occurring during fish storage and qualitatively and quantitatively characterise fish metabolites determining fish quality.
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1. Introduction

Fish is an important nutritive source for human consumption. The world fish food supply has grown dramatically in the last years, with an average growth rate of 3.2 percent per year in the period 1961–2009, outpacing the increase of 1.7 percent per year in the world's population (FAO, 2012). Atlantic salmon (*Salmo salar*) is a popular food product. Norway and Chile are the world's two leading producers of farmed salmon and salmon aquaculture is the one of the fastest growing food production systems in the world (FAO, 2012).

Fish is highly perishable and temperature is highly important in determining its shelf-life. Freshness is one of the most important attributes when assessing fish quality. A wide variety of chemical and physical methods have been used to assess the freshness of fish during storage (Béné, Hayman, Reynard, Luisier, & Villettaz, 2001; Olafsdóttir et al., 1997). One of the indices that increase with time during fish storage and is used extensively as a commercial freshness indicator is the *K*-index (Ehira & Uchiyama, 1987). In some markets, *K*-values are used as quality criteria to determine the consumption limit for raw and chilled fish. The *K*-index is defined as the percentage of the amount of inosine (Ino) and hypoxanthine (Hx) to the total amount of ATP and its degradation products (Tejada, 2009). The *K*-index can be calculated as follows (Ehira & Uchiyama, 1974; Saito, Arai, & Matuyoshi, 1959):

$$K(\%) = ([\mathrm{Ino}] + [\mathrm{Hx}])/([\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}] + [\mathrm{IMP}] + [\mathrm{Ino}] + [\mathrm{Hx}]) * 100$$

A higher *K*-index corresponds to less fresh fish. The rate of increase in *K*-index varies between different species (Tejada, De las Heras, & Kent, 2007). Within one species, *K*-value depend on size and storage temperature (Ehira, 1976; Uchiyama, Ehira, Uchiyama, & Masuzawa, 1978). The *K*-index of 20% has been set as the limit for consumption of raw fish (Hamada-Sato, Usui, Kobayashi, Imada, & Watanabe, 2005). Maximum *K*-index at the rejection of the Atlantic salmon for consumption is 70–80% (Erikson, Beyer, & Sigholt, 1997).

Recently, nuclear magnetic resonance (NMR) technique was employed for the determination of *K*-index in Bogue fish (Ciampa, Picone, Laghi, Nikzad, & Capozzi, 2012). NMR is a powerful tool for food quality evaluation. It allows simultaneous characterisation of wide number of components in the food samples. High resolution NMR is suitable for *K*-index determination since this technique is straightforward and highly reproducible

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and allows direct quantitative monitoring of substances determining the *K*-index. In addition, different fish metabolites determining fish quality, taste, etc. may be simultaneously monitored and qualitatively described making NMR spectroscopy a valid, and in many aspects unique, tool to characterise the metabolic profile and properties of fish (Aursand et al., 2009; Castejon et al., 2010; Ciampa et al., 2012; Gribbestad, Aursand, & Martinez, 2005; Mannina et al., 2008).

This study was aimed to demonstrate of the possibility of NMR spectroscopy to monitor different metabolites and biochemical processes that take place in farmed salmon (*S. salar*) fillets stored at 0 and 4 °C.

2. Materials and methods

2.1. Sample preparation

Two farmed Atlantic salmon (*S. salar*) from the same supplier in Mid Norway were purchased 5 days after slaughter at a local fish store in Trondheim. The samples were brought to the laboratory at the Department of Biotechnology, Norwegian University of Science and Technology (NTNU) within one hour. The fish was filleted and the fillets were divided in pieces of around 25 g and placed in separate plastic bags. The samples from the first salmon were stored at 4 °C while the samples from the second was stored in ice (fish-to-ice ratio 2:1) in a cold room at 4 °C (hereafter called "0 °C storage").

2.2. Experimental design

The effect of the storage at two different temperatures on the freshness and post-mortem catabolism of salmon muscle were studied by analyzing the samples stored at 4 and 0 °C as a function of storage time. Time 0 is the time the samples arrived in the lab. Samples were taken after: 0 (T_0), 2 (T_1), 4 (T_2), 7 (T_3), 10 (T_4) and 14 (T_5) days.

2.3. Chemicals

Deuterium oxide (D_2O, 99.9%) was purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP, 98 atom% D) from Armar Chemicals (Dottingen, Switzerland), Trichloroacetic acid (TCA) from *Sigma–Aldrich*. Potassium hydroxide from *Sigma–Aldrich* was used for pH adjustments.

2.4. Trichloroacetic acid extraction

The water soluble polar metabolites were extracted from the fish samples using TCA at 4 °C as previously described (Ciampa et al., 2012). Shortly, 25 g of fish muscle were homogenised in 50 ml of 7.5% TCA using a vertical homogeniser (Ultra-Turrax, Ika). The homogenate was filtered through a filter paper (Whatman, N°4; Little Chalfont, UK). The pH of the filtrate was adjusted to pH 7.0 using 9 M KOH. The filtrate was stored at −80 °C until NMR measurements were performed.

2.5. Sample preparation for NMR analysis

1 ml aliquot of the supernatant in an Eppendorf microfuge tube was centrifuged at 20,000×g for 5 min in order to remove the potassium trichloroacetate precipitate. 800 μL of the centrifuged sample were placed in a standard 5 mm NMR tube, adding 160 μL of TSP (final concentration, 1 mM).

2.6. NMR experiments

1D ^{1}H, 2D ^{1}H–^{1}H TOCSY and ^{1}H–^{13}C HSQC NMR spectra were acquired at 298 K with a Bruker Avance 600-MHz spectrometer equipped with 5-mm z-gradient TXI (H/C/N) cryoprobe at the NMR center of the Faculty of Natural Sciences and Technology at the Norwegian University of Science and Technology in Trondheim.

The 1D ^{1}H experiment (Bruker, noesygppr1d pulse sequence) had the following settings: 512 number of scans, a recycle delay of 5 s; a 90° pulse of 7.8 μs; 32 K data points; presaturation power PL9 = 42.0 dB; spectral width 12 ppm; acquisition time 2.28 s; mixing time of 10 ms. 2D ^{1}H–^{1}H TOCSY experiment (Bruker mlevphpr pulse sequence) had the following setting: TPPI phase sensitive mode; spectral width 12 ppm in both dimensions, a 90° pulse of 7.8 μs, relaxation delay – 2 s; mixing time – 100 ms, 4 K data points in F2 and 512 increments in F1. The 2D ^{1}H–^{13}C HSQC spectrum was registered in the echo–antiecho phase-selective mode with the following parameters: 7.8 μs 90° pulse, 12 and 200 ppm spectral widths in the proton and carbon dimensions, respectively, ^{1}H–^{13}C coupling constant was 145 Hz. The spectra were calibrated taking the ^{1}H and ^{13}C chemical shifts of TSP signal equal to 0 ppm.

Each acquired spectrum was processed with TopSpin 3.x (Bruker, Germany) and MestReC 4.9.8.0 softwares (Mestreab Research SL, Spain) by manually adjusting phase and base-line and applying a line broadening factor of 0.5 Hz. NMR assignment was performed using both the registered experiments and the published data for the reference standards (BMRB, HMDB, YMDB, ECMDB databases), literature data (Fan, 1996; Fan & Lane, 2008). The metabolites proton signals in the range between 0.0 ppm and 12.0 ppm were integrated and normalised to the resonance of the TSP signal.

3. Results and discussion

3.1. NMR spectra

The 1D and 2D NMR spectra of the two fish at sampling point T_0 time were similar, although differences in the relative intensities of some resonances were observed. Fig. 1 shows a representative 1D ^{1}H and 2D ^{1}H–^{1}H TOCSY NMR spectra with assignment of the most intensive peaks.

As mentioned above, the metabolite assignment was achieved using the published data for the reference standards (BMRB, HMDB, YMDB, ECMDB), literature data (Fan & Lane, 2008) and performing *ad hoc* 2D NMR experiments (TOCSY, HSQC). Different polar metabolites, amino acids, dipeptides, organic acids, carbohydrates and others, were assigned with this approach. All signal assignments are reported in Table 1 (for the convenience, the assignment with the increasing values of chemical shifts, is reported in the Supplementary Table 1).

3.2. High-field NMR spectral region

The high field region of the analyzed spectra of salmon extracts (0.8–3.0 ppm, Fig. 2A) contains signals belonging to the aliphatic groups. The aliphatic signals of the following amino acids were assigned in this region: isoleucine (Ile), leucine (Leu), valine (Val), threonine (Thr), alanine (Ala), lysine (Lys), glutamate (Glu), glutamine (Gln), methionine (Met) and β-alanine (βAla). However, the major signals in the region belong to lactic acid. Acetic and succinic acids signals were also detected in this region. The differences in the spoilage process at 0 and 4 °C can be observed by NMR spectroscopy analyzing the high-field (upfield) regions of the acquired spectra. In particular, for the samples at

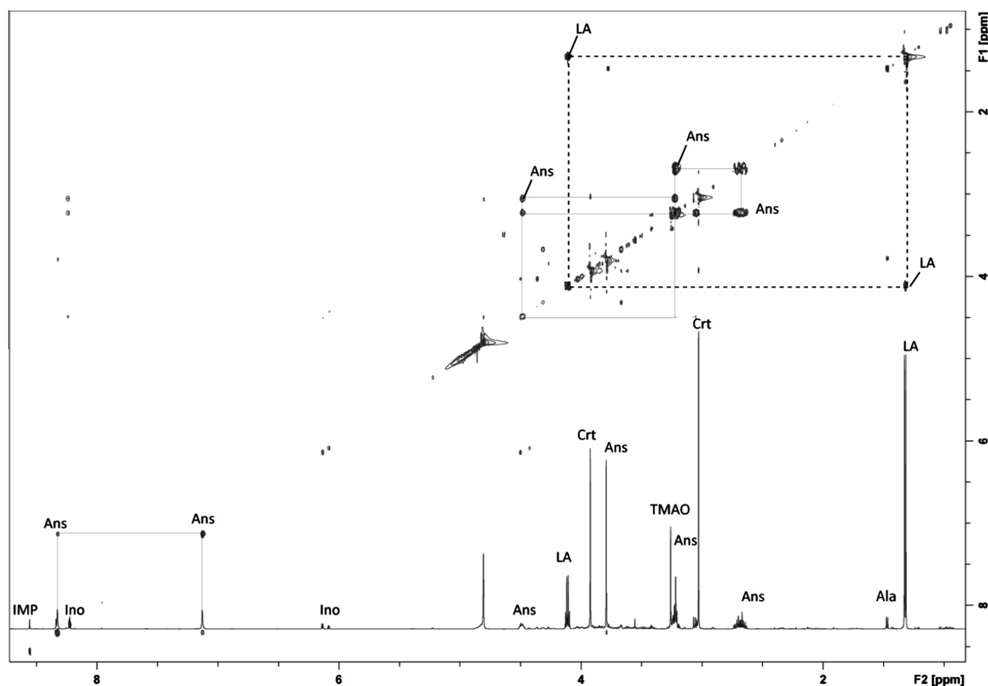

Fig. 1. 1D ^{1}H and 2D ^{1}H–^{1}H TOCSY NMR spectra of Atlantic salmon (*Salmo salar*) muscle TCA extract at 298 K (T_0).

4 °C, formation of ethanol and 2,3-butanediol can be observed (triplet at 1.18 and doublet at 1.14 ppm of ethanol and 2,3-butanediol, respectively, Fig. 2A). In addition, formation of acetic acid is much more evident for the higher temperature storage (4 °C, singlet at 1.91 ppm). It should be noted that for the 4 °C storage the formation of many other (unassigned) substances may be monitored. In this spectral region the doublets at 1.22; 1.37; 1.43 ppm, as well as the singlets at 1.24 and 2.22 ppm can be observed.

3.3. Mid-field NMR spectral region

In the midfield region of the NMR spectra (3.0–5.5 ppm, Fig. 2B) the α-protons of the amino acids contribute to the observed spectra with strong overlapping signals. The strongest peaks in this region were assigned to protons of creatine/phosphocreatine, anserine, choline and lactic acid. In analogy with the high-field region, significant intensity variations between samples at 4 and 0 °C were observed also in this region. For example, the singlet of trimethylamine oxide (TMAO) at 3.26 ppm (Fig. 2B) significantly decreases in intensity for the sample stored at 4 °C. Likewise, the signal belonging to the AMP (dd at 4.37 ppm, Fig. 2B) also decreases more rapidly at higher storage temperature. The spectral region between 4.1 and 5.5 ppm (Fig. 2C) is characterised also by the presence of sugar signals. The decreasing intensities of the glycogen (singlet at 5.39 ppm) and α-saccharides signals (doublets at 5.23; 5.45) during the storage is clearly seen in Fig. 2C.

3.4. Low-field NMR spectral region

In the low-field NMR spectral region (9.5–5.5 ppm) some of the ATP degradation metabolites can be observed (Fig. 3). Signals belonging to ATP, AMP, Ino and Hx were detectable at different time points. Through quantitative monitoring of these nucleotides it is possible to calculate the *K*-index, an important parameter connected with fish freshness (for details see below). In addition, nicotinic acid and niacinamide were assigned in the low-field spectral region for all samples and all time points.

3.5. Quantitative analysis of metabolites affecting the sensorial properties of fish

Some of the most important classes of metabolites influencing flavor, already described in fish muscle, are: free amino acids, peptides, guanidine compounds, nucleotides and organic acids. The distribution of these compounds varies with the taxonomic family of fish and can be different for wild and farmed fish (Aoki, Takada, & Kunisaki, 1991). The free amino acid content of aquatic organisms range from about 0.5 to 2% of muscle weight (Ruiter, 1995). Farmed fish tend to contain less free amino acids than wild fish (Haard, 1992; Simpson & Haard, 1999). The concentration of some amino acids determined for reference samples at T_0 are listed in Table 1 and the changes of concentrations of some metabolites measured during the storage of salmon fillet are reported in the Table 2. The different amounts of amino acids at T_0 between the two fishes could be due to individual variability.

The most abundant amino acids found in this study are creatine, taurine, lysine and threonine, in decreasing order. Among the identified amino acids Gly, Ala, Ser, Thr contribute to sweet taste, while the Arg, Leu, Val, Met, Phe, His and Ile impart bitter taste (Sikorski, 1990).

For most of the amino acids (Table 2), only small changes were observed between day 1 and day 4 while the concentration increased between the sixth and fourteenth day of storage. In this second phase, bacterial spoilage becomes more important and the activity of the bacteria accelerates the rate of protein hydrolysis with further release of free amino acids in fish muscle. However, bacteria can also metabolize free amino acids. The comparative analysis of the amino acid concentration at T_0 and subsequent time points shows that for glutamate and glycine, the concentration decreases during the storage at 4 °C. The decrease in concentration indicates that their release from muscle proteins was slower than their enzymatic degradation by i.e. bacteria. In addition, rapid chilling of fish and proper icing reduce the rate of spoilage. The only amino acids that increase during storage at 0 °C are glutamate and methionine. For the latter, the results are confirmed by other studies (Jiang & Lee, 1985).

Table 1
Signal assignments for ^1H NMR spectrum of Atlantic salmon (*Salmo salar*) TCA muscle extract.

Compound[a]	δ (ppm)	Mult	Conc. (mg/100 g) (4 °C)	Conc. (mg/100 g) (0 °C)	Assignment	TOCSY (ppm)
	Essential amino acids					
Isoleucine (Ile)	0.93	t			CH_3 (HD1)[c]	1.00/1.46/1.97
	1.00[b]	d			CH_3 (HG_2)	0.93/1.46/1.97
	1.46	m	1.95 ± 0.05	1.97 ± 0.09	CH_2 (HG_1)	–
	1.97	m			CH (HB)	1.00/1.46/3.66
	3.66	d			CH (HA)	1.00/1.97
Leucine (Leu)	0.95	t			Two CH_3 (HD)	1.70/3.73
	1.70	m	7.96 ± 0.97	5.91 ± 0.87	CH/CH_2 (HG/HB)	0.95/3.73
	3.73	m			CH (HA)	0.95/1.70
Valine (Val)	0.98	d			CH_3 (HG_1)	1.03/2.27/3.61
	1.03	d	3.55 ± 0.78	2.96 ± 0.57	CH_3 (HG_2)	0.98/2.27/3.61
	2.27	m			CH (HB)	0.98/1.03/3.61
	3.61	d			CH (HA)	0.98/1.03/2.27
Threonine (Thr)	1.32	d			CH_3 (HG_2)	–
	3.59	d	28.11 ± 1.52	5.70 ± 1.02	CH (HA)	1.32/4.27
	4.27	m			CH (HB)	1.32/3.59
Lysine (Lys)	1.47	–			CH_2	Overlap
	1.71	m	12.49 ± 1.13	13.90 ± 1.07	CH_2 (HD)	1.47/1.90/3.02/3.76
	1.90	m			CH_2 (HB)	1.47/3.02/3.76
	3.02	–			CH_2 (HE)	Overlap
	3.76	t			CH (HA)	1.47/1.90
Methionine (Met)	2.12	m			CH_3/CH_2 (HE/HB)	2.19/2.64/3.95
	2.19	m	2.77 ± 0.19	2.90 ± 0.18	CH_3/CH_2 (HE/HB)	2.12/2.64/3.95
	2.64	t			CH_2 (HG)	2.12/2.19/3.95
	3.95	dd			CH (HA)	2.12/2.19/2.64
Phenylalanine (Phe)	7.31	d			Two CH (HD) (H_2/H_6)	7.36/7.42
	7.36	m	0.61 ± 0.05	1.02 ± 0.03	CH/CH (HZ) (H_4)	7.31/7.42
	7.42	m			CH/CH (HE) (H_3/H_5)	7.31/7.36
Histidine (His)	7.11	s	4.61 ± 0.67	8.58 ± 0.69	CH (HD_2) (H_5)	7.95
	7.95	s			CH (HE_1) (H_2)	7.11
	Non-essential amino acids					
Alanine (Ala)	1.47	d			CH_3 (HB)	3.78
	3.78	q	18.98 ± 1.07	13.30 ± 1.16	CH (HA)	1.47
Glutamate (Glu)	2.12	m			CH_2 (HB)	2.06/2.35/3.75
	2.06	m			CH_2 (HB)	2.12/2.35/3.75
	2.35	m	13.80 ± 0.47	7.93 ± 0.57	CH_2 (HG)	2.06/2.12/3.75
	3.75	dd			CH (HA)	2.06/2.12/2.35
Glutamine (Gln)	2.17	m			CH_2 (HB)	2.53/3.77
	2.53	m	2.85 ± 0.18	2.17 ± 0.47	CH_2 (HG)	2.17/3.77
	3.77	t			CH (HA)	2.17/2.53
Glycine (Gly)	3.55	s	11.70 ± 0.77	7.81 ± 0.55	CH_2 (HA)	
Tyrosine (Tyr)	6.89	d			CH (HE)	7.19
	7.19		2.36 ± 0.18	1.00 ± 0.37	Only in T5	6.89
	7.20	d			CH (HD)	6.89
Aspartate (Asp)	2.66	dd			CH_2 (HB)	
	2.80	dd			CH_2 (HB)	2.66
	3.86	dd			CH (HA)	2.66/2.80 in T5; 4 °C
β-Alanine (βAla)	2.55	t			CH_2 (H_4)[d]	3.18
	3.18	t	1.74 ± 0.37	1.32 ± 0.26	CH_2 (H_5)	2.55
Taurine (Tau)	3.25	t			CH_2 (H_5)	3.42
	3.42	t	65.1 ± 1.97	14.18 ± 1.23	CH_2 (H_6)	3.25
Creatine/phospho(Crt)	3.03	s			CH_3 (H_6)	
	3.92	s	177.31 ± 3.0	101.55 ± 4.0	CH_2 (H_4)	
GABA	1.89	m			CH_2 (H_5)	2.29/3.02
	2.29	t			CH_2 (H_4)	1.89/3.02
	3.02	t			CH_2 (H_6)	–
1-Methyl-histidine	3.66	s			CH_3 (H_6)	
	7.15	s	0.63 ± 0.04	0.31 ± 0.01	CH (H_2)	8.04
	8.04	s			CH (H_4)	7.15
	Dipeptides					
Anserine (Ans)	2.67	m			CH_2 (H_{14})	34.80
	3.06	dd			CH_2 (H_7)	28.31
	3.23	m	227.0 ± 3.38	131.0 ± 3.57	Two CH_2 (H_7/H_{14})	38.348
	3.8	s			CH_3 (H_6)	
	4.48	dd			CH (H_8)	HSQC: 56.17
	7.13	s			CH (H_5)	HSQC: 122.04
	8.33	s			CH (H_2)	HSQC: 138.37

(*continued on next page*)

Table 1 (*continued*)

Compound[a]	δ (ppm)	Mult	Conc. (mg/100 g) (4 °C)	Conc. (mg/100 g) (0 °C)	Assignment	TOCSY (ppm)
	Organic acids					
Acetic acid (AA)	1.91	s	1.04 ± 0.22	0.56 ± 0.090	CH_3	
Lactic acid (LA)	1.32	d			CH_3	HSQC: 22.8
	4.11	q	473.0 ± 8.97	258.0 ± 5.87	CH	HSQC: 71.2
Succinic acid (SA)	2.40	s	0.38 ± 0.09	0.0	CH_2/CH_2	
Fumarate	6.51	s			Two CH	
Formic acid (FA)	8.45	s			CH	
	Carbohydrates					
Glycogen	3.42	m			Two CH	
	3.60	m			n CH	
	3.69					
	3.84	m	1.86 ± 0.22	3.1 ± 0.57		
	3.96	Br. s.			Two CH (H_4/H_9)	
	5.39	br. s.			Four CH	3.42/3.60/3.69/3.84/3.96
Maltose	3.42	t			CH (H_5)	
	3.58	m	1.27 ± 0.24	6.4 ± 0.27	Two CH (H_3/H_{13})	
	3.70	m			CH (H_6)	
	5.40	d			CH (H_2)	3.42/3.58/3.70
α-D-glucose (αGlc)	3.40	m			CH (H_5)	
	3.49	m			n CH	
	3.77	m			n CH	
	3.90	dd			CH_2 (H_{11})	
	5.45	d			CH (H_2)	3.40/3.49/3.77/3.90
β-D-glucose (βGlc)	3.24					
	3.40	m			CH (H_5)	
	3.47	m			n CH	
	4.63	d			CH (H_2)	3.24/3.40/3.48
α-D-glucose-6-phosphate (αG6P)	3.41					
	3.55	ddd			CH_2 (H_{11})	
	3.71	t			CH (H_4)	
	3.83					
	3.88	ddd	17.1 ± 1.27	10.7 ± 1.34	CH (H_6)	
	3.94					
	4.03	m			CH_2 (H_{11})	
	5.23	d			CH	3.41/3.55/3.71/3.83/3.94/4.03
β-D-glucose-6-phosphate (βG6P)	3.26					
	3.48					
	3.56					
	4.00					
	4.65	d			CH	3.26/3.48/3.56/4.00
	Nucleotides					
ATP	8.13	s			CH (H_{12})	
	8.49	s			CH (H_7)	
AMP	4.03	dd			CH_2 (H_{17})	
	4.37	dd			CH (H_5)	
	4.50	dd			CH (H_3)	
	6.14	d			CH (H_2)	
IMP	8.2	s	99.09 ± 2.97	71.40 ± 4.57	CH (H_7)	
	8.56	s			CH (H_{12})	
Inosine (Ino)	3.84	dd			CH_2 (H_{17})	
	3.91	dd			CH_2 (H_{17})	
	4.27	dd			CH (H_5)	
	4.43	dd	94.18 ± 1.51	59.80 ± 1.32	CH (H_4)	
	4.75	s			CH (H_3)	
	6.09	d			CH (H_2)	
	8.23	s			CH (H_7)	
	8.34	s			CH (H_{12})	
Hypoxanthine (Hx)	8.18	s			CH (H_2)	8.20
	8.20	s	2.28 ± 0.30	1.01 ± 0.05	CH (H_7)	8.18
NAD	4.23	m				
	4.38	m				
	8.84	d	1.20 ± 0.02	1.28 ± 0.03	CH (H_{39})	9.14/9.33
	9.14	d			CH (H_{37})	8.84/9.33
	9.33	s			CH (H_{35})	8.84/9.14
	Biogenic amines					
Putrescine	1.79	m			Two CH_2 (H_3/H_4)	3.09
	3.09	t			Two CH_2 (H_2/H_5)	1.79
Tyramine	2.91	t			CH_2	3.24
	3.24	t			CH_2	2.91
	6.88	d			Two CH	7.19

Table 1 (*continued*)

Compound[a]	δ (ppm)	Mult	Conc. (mg/100 g) (4 °C)	Conc. (mg/100 g) (0 °C)	Assignment	TOCSY (ppm)
	7.19	d			Two CH	6.88
Cadaverine	1.46	m			CH_2 (H_4)	1.71/3.01
	1.71	quin			Two CH_2 (H_3/H_5)	1.46/3.01
	3.01	t			Two CH_2 (H_2/H_6)	1.46/1.71
	Others					
Nicotinic acid (NA)	7.52	dd			CH (H_5)	8.25/8.60/8.93
	8.25	m			CH (H_4)	
	8.60	dd			CH (H_6)	7.52/8.25/8.93
	8.93	d			CH (H_2)	7.52/8.25/8.60
Niacinamide (NAM)	7.59	dd			CH (H_5)	8.25/8.70/8.93
	8.25	m			CH (H_4)	
	8.70	dd	1.36 ± 0.04	3.00 ± 0.03	CH (H_6)	7.59/8.25/8.93
	8.93	d			CH (H_2)	7.59/8.25/8.70
Nicotinamide Ribotide (NAR)	9.00	d			CH (H_9)	9.34
	9.34	d			CH (H_{11})	9.00
	9.57	s			CH (H_7)	9.34
Trimethylamine (TMA-N)	2.89	s	0.003 ± 0.0001	0.003 ± 0.0001	CH_3	HSQC: 47.25
Trimethylamine oxide (TMAO-N)	3.26	s	4.74 ± 0.5	2.54 ± 0.27	CH_3	
Formalehyde	3.29	s			CH	
	3.31	s			CH	
1.3-Diaminopropane. polyamine	2.11	m			CH_2 (H_3)	3.11
	3.11	t			Two CH_2 (H_2/H_4)	2.11
Choline (Cho)	3.22	s			Three CH_3	
	3.51	t	5.66 ± 0.12	2.64 ± 0.13	CH_2 (H_3)	4.05
	4.05	ddd			CH_2 (H_2)	3.51
Ethanol (EtOH)	1.18	t			CH_3	
	3.66	q	0.0	0.0	CH_2	
2.3-Butanediol	1.14	d			Two CH_3	3.62/3.72
Uracil	5.80	d			CH (H_5)	
	7.53	d			CH (H_6)	

[a] Some of the metabolites were only present at certain time points.
[b] Not overlapping signals easier for integration are presented with underscore.
[c] In the protein amino acids assignment the biochemical designation was used.
[d] For other metabolites organic nomenclature (HMDB) was used. Mult. in the heading of the table stands for multiplicity.

Nucleotides, like adenosine triphosphate (ATP) and nicotinamide adenin dinucleotide (NAD^+/NADH) are present in both samples (Table 1) and contribute to a meaty taste (Sikorski, 1990). Normally, dark muscle contains about two times more of these compounds than white muscle. The average concentrations of ATP and NAD at time T_0 were 4.00 and 1.24 mg/100 g, respectively. Creatine, lactate and succinate are other taste-active components (Schlichtherle-Cerny & Grosch, 1998). In particular, creatine (Cr) and phosphocreatine (PCr) contribute to the thickness and mouthfulness (Shah, Ogasawara, Egi, Kurihara, & Takahashi, 2010).

3.6. Content of carbohydrates and of their glycolytic metabolites

A number of signals from sugar moieties were previously assigned from a perchloric acid extract from Atlantic salmon (Castejon et al., 2010; Gribbestad et al., 2005). In the absence of oxygen, the main carbohydrate of the fish muscle, glycogen, undergoes anaerobic glycolysis to lactic acid. During *post-mortem* storage, two possible pathways related to the degradation of glycogen in fish muscle are active (Eskin & Shahidi, 2012): hydrolytic (amylolytic, *via* Maltose) pathway or via the phosphorolytic (glycolytic) pathway. NMR can be used to detect characteristic metabolites within each pathway. An increase in amount of maltose was observed for the samples at 4 °C after 2 storage days (Table 2). The glycogen decrease was directly observed in our study during the fish storage (Fig. 2C).

The final product of glycolysis is lactic acid, which tends to increase during post mortem degradation when glycogen is completely degraded, and with decrease of the maltose, after about 7–8 storage days (Table 2).

The changes in the concentration of sugar and/or sugar phosphates in the muscle contribute to the gradual loss of the sweet, meaty tint of the flavor of very fresh fish (Sikorski, 1990). The decrease in carbohydrates is probably due to alcoholic fermentation processes which lead to the production of organic acids such as acetate and succinate (only at 4 °C) and alcohols (i.e. ethanol and 2,3-butanediol) with higher rate at 4 °C. A higher production of these substances was observed with increasing storage time (Table 2).

3.7. The measurement of changes in the amounts of physiologic dipeptides

Free histidine and histidine-derived dipeptides, like β-alanylhistidine (carnosine) and β-alanyl-1-methylhistidine (anserine), are normally present in fish muscle. They are important due to their buffering ability and other important biological functions (Van Waarde, 1988). The relative concentration of these compounds is different for the different fish classes, and for *Salmonidae* anserine is the major histidine-related substance (Van Waarde, 1988).

Histidine (His) was found in relatively low concentrations (4.6 and 8.6 mg/100 g in the samples at 4 and 0 °C, respectively). The anserine was found in significant amount (227 and 131 mg/100 g for samples at 4 and 0 °C, respectively), while no carnosine was detected.

In this work, different rates of increase for β-alanine and 1-methyl-histidine (anserine degradation products) were detected by NMR for salmon samples stored at 4 and 0 °C (Table 2). The obtained data indicate that the concentration of β-alanine increased after 2 days of storage both for samples at 4 and 0 °C

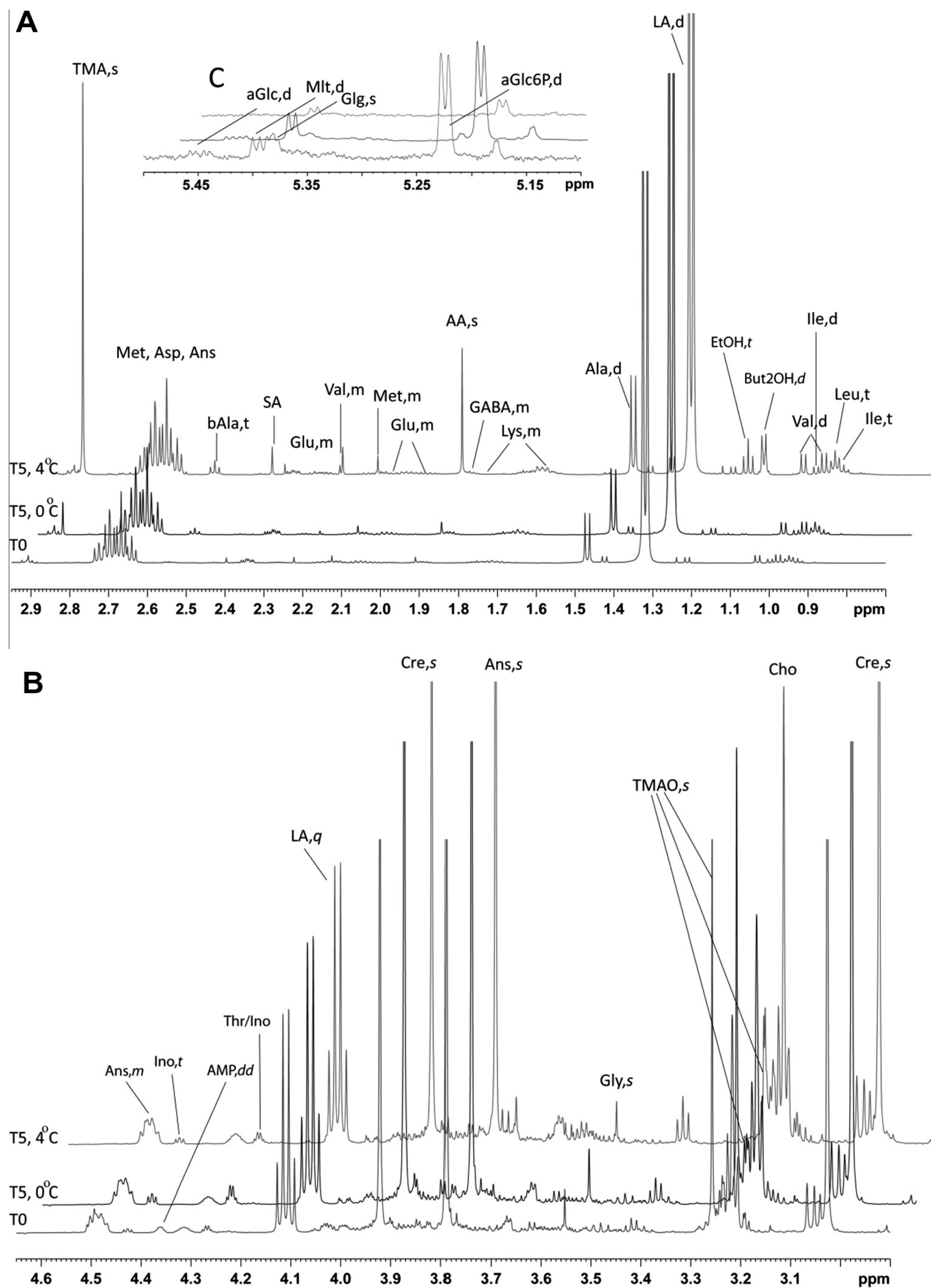

Fig. 2. The high-field (A) and mid-field (B and C) regions of 298 K ^{1}H NMR spectra of Atlantic salmon (*Salmo salar*) muscle TCA extract at T_0 (bottom) and T_5 (middle – 0 °C; top – 4 °C) time points.

with a higher rate of increase at 4 °C (Table 2). The observed increase in β-alanine and 1-methyl-histidine could be due to degradation of anserine by anserinase (Van Waarde, 1988).

3.8. The evolution of the energy related vitamin B3

The vitamin content of fish varies with species, age, season and fishing location. Among water soluble vitamins only niacin (vitamin B3) was found in the analyzed salmon samples. Our NMR investigations allowed detection of two forms of vitamin B3 in salmon muscle – niacinamide (NAM) and nicotinic acid (NA) (Table 1). Niacinamide was detected in all samples at every time point. The nicotinic acids' signals were detectable only for the sample at 4 °C at T_5 time point. It was previously argued (Brækkan, 1959) that the niacin is present in almost the same concentrations in both types of muscle and the most active and energetic fish may show the highest content of niacin (Brækkan, 1959). The following values for the content of niacin in fish have previously been reported: Tunas, 0–23.4 mg/100 g; Herrings, 0.6–9.6 mg/100 g; Halibuts, 2.8–14.2 mg/100 g; Codfishes, 0.2–6.7 mg/100 g (Sikorski, 1990) and for salmonidae 8.8 mg/100 g (Brækkan, 1959). The content of niacin in salmon muscle TCA

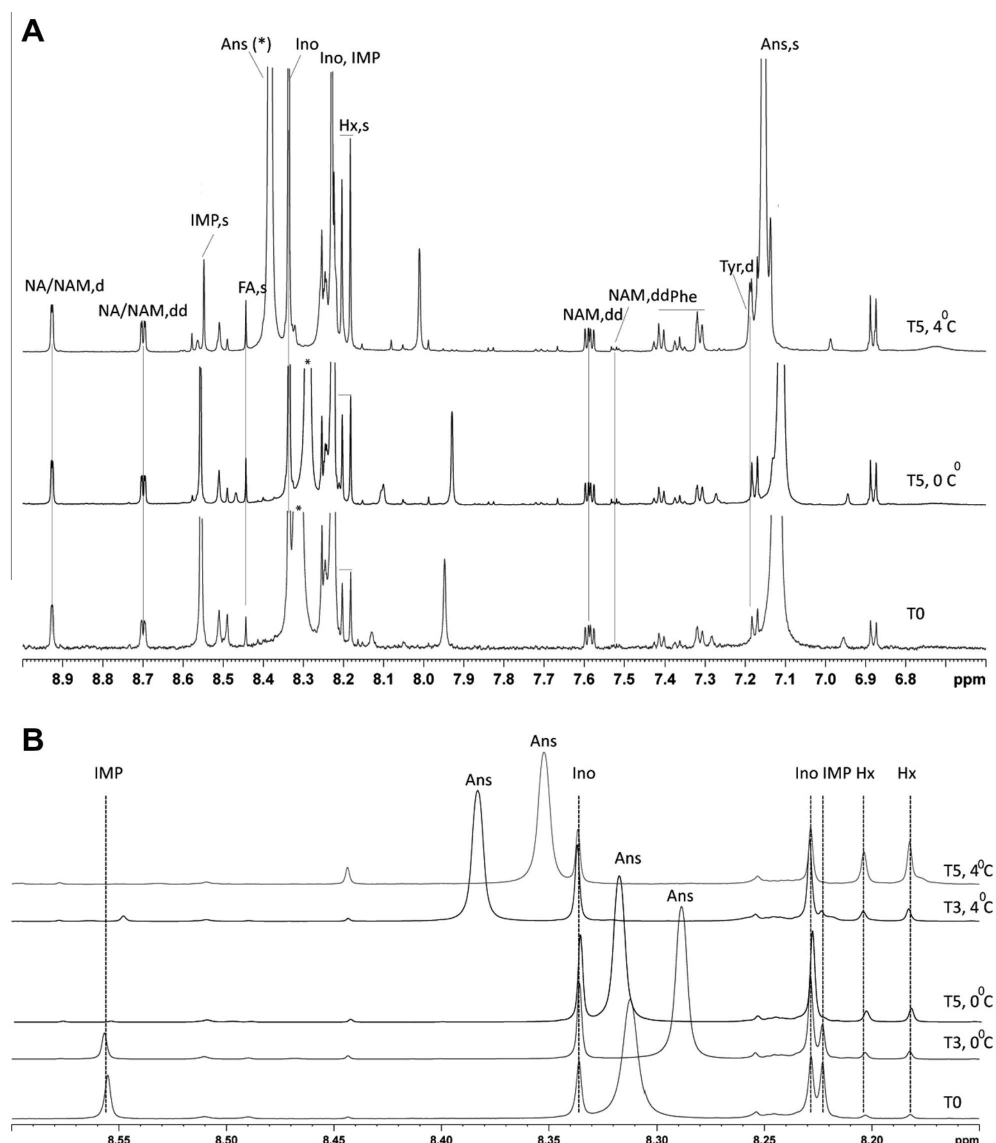

Fig. 3. The low-field region of 298 K ^{1}H NMR spectra of Atlantic salmon (*Salmo salar*) muscle TCA extracts: (A) T_0 (bottom) and T_5 (middle – 0 °C; top – 4 °C) time points; (B) ATP degradation metabolites at T_0, T_3 and T_5 time points.

extract in our studies was 1.36 mg/100 g in samples at 4 °C and 3.00 mg/100 g for those at 0 °C. These data are in the same range as values found in the literature.

3.9. The measurement of metabolites to calculate the fish freshness K-index

Adenosine triphosphate (ATP) and its breakdown products have been found to correlate well with the loss of the freshness in a wide range of species (Jones, Murray, Livingston, & Murray, 1964; Spinelli, Eklund, & Miyauchi, 1964). Most of the ATP degradation products were detected in all samples (Fig. 3B) in this study.

In the ^{1}H NMR spectra in Fig. 3B, the singlets for hypoxanthine (Hx) can be seen at 8.20 and 8.18 ppm. The singlets at 8.34 and 8.23 ppm belonging to inosine (Ino) are detectable for all the registered time points at both storage temperatures. IMP can be assigned as the singlets at 8.22 and 8.56 ppm and can be detected at time points T_0–T_3 during storage at 4 °C and for all time points in samples at 0 °C (Fig. 3B). The concentrations of ATP degradation products at different time points are reported in Table 2 and in Fig. 4.

According to our data, the salmon sample kept at 4 °C exceeds the value 80% for *K*-index from time point T_3 (Fig. 4). It seems that the time between T_2 and T_3 time points is crucial for this sample. The *K*-index exceeds the 80% value at T_3 for both storage temperatures. As expected, the sample stored at 0 °C has longer shelf-life. At time T_3 the *K*-index is 69% reaching 83% at T_4. The latter value is higher than the acceptable maximum *K*-values for the rejection of Atlantic salmon at 70–80% (Erikson et al., 1997). Based on our data the shelf life of Atlantic salmon is 12 days at 4 °C storage and 15 days at 0 °C storage.

3.10. The measurement of the precursor of fishy smell trimethylamine oxide

Trimethylamine oxide (TMAO) is present in many fish species [43]. During spoilage of fish, TMAO is converted to trimethylamine (TMA, a volatile substance with unpleasant specific "fishy" odor and very low odor threshold (Hebard, Flick, & Martin, 1982)) and formaldehyde (Van Waarde, 1988). In addition, formaldehyde can contribute to formation of crosslinkages between muscle proteins, resulting in release of a large amount of moisture from the muscle

Table 2
The content of some Atlantic salmon (*Salmo salar*) metabolites in fish muscle measured during the storage at different temperatures.

Metabolite	T (°C)	Concentration (mg/100 g) at different time point					
		T_0 (0 days)	T_1 (2 days)	T_2 (4 days)	T_3 (7 days)	T_4 (10 days)	T_5 (14 days)
	Essential amino acids						
Isoleucine (Ile)	0	2.0 ± 0.1	1.70 ± 0.1	2.0 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.2 ± 0.1
	4	1.9 ± 0.1	1.3 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.9 ± 0.1	3.8 ± 0.1
Leucine (Leu)	0	5.9 ± 1.0	5.1 ± 0.8	6.5 ± 0.6	8.1 ± 0.6	8.6 ± 1.0	9.2 ± 1.0
	4	8.0 ± 1.0	5.1 ± 0.7	6.9 ± 0.5	10.9 ± 0.9	12.0 ± 0.6	19.9 ± 0.9
Valine (Val)	0	3.0 ± 0.6	2.6 ± 0.8	3.5 ± 0.4	4.2 ± 0.9	4.6 ± 0.7	5.00 ± 0.6
	4	3.6 ± 0.8	2.3 ± 1.0	3.2 ± 0.5	4.5 ± 0.7	5.4 ± 0.6	8.7 ± 0.7
Threonine (Thr)	0	5.7 ± 1.0	5.0 ± 0.8	7.7 ± 0.6	8.9 ± 0.5	11.5 ± 0.8	12.0 ± 1.0
	4	28.1 ± 1.5	17.7 ± 1.7	25.2 ± 1.5	36.2 ± 1.0	28.0 ± 1.1	24.4 ± 1.1
Lysine (Lys)	0	13.9 ± 1.1	14.1 ± 1.1	21.9 ± 1.4	19.1 ± 1.1	24.3 ± 1.3	24.2 ± 1.6
	4	12.5 ± 1.1	7.9 ± 1.1	11.8 ± 2.5	14.7 ± 2.3	26.6 ± 1.6	45.4 ± 1.3
Methionine (Met)	0	2.9 ± 0.2	2.9 ± 0.2	4.1 ± 0.2	4.1 ± 0.2	4.3 ± 0.2	3.8 ± 0.2
	4	2.8 ± 0.2	1.7 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	2.8 ± 0.2	4.8 ± 0.2
Phenylalanine (Phe)	0	1.0 ± 0.0	1.0 ± 0.0	1.2 ± 0.0	1.4 ± 0.0	2.1 ± 0.3	2.4 ± 0.6
	4	0.6 ± 0.1	0.6 ± 0.0	0.2 ± 0.1	0.9 ± 0.0	2.7 ± 0.4	5.0 ± 0.3
Histidine (His)	0	8.6 ± 0.7	5.4 ± 0.7	10.4 ± 0.6	8.4 ± 0.6	10.5 ± 0.7	11.2 ± 0.5
	4	4.6 ± 0.7	2.7 ± 0.6	4.5 ± 0.7	5.3 ± 0.7	4.6 ± 0.7	4.8 ± 0.7
	Non-essential amino acids						
Alanine (Ala)	0	13.3 ± 1.2	10.4 ± 1.3	16.6 ± 1.3	18.8 ± 1.1	18.2 ± 1.3	17.7 ± 1.2
	4	19.00 ± 1.1	11.9 ± 1.0	16.5 ± 1.2	21.1 ± 1.0	22.1 ± 1.1	31.0 ± 0.7
Glutamate (Glu)	0	7.9 ± 0.6	7.5 ± 0.6	13.6 ± 0.6	14.5 ± 0.6	13.4 ± 0.7	11.00 ± 0.6
	4	13.8 ± 0.5	8.0 ± 0.6	9.9 ± 0.6	8.1 ± 0.6	7.5 ± 0.6	9.8 ± 0.5
Glutamine (Gln)	0	2.2 ± 0.5	2.1 ± 0.6	4.3 ± 0.6	5.00 ± 0.1	6.1 ± 0.3	6.3 ± 0.6
	4	2.9 ± 0.2	2.00 ± 0.2	3.6 ± 0.1	7.9 ± 0.1	9.00 ± 0.1	12.5 ± 0.2
Glycine (Gly)	0	7.8 ± 0.5	6.8 ± 0.6	10.6 ± 0.6	8.8 ± 0.5	11.4 ± 0.5	9.7 ± 0.6
	4	11.7 ± 0.8	5.6 ± 0.7	7.0 ± 0.7	7.6 ± 0.8	6.9 ± 0.6	8.5 ± 0.6
Tyrosine (Tyr)	0	1.0 ± 0.4	1.4 ± 0.5	3.1 ± 0.4	3.3 ± 0.2	3.9 ± 0.5	3.4 ± 0.5
	4	2.4 ± 0.2	0.3 ± 0.3	2.2 ± 0.5	2.4 ± 0.3	3.4 ± 0.3	7.8 ± 0.2
β-Alanine (β-Ala)	0	1.3 ± 0.3	1.3 ± 0.3	2.6 ± 0.3	3.0 ± 0.4	3.7 ± 0.5	3.8 ± 0.8
	4	1.7 ± 0.4	1.2 ± 0.5	2.2 ± 0.5	4.8 ± 0.2	5.5 ± 0.4	7.6 ± 0.4
Taurine (Tau)	0	14.2 ± 1.2	8.9 ± 1.3	15.1 ± 1.1	22.3 ± 1.2	15.9 ± 1.5	19.3 ± 1.4
	4	32.6 ± 2.0	10.4 ± 1.0	16.9 ± 1.5	32.8 ± 1.3	25.6 ± 1.6	35.5 ± 0.7
Phosphocreatine–Creatine (P-Crt)	0	101.6 ± 4.0	81.7 ± 3.8	131.1 ± 3.6	145.5 ± 4.1	137.1 ± 3.2	127.3 ± 4.5
	4	177.3 ± 3.0	106.7 ± 4.0	124.3 ± 4.0	141.1 ± 3.3	134.3 ± 3.5	150.1 ± 3.7
1-Methyl-histidine	0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
	4	0.6 ± 0.0	0.6 ± 0.0	0.8 ± 0.0	1.8 ± 0.0	0.9 ± 0.1	0.9 ± 0.1
	Dipeptides						
Anserine	0	131.0 ± 3.6	108.0 ± 3.8	157.0 ± 3.6	170.0 ± 3.1	166.0 ± 3.3	170.0 ± 3.6
	4	227.0 ± 3.4	132.3 ± 3.0	178.0 ± 3.5	201.7 ± 3.3	170.4 ± 3.6	218.0 ± 3.7
	Organic acids						
Acetic acid	0	0.6 ± 0.1	0.6 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	1.0 ± 0.1	1.2 ± 0.1
	4	1.0 ± 0.2	0.5 ± 0.2	0.7 ± 0.1	1.7 ± 0.4	7.5 ± 0.9	11.1 ± 0.9
Lactic acid	0	258.5 ± 5.9	206.2 ± 5.8	314.7 ± 5.6	365.2 ± 5.1	340.0 ± 5.2	308.1 ± 5.5
	4	473.0 ± 9.0	292.2 ± 8.0	356.5 ± 8.5	400.1 ± 8.3	355.2 ± 8.6	414.7 ± 8.7
Succinic acid	0	0.0	0.0	0.0	0.0	0.0	0.0
	4	0.4 ± 0.1	0.9 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	1.8 ± 0.1	3.6 ± 0.1
	Carbohydrates						
Glycogen	0	3.1 ± 0.6	3.1 ± 0.5	2.4 ± 0.5	2.2 ± 0.1	0.9 ± 0.3	0.8 ± 0.2
	4	1.9 ± 0.2	5.1 ± 0.4	1.2 ± 0.2	0.0	0.0	0.0
Maltose	0	6.4 ± 0.3	6.4 ± 0.2	5.0 ± 0.4	4.5 ± 0.1	1.8 ± 0.3	1.7 ± 0.3
	4	1.3 ± 0.2	11.1 ± 0.3	10.0 ± 0.3	9.9 ± 0.3	1.8 ± 0.2	0.0
αD-glucose (α-GLC)	0	10.7 ± 1.3	8.6 ± 1.6	14.5 ± 1.5	16.5 ± 1.1	12.5 ± 1.1	9.6 ± 1.2
	4	17.1 ± 1.3	12.6 ± 1.2	15.4 ± 1.5	11.8 ± 1.3	3.9 ± 0.6	1.4 ± 0.2
	Nucleotides						
Inosine 5-monophosphate (IMP)	0	71.4 ± 4.6	58.2 ± 2.5	51.6 ± 3.9	47.2 ± 3.9	11.5 ± 1.9	2.00 ± 0.3
	4	99.1 ± 3.0	51.5 ± 2.5	44.9 ± 2.9	6.5 ± 1.9	0.0	0.0
Hypoxanthine (Hx)	0	1.0 ± 0.1	2.1 ± 0.0	3.6 ± 0.6	4.6 ± 0.6	6.6 ± 1.6	7.9 ± 2.0
	4	2.3 ± 0.3	2.3 ± 0.8	4.3 ± 0.6	7.8 ± 0.9	19.3 ± 1.6	26.1 ± 2.0
	Others metabolites						
Trimethylamine (TMA-N)	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.2
	4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	2.1 ± 0.1	2.5 ± 0.1
Trimethylamine oxide (TMAO-N)	0	3.5 ± 0.3	3.3 ± 0.3	3.1 ± 0.4	3.3 ± 0.1	3.5 ± 0.3	3.3 ± 0.3
	4	4.7 ± 0.5	3.4 ± 0.4	3.2 ± 0.2	3.4 ± 0.2	1.0 ± 0.2	1.1 ± 0.2
Choline (Cho)	0	2.6 ± 0.1	2.2 ± 0.2	3.2 ± 0.3	3.9 ± 0.1	3.5 ± 0.3	3.3 ± 0.2
	4	5.7 ± 0.1	3.7 ± 0.2	4.3 ± 0.2	4.8 ± 0.3	4.5 ± 0.2	4.7 ± 0.2
Ethanol (EtOH)	0	0.0	0.0	0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0
	4	0.0	0.0	0.0	0.4 ± 0.0	1.7 ± 0.3	4.9 ± 0.6
2-3 Butanediol	0	0.0	0.0	0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
	4	0.0	0.0	0.0	0.6 ± 0.0	7.4 ± 1.6	9.1 ± 1.4

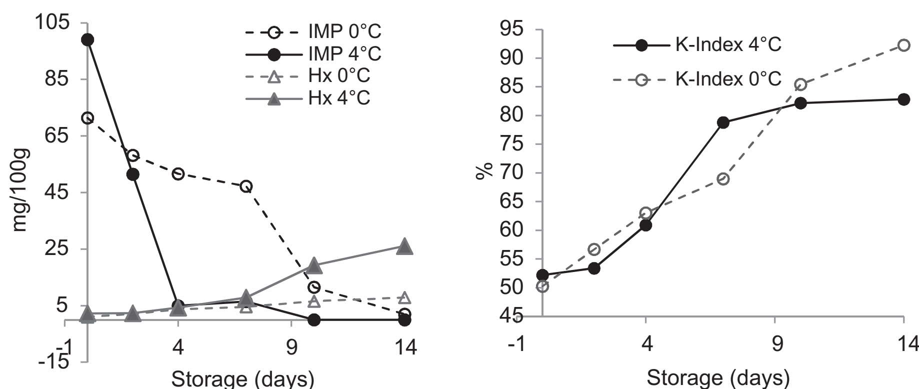

Fig. 4. Changes in concentration of IMP, Hx relative to *Salmo salar* samples as well as the *K*-index (%) during the fish storage at 4 and 0 °C (IMP in black, cycle; Hx in gray, triangle; 0 °C dash line; 4 °C solid line).

during cooking and in a dry and spongy product (Van Waarde, 1988). The TMAO content in fish increases with the age, size and decreases with a drop of salinity. In addition, the TMAO concentration is season and diet-dependent (Van Waarde, 1988). For young salmon the TMAO has a exogenous origin (Benoit & Norris, 1945). Moreover, TMA can be formed from choline during bacterial spoilage (Van Waarde, 1988). For both temperatures investigated in this study, the presence of TMAO (characteristic singlet at 3.26 ppm, Table 1) was detected in salmon samples at time T_0. The increase in the TMA concentration showed a good correlation with the decrease in TMAO concentration (Table 2).

After 7 days a significantly higher quantity of TMA is produced at 4 °C compared to samples stored at 0 °C (Table 2). This is caused by the slower conversion of TMAO into TMA at 0 °C compared to at higher temperatures. At 0 °C the conversion of TMAO into TMA is replaced by a slow enzymatic reaction which leads to the formation of dimethylamine (DMA) and formaldehyde (Huss, 1995).

3.11. Biogenic amines

The most significant biogenic amines occurring in foods are histamine, putrescine, cadaverine, tyramine, tryptamine, 2-phenylethylamine, spermine, spermidine and agmatine (Paleologos, Savvaidis, & Kontominas, 2004; Özogul & Özogul, 2006). Biogenic amines are dangerous for consumers, because, unlike TMA-N or other compounds (methylmercaptan and dimethylsulphide) that are strongly related to development of unpleasant odors, some of these metabolites such as histamine are odorless but still have a harmful effect for consumers. In Atlantic salmon, using a NMR approach, we observed the formation of tyramine and cadaverine due to decarboxylation of the amino acids tyrosine and lysine, respectively, during post mortem storage. These metabolites have a chemical structure very similar to their precursor amino acids. These compounds were qualitatively but not quantitatively monitored by NMR, because their signals often overlap with those corresponding to the precursor compounds and thus individual quantification is difficult. The formation of these biogenic amines has been observed only during storage at 4 °C after about 7 days of storage (T_3 time point).

4. Conclusion

Production of safe and tasty foods with a high nutritive value requires reliable, accurate and sensitive analytic protocols that can analyze many compounds simultaneously.

NMR spectroscopy seems to collect all these advantages, providing a well-defined molecular profile in a few minutes, requiring a simple extraction procedure which uses very small volumes of environment friendly solvent (water) and, considering its high-throughput applicability, the cost per analysis is sufficiently restrained. This work shows the significant potential and strength of high resolution NMR spectroscopy in detection and consequent assignment of various salmon metabolites upon different fish storage conditions.

In this study, 49 metabolites were unequivocally assigned, and the content of 31 of them was measured simultaneously. Furthermore, other unassigned signals, detected in this study, undergo a significant change in their corresponding area during the storage. Altogether, assigned and unassigned signals constitute a rich set of biomarkers, the so called molecular profile, suitable to follow the freshness loss of raw salmon fillets, a process which is composed by two different phenomena, i.e. the *post mortem* autogenic enzymatic hydrolysis and the spoilage due to the microbial proliferation.

NMR spectroscopy can provide a one shot picture of the fish "chemical" quality. Further studies should include studies of correlation with sensorial, nutritional and technological quality (Laghi, Picone, & Capozzi, 2014). The potential of NMR spectroscopy entitle the technique an important role in the new Foodomics science, the field where food scientists, technologists and nutritionist joint their efforts to strengthen the link between food, process induced changes, quality and health.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.03.037.

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