Food Chemistry 211 (2016) 803–811

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Quality changes of salmon by-products during storage: Assessment and quantification by NMR

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ARTICLE INFO

Article history:
Received 19 August 2015
Received in revised form 10 May 2016
Accepted 13 May 2016
Available online 14 May 2016

Chemical compounds studied in this article:
Creatine (PubChem CID: 586)
Phosphocreatine (PubChem CID: 9548602)
Taurine (PubChem CID: 1123)
Anserine (PubChem CID: 112072)
Trimethylamine N-oxide (PubChem CID: 1145)
Trimethylamine (PubChem CID: 1146)
Cadaverine (PubChem CID: 273)
Putrescine (PubChem CID: 1045)
Tyramine (PubChem CID: 5610)
Histamine (PubChem CID: 774)

Keywords:
Salmon by-products
Fish metabolites
NMR of fish by-products

ABSTRACT

Safe utilization of fish by-products is an important task due to increasing fish consumption. It can provide new valuable food/feed and will increase the economical profit and sustainability of the fishery industry. NMR spectroscopy is a reliable tool able to monitor qualitative and quantitative changes in by-products. In this work the trichloroacetic acid extracts of salmon backbones, heads and viscera stored at industrially relevant temperatures (4 and 10 °C) were studied using NMR. Twenty-five metabolites were detected and the possibility of salmon by-products utilization as a source of anserine, phosphocreatine and taurine was discussed. Statistical data elaboration allowed determining the main processes occurring during by-products storage: formation of trimethylamine and biogenic amines, proteolysis and different types of fermentations. By-products freshness was evaluated using a multi-parameter approach: the trimethylamine and biogenic amines concentration changes were compared with K_i and H-values and safe temperatures and times for storage of salmon by-products were proposed.

1. Introduction

The increasing world population leads to a larger demand for fish and fishery products worldwide. Thus development of sustainable fish farming practices gains an important role as one of the measures to preserve limited natural fish resources. The global production of farmed salmon in 2011 reached 1.93 million tonnes and most of the fish are filleted with the fillet yielding about 55% of the total fish (FAO, 2014). Consequently, salmon processing generates considerable quantities of residual material. Viscera and trimmings account for about 50% of the salmon by-products, while heads and backbones make for about 7 and 8%, respectively (Olafsen, Richardsen, Nystøyl, Strandheim, & Kosmo, 2014). This material contains valuable high-quality protein, fatty acids, vitamins, and minerals, among others. The market for products with nutritional and health beneficial ingredients is growing. Fish proteins and hydrolysates can supply the increasing demand for animal-derived proteins and can also be used as valuable nutritional ingredients in different products and diet formulations (FAO, 2014). The utilization of fish by-products may become an important industry: heads and backbones left after filleting can be used as raw materials for the production of sausages, cakes, gelatin, sauces and viscera can be used as a potential source of protein hydrolysates (FAO, 2014). However, the fast decomposition of fish by-products may become a challenge for their successful market penetration. With prolonged storage time and increased temperature, freshness and quality of by-products irreversibly decrease and hygienic regulations may restrict their use due to food safety and quality issues. Consequently, accurate qualitative

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http://dx.doi.org/10.1016/j.foodchem.2016.05.088
0308-8146/

and quantitative monitoring of these changes is important for further by-products processing and utilization.

Quality changes of marine products can be detected by sensory evaluation and/or by using classical standard analytical methods. However, these methods are time and human source demanding and can be not sensitive enough. Therefore, more advanced and sensitive techniques for quality and quantity evaluation are needed. In this work nuclear magnetic resonance (NMR) spectroscopy was used to qualitatively and quantitatively monitor water-soluble metabolites present in salmon by-products and detect their changes during storage. Principal component analysis (PCA) was used to evidence the most important changes in by-products composition and concentration. The data obtained from this study show that it is possible to detect high-added value compounds in salmon by-products and to determine their safe processing using NMR spectroscopy.

2. Materials and methods

2.1. Sample preparation

Fresh farmed salmons (*Salmo salar*) from Salmar/Nutrimar (Norway) were used for the tests. Heads and backbones from more than 15 fish were collected from the filleting lines. These rest raw materials and whole fish were packed separately, placed on ice and transported to the lab (SINTEF, Trondheim, Norway) immediately after production. To obtain the salmon viscera, fish were hand filleted on slaughter day (day 0). The obtained viscera were kept on ice (max 1 h) until further processing. Heads were minced in a Hobart mincer using 10 mm holes. The minced heads (MH) were kept on ice (max 1 h) until further processing. The whole heads (WH), whole backbones and viscera were placed in 10 L buckets with a cover and stored at different temperatures (4 and 10 °C) for up to 7 days. At sampling day, the whole samples (heads, backbones and viscera) were minced separately in a Hobart mincer using 10 mm holes to produce a homogenous batch of each by-product. The minced raw materials were kept on ice (max 1 h) until TCA extraction (see below).

2.2. Sampling

Chemical quality and stability of the analyzed by-products were followed at two storage temperatures: 4 and 10 °C for up to 7 days. Sampling was carried out at: 0 (T_0), 1 (T_1), 2 (T_2), 3 (T_3) and 7 (T_7) days after slaughter.

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 (St. Louis, MO, USA). Potassium hydroxide from Sigma-Aldrich was used for pH adjustments.

2.4. TCA extract preparation

Five grams of each of the mentioned by-product samples were subjected to two-steps TCA-extraction. In more details: firstly, the sample was homogenized with 4:1 (v/w) volumes of 7.5% (w/v) ice-cold trichloroacetic acid, centrifuged and the supernatant was collected. Secondly, the remaining insoluble fish residues were further homogenized with TCA as described above. Supernatants from these two extractions were combined and the pH of the resulting solutions was adjusted to pH 7.0 using 9 M KOH. All extraction steps were carried out at 4 °C. The extracts were divided into 1.5 ml aliquots and stored at −80 °C until analysis.

2.5. Sample preparation for NMR analysis

900 μl of the sample extract were mixed with 100 μl of 1 mM TSP in 20 mM phosphate buffer, pH 7.0 in an Eppendorf tube and centrifuged at 20,000*g* for 5 min. 600 μl of the centrifuged sample were transferred in a standard 5 mm NMR tube.

2.6. NMR experiments

1D ^{1}H, 2D ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) NMR spectra were acquired at 300 K with a Bruker Avance 600-MHz spectrometer equipped with 5-mm z-gradient TXI (H/C/N) cryoprobe. All the NMR experiments mentioned above were acquired with the standard Bruker pulse sequences noesygppr1d; mlevgpphprzf; hsqcetgpprsisp2.2 and hmbcgplpndprqf, correspondingly and using the standard settings. For metabolites quantification the 1D ^{1}H experiments (Bruker, noesygppr1d pulse sequence) with the following setting were used: number of scans ns = 4; TD = 32 K; sw = 20 ppm; aq = 1.36 s and constant receiver gain (RG = 28.5). All spectra were processed by carrying out phase and baseline correction, integration and signal-to-noise (S/N) calculation using the TopSpin 3.2 software (Bruker, Germany). NMR assignment was performed using both the registered experiments and the available NMR databases (Biological Magnetic Resonance Data Bank (BMRB) and the Human Metabolome Database (HMDB)). The ERETIC2 (Electronic Reference To access In vivo Concentrations, Topspin 3.2, Bruker) quantification tool and a custom Python script were used for metabolites quantification. The spectra were calibrated against an external standard assigning a chemical shift of 0 ppm to the TSP signal in both ^{1}H and ^{13}C dimensions.

2.7. Data analysis

2.7.1. Principal component analysis (PCA)

NMR spectra were examined using TopSpin 3.2 and AMIX-Viewer, version 3.9.14 (Bruker BioSpin, Billerica, MA, USA). Normalized to the average mass of the extracted salmon tissues and volume of extractions ^{1}H NMR spectra were overlaid to detect inconsistencies in water suppression, shimming or baseline correction. PCA of 1D ^{1}H NMR spectra was performed with the Statistics toolbox within the AMIX software using the following settings: variable size bucketing, 12–0.05 ppm spectral width and water signal exclusion. Manual bucketing was performed in order to include all peaks in the relative bucket for a total of 74 buckets. Strongly overlapped spectral features were excluded from analysis.

2.7.2. Error analysis

Signal-to-noise ratios (S/N) were detected using the TopSpin software, where the S/N ratio is calculated using the following formula: $S/N = I_{max}/(2\sigma_{noise})$; where I_{max} is the maximum amplitude of the peak and σ_{noise} is the standard deviation of the noise region (12–11 ppm). It was assumed that only the signals with S/N ratio greater than 3 can be considered as reliably detectable (Maniara, Rajamoorthi, Rajan, & Stockton, 1998).

The value and the uncertainty for the individual metabolite concentrations C_i were calculated with a custom analysis script using Python 2.7 with libraries *Numpy* and *Matplotlib*. For each peak

integral P_i the uncertainty was estimated using the following equation[1]:

$$\delta P_i = \sqrt{N_i} \cdot \sigma_{noise}$$

with the absolute uncertainty of the peak integral δP_i for peak i, number of sample points within the integration region N_i and the standard deviation of the background σ_{noise} attributed to independent, random noise. The value of σ_{noise} was calculated using the sample standard deviation since its value is an estimate for the entire spectrum from a sample located in the noise region (11–12 ppm). Good agreement with the standard deviation value was found throughout the spectra in different background regions (data not shown).

In order to obtain the concentration of the metabolite C_i in the NMR sample tube, its peak integral P_i was compared to the peak integral of the reference (TSP) substance P_R with known concentration C_R using the following equation:

$$C_i = A_i \cdot (n_R/n_i) \cdot C_R$$

In the above equation, the peak ratio $A_i = (P_i/P_R)$ was determined using either TopSpin or the custom Python script (both yield the same values within the given uncertainty range). The ratio of the proton numbers for the metabolite compound n_i and of the reference substance n_R is a constant. All other quantities have respective absolute uncertainties, namely δA_i for the peak ratio and δC_R for the reference material concentration.

In order to obtain the uncertainty of the metabolite concentration δC_i standard error propagation was used (Taylor, 1997). Since the above equation is based on multiplicative factors and the errors are assumed independent and random the relative errors are added quadratically:

$$\left(\frac{\delta C_i}{C_i}\right) = \sqrt{\left(\frac{\delta A_i}{A_i}\right)^2 + \left(\frac{\delta C_R}{C_R}\right)^2}$$

The relative error for the reference concentration $(\delta C_R/C_R)$ was estimated using the standard deviation of the TSP reference peak integral values $P_R^{(k)}$ for all obtained NMR spectra. For the peak ratio the relative error was calculated using the individual peak errors δP_i and δP_R as:

$$\left(\frac{\delta A_i}{A_i}\right) = \sqrt{\left(\frac{\delta P_i}{P_i}\right)^2 + \left(\frac{\delta P_R}{P_R}\right)^2}$$

The reported uncertainties (Table S1, ΔC (%)) correspond to a one σ confidence interval with assumed Gaussian distributed errors attributed to the above described sources of uncertainties.

2.8. Experimental design

In summary, by-products from more than fifteen farmed salmons (*S. salar*) were used in the investigation. By-products included backbones, viscera, whole heads and minced at day zero heads. These samples were kept at two different temperatures (4 and 10 °C) for up to 7 days. TCA extracts of all analyzed by-products were prepared at: 0 (T_0), 1 (T_1), 2 (T_2), 3 (T_3) and 7 (T_7) days after slaughter. NMR spectra were acquired for each tissues/temperature/time points for a total of 35 spectra.

[1] The result for δP_i is obtained by applying Gaussian error propagation: The peak integral P_i is the sum of N_i data points x_j. Each data point x_j has an independent, random uncertainty δx_j. In the next step we add the uncertainties for the quantity P_i quadratically: $\delta P_i = \sqrt{\sum_j^{N_i} (\delta x_j)^2}$. Since the sample point error can be assumed to follow a Gaussian distribution with standard deviation σ_{noise} one can simplify the above equation to $\delta P_i = (\sqrt{N_i} \cdot \sigma_{noise})$ using $\delta x_j = \sigma_{noise}$.

3. Results and discussion

Seafood by-products contain a number of high-added value metabolites. However, their composition and concentration may change during storage. Therefore, accurate qualitative and quantitative monitoring of molecular changes in fish raw material is very relevant for further by-products processing and utilization. The quantification of such metabolites can be used as a decision-making tool for future choices of high-added value product sources.

In Fig. 1 the 1D ^{1}H NMR spectra of the TCA extracts of salmon heads (panel A), viscera (panel B) and backbones (panel C) at T_0 are shown. Signals of the most important by-product metabolites are shown in the spectra: Cholic acid derivatives, amino acids, lactic acid, phospho/creatine (PCr), trimethylamine oxide (TMAO), taurine, anserine, sugars, products of ATP degradation and formic acid. Their concentrations at each time point are reported in Table 1 (in moles) and in the Table S1 (in milligrams). The metabolites concentration expressed in mmol was used for the PCA and their concentration in mg was used for comparison with the available literature data.

3.1. Error analysis

The presented error analysis for quantitative NMR results is of particular interest for experiments where it not possible to obtain multiple samples to analyze due to low sample quantities or limited NMR time.

TSP is a suitable external standard for salmon by-products NMR analysis. No TSP signal overlapping was observed, nor its interaction with the sample components. The pipetting error was estimated as 2.7% based on the TSP peak integral value standard deviation, and was included as part in the reported measurement error (Tables S1, ΔC). The used experimental protocol (both extraction and NMR experiment) allowed monitoring and quantifying the most important post-mortem processes in salmon by-products storage (discussed below).

3.2. Main bioactive metabolites assessment

3.2.1. Phospho/creatine, taurine and anserine

The main metabolites in the investigated salmon by-product extracts are phospho/creatine (both phosphorylated and not phosphorylated forms – PCr), taurine, and anserine (Fig. 1 and Tables 1, S1). The concentration of these bioactive compounds within by-products is of relevance as it can affect the further industrial usage of salmon by-products. The food and pharmaceutical industries use these substances as a component of functional foods and cosmetics. The amount of these metabolites in different by-products is reported in Tables 1 and S1. Creatine is an amino acid that can exist also in the phosphorylated form. This compound is largely used in the production of various food, drugs and food supplements (Sullivan, Geiger, Mattson, & Scheff, 2000). A significant quantity of creatine was reported in herring flesh (650 mg/100 g), beef (450 mg/100 g), pork meat (500 mg/100 g) and salmon muscles (450 mg/100 g) (Balsom, Söderlund, & Ekblom, 1994).

Among the analyzed salmon by-products, backbones at T_0 contain the highest PCr concentration (397 mg/100 g) followed by heads (310 mg/100 g) and viscera (45 mg/100 g) (Table S1). It should be noted the concentration of PCr significantly decreases at T_7 in minced heads (10 °C) and backbones (4 and 10 °C). On the other hand, it was detected that while PCr decreased, the concentration of creatinine increased at T_7 in backbones, minced and whole heads stored at 10 °C (Tables 1 and S1). The degradation of PCr to creatinine is both a pH and temperature dependent

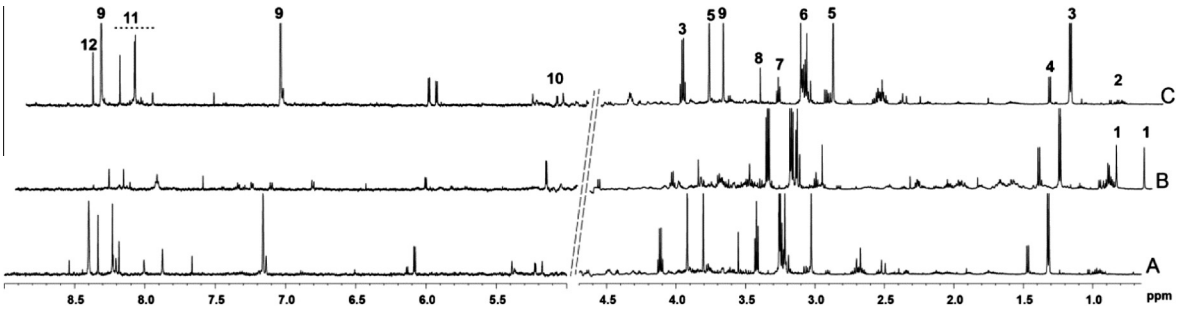

Fig. 1. 1D 600 MHz ^{1}H NMR spectra of Atlantic salmon (*Salmo salar*) heads (A), viscera (B) and backbones (C) TCA extract at 300 K (T_0 time point). For convenience, the high- and low field regions of the spectra are reported at a different scale. Signals assignment is as follows: 1 – cholic acid derivatives; 2 – methyl groups of amino acids; 3 – lactic acid; 4 – alanine; 5 – PCr; 6 – TMAO; 7 – taurine; 8 – glycine; 9 – anserine; 10 – sugars; 11 – products of ATP degradation; 12 – formic acid.

process. Creatinine is formed at higher temperatures and in an acidic environment (Lempert, 1959). Considering the above, it can be concluded that the sharp decrease in PCr concentration in salmon backbones and in particular in minced heads is due to the creatinine formation from creatine. Adding the concentrations of PCr and creatinine gives a value close to the average PCr concentration for the corresponding tissue. For example, for the minced heads (T_7, 10 °C) the summed concentration of PCr and creatinine is 1.39 mmol/100 g that is close to the average of the PCr concentration in minced heads during the first three days of storage at 10 °C – 1.43 mmol/100 g. The fact that the concentration of PCr in salmon backbones and heads is similar to its concentration in muscle and that this value does not significantly vary for more than 3 storage days indicates that salmon backbones can be considered as a good phosphocreatine source.

Taurine plays an important role in many physiological processes and has several positive effects related to its antioxidant properties (Bouckenooghe, Remacle, & Reusens, 2006; Zulli, 2011). Taurine concentration varies significantly between different raw materials and foods, being the highest in marine and animal sources (Ferraro et al., 2010; Laidlaw, Grosvenor, & Kopple, 1990). Scallops contain the highest amount of taurine (827 mg/100 g) (Laidlaw et al., 1990), while in salmon muscle the concentration is 70 mg/100 g and in salmon stomachs and intestines it is 380 mg/100 g (Dragnes, Stormo, Larsen, Ernstsen, & Elvevoll, 2009).

The analyzed salmon by-products contain at T_0: 63, 193 and 348 mg of taurine in 100 g of backbones, heads and viscera, respectively (Table S1). These values are in good agreement with the literature (Dragnes et al., 2009; Ferraro et al., 2010; Laidlaw et al., 1990). The salmon viscera appear to be the most promising source of taurine with an average three days metabolite concentration of 384 ± 29 and 403 ± 41 mg/100 g at 4 and 10 °C storage temperature, respectively.

Anserine is an important tissue buffering component that influences cellular transport and water balance (Abe, 2000) and works as an effective *in vivo* antioxidant (Nagasawa, Yonekura, Nishizawa, & Kitts, 2001). The concentration of anserine in salmon muscles varied between 400 (Crush, 1970), 566 (Shirai, Fuke, Yamaguchi, & Konosu, 1983) and 679 mg in 100 g (Aursand, Jorgensen, & Grasdalen, 1995) revealing differences in the amount of the metabolite of up to 50%. This large variation can be due to a broad range of reasons connected to different fish age, gender, seasons etc.

In our study anserine was found in salmon heads, backbones, but not in viscera. The initial concentration of this metabolite was higher in backbones than in heads (249 *vs* 163 mg/100 g respectively). Albeit only slightly, the amount of anserine varies during the storage: it increases in backbones until T_3 and drops at T_7, while decreases for minced and whole heads until T_3 and grows until T_7 not exceeding the initial value (Tables 1 and S1). The drop in anserine levels can be linked to increasing levels of beta-alanine during by-product storage (Van Waarde, 1988). This decrease has been attributed to the hydrolytic cleavage of muscle anserine. This observation is in good correlation with the detected increase of beta-alanine in salmon by-products in the study, in particular in minced heads. It should be noted that even if viscera do not contain anserine they still have some amounts of beta-alanine (Tables 1 and S1).

3.3. Post mortem processes monitoring

3.3.1. Principal component analysis (PCA)

The data reported above show that the salmon by-products are a valuable source of PCr, taurine and anserine. Another major by-product metabolite is lactic acid. The concentration of lactic acid greatly changes during storage (Tables 1 and S1). For instance, in viscera stored at 10 °C the amount of lactic acid increases due to post-mortem glycolysis from 1.33 (T_0) to 3.22 mg/100 g (T_7), i.e. almost 2.5 times.

In order to detect other, subtler changes in concentrations of minor metabolites, PCr, taurine, anserine and lactic acid were excluded from the PCA (Fig. 2). A good by-product separation can be observed for the first two principal components (PC1 and PC2) that explain respectively 80 and 15% of the data. The PCA plot points to different processes occurring during by-products storage: (i) formation of trimethylamine (TMA) and the corresponding reduction of trimethylamine oxide (TMAO); (ii) proteolysis with an increase of free amino acids concentration; (iii) formation of acetic and succinic acids; (iv) formation of ethanol and 1,3-propanediol; (v) degradation of cholic acid derivatives; (vi) formation of biogenic amines. The formation of TMA and proteolysis according to the PCA model are the most important temperature dependent processes occurring during by-products storage (Fig. 2).

3.3.2. Reduction of TMAO and formation of TMA

Trimethylamine N-oxide (TMAO) is one of the most important osmolytes and can act as a protein stabilizer and enhance protein folding (Yancey, 2005). It was found in all marine teleosts and in particular in *S. salar* (38 mg/100 g) (Anthoni, Børresen, Christophersen, Gram, & Nielsen, 1990; Chung & Chan, 2009). The TMAO level in fish varies with the season, size, age and environmental conditions (Hebard, Flick, & Martin, 1982). It has to be noted that the differences in TMAO levels among individual fish of the same species may sometimes be larger than the differences among the species (Hebard et al., 1982). Trimethylamine (TMA) is a compound formed by the bacterial reduction of TMAO or by the breakdown of choline or other trimethylalkylammonium compounds, such as carnitine or betaine during the spoilage of fish (Hebard et al., 1982; Sikorski, 1990; Van Waarde, 1988). TMA plays

Table 1
Concentration changes of some metabolites during salmon by-products storage (number of mmol in 100 g of wet by-product). T0, T1, T2, T3 and T7 indicate storage time in days. WH – whole heads, MH – minced heads.

Metabolites	Tissue	Storage time, temperature								
		Concentration, mmol/100 g								
		T0	T1		T2		T3		T7	
			4 °C	10 °C	4 °C	10 °C	4 °C	10 °C	4 °C	10 °C
PCr	Backbones	1.882	2.191	2.236	2.322	2.324	2.518	2.475	2.140	1.967
	WH	1.467	1.118	1.344	0.948	1.096	1.047	1.259	1.169	1.283
	MH		1.370	1.391	1.467	1.488	1.453	1.358	1.625	0.709
	Viscera	0.215	0.274	0.312	0.219	0.305	0.284	0.274	0.246	0.331
Creatinine	Backbones	–	–	–	–	–	–	–	–	0.158
	WH	–	–	–	–	–	–	–	–	0.136
	MH		–	–	–	–	–	–	–	0.681
Taurine	Backbones	0.501	0.599	0.628	0.581	0.702	0.617	0.621	0.638	0.482
	WH	1.540	1.494	1.545	1.348	1.694	1.620	1.626	1.824	1.546
	MH		1.670	1.531	1.505	1.415	1.625	1.320	1.652	1.583
	Viscera	2.781	3.262	3.141	2.905	3.691	3.319	3.274	2.863	2.949
Anserine	Backbones	1.038	1.182	1.238	1.266	1.267	1.428	1.252	1.060	1.113
	WH	0.679	0.412	0.506	0.335	0.326	0.322	0.402	0.323	0.528
	MH		0.411	0.385	0.350	0.256	0.192	0.266	0.219	0.304
b-Alanine	Backbones	0.079	0.126	0.148	0.200	0.201	0.222	0.220	0.334	0.269
	WH	0.136	0.165	0.183	0.195	0.245	0.215	0.286	0.371	0.318
	MH		0.334	0.332	0.407	0.456	0.565	0.456	0.612	0.515
	Viscera	0.112	0.102	0.216	0.171	0.276	0.237	0.199	0.228	0.257
Lactic acid	Backbones	2.806	3.570	3.678	3.817	3.810	4.071	4.348	3.631	3.637
	WH	2.364	1.824	2.138	1.682	1.948	1.790	2.188	2.109	1.556
	MH		2.526	2.826	2.859	3.141	3.138	2.782	3.259	2.826
	Viscera	1.335	1.829	1.852	2.133	2.628	2.134	2.290	2.328	3.225
TMAO	Backbones	0.334	0.378	0.407	0.409	0.438	0.415	0.401	0.039	0.037
	WH	0.371	0.330	0.375	0.325	0.353	0.340	0.357	0.105	–
	MH		0.351	0.324	0.379	–	0.101	–	–	–
	Viscera	0.026	0.003	–	–	–	–	–	–	–
TMA	Backbones	–	–	–	0.010	–	0.029	0.019	0.324	0.399
	WH	–	–	–	–	0.003	0.003	0.036	0.295	0.673
	MH		0.003	0.009	0.009	0.274	0.232	0.503	0.712	0.957
	Viscera	–	–	0.003	0.001	0.030	0.047	0.161	0.228	0.689
Choline	Backbones	0.053	0.032	0.037	0.038	0.073	0.048	0.048	0.059	0.046
	WH	0.045	0.055	0.070	0.074	0.100	0.081	0.107	0.143	0.118
	MH		0.069	0.070	0.066	0.110	0.101	0.028	0.027	0.018
	Viscera	0.069	0.142	0.312	0.190	0.325	0.247	0.218	0.193	0.147
Alanine	Backbones	0.281	0.276	0.323	0.324	0.337	0.386	0.382	0.501	0.575
	WH	0.362	0.348	0.367	0.363	0.471	0.358	0.456	0.608	0.791
	MH		0.377	0.402	0.416	0.502	0.505	0.522	0.657	0.847
	Viscera	0.465	0.732	1.359	1.143	2.224	1.868	2.120	2.433	4.244
Hypoxanthine	Backbones	0.018	0.039	0.092	0.076	0.073	0.125	0.124	0.255	0.334
	WH	0.109	0.128	0.140	0.139	0.208	0.206	0.179	0.238	0.355
	MH		0.163	0.192	0.237	0.292	0.293	0.285	0.356	0.433
	Viscera	0.026	0.071	0.112	0.086	0.148	0.133	0.189	0.211	0.349
Inosine	Backbones	0.132	0.292	0.314	0.286	0.301	0.309	0.306	0.098	–
	WH	0.199	0.147	0.148	0.112	0.118	0.098	0.125	0.057	–
	MH		0.120	0.105	0.104	0.046	0.050	–	–	–
	Viscera	0.069	0.051	0.052	0.048	0.049	–	–	–	–
IMP	Backbones	0.141	0.039	0.018	0.029	0.009	–	–	–	–
	WH	0.045	–	–	–	–	–	–	–	–
	MH		–	–	–	–	–	–	–	–
Acetic acid	Backbones	0.026	0.047	0.055	0.076	0.064	0.077	0.076	0.481	0.956
	WH	0.027	0.037	0.044	0.037	0.045	0.054	0.098	0.551	1.838
	MH		0.043	0.044	0.057	0.283	0.252	0.874	1.232	3.286
	Viscera	0.043	0.213	0.138	0.105	0.364	0.408	1.041	1.493	4.079
Succinic acid	Backbones	0.018	0.016	0.009	0.010	0.009	0.019	0.019	0.079	0.204
	WH	0.018	0.009	0.009	0.009	0.018	0.009	0.027	0.105	0.391
	MH		0.009	0.017	0.009	0.046	0.030	0.190	0.228	0.469
	Viscera	0.043	0.061	0.087	0.076	0.226	0.284	0.587	0.940	2.076
Formic acid	Backbones	–	–	–	–	–	–	0.019	0.196	0.371
	WH	0.018	0.037	0.044	0.028	0.036	0.009	0.027	0.133	0.882
	MH		0.026	0.026	0.009	0.046	0.030	0.285	0.511	0.755
	Viscera	0.026	0.091	0.043	0.057	0.128	0.199	0.435	0.571	1.360

(*continued on next page*)

Table 1 (*continued*)

Metabolites	Tissue	Storage time, temperature								
		Concentration, mmol/100 g								
		T0	T1		T2		T3		T7	
			4 °C	10 °C	4 °C	10 °C	4 °C	10 °C	4 °C	10 °C
Ethanol	Backbones	–	–	–	–	–	–	–	0.137	0.353
	WH	–	–	–	–	–	–	–	0.181	0.446
	MH		–	–	–	0.064	0.040	0.361	0.548	1.123
	Viscera	0.095	–	–	–	0.118	0.114	0.265	0.369	1.112
2,3-Butanediol	Backbones	–	–	–	–	–	–	–	0.049	0.083
	WH	–	–	–	–	–	–	0.009	0.038	0.055
	MH		–	–	–	0.027	0.020	0.038	0.046	0.074
	Viscera	–	–	–	–	0.020	0.019	0.047	0.079	0.147
1,3-Propanediol	WH	–	–	–	–	–	–	–	0.105	1.173
	MH		–	–	–	–	–	0.256	0.456	1.924
Butyric acid	WH	–	–	–	–	–	–	–	–	0.191
	MH		–	–	–	–	–	–	–	0.156
Tyramine	Backbones	–	–	–	–	–	0.010	–	0.010	0.019
	WH	–	–	–	–	0.009	0.018	0.018	0.019	0.055
	MH		–	–	–	–	0.020	0.019	0.027	0.037
	Viscera	–	–	–	–	–	–	0.114	0.307	0.900
Histamine	Viscera	–	–	0.104	0.095	0.226	0.199	0.360	0.290	0.505
Cadaverine	Backbones	–	–	–	–	–	–	–	0.128	0.269
	WH	–	–	–	–	–	–	–	0.209	0.600
	MH		–	–	–	–	–	0.209	0.301	0.681
	Viscera	–	–	–	–	–	–	0.814	1.291	3.326
Putrescine	Backbones		–	–	–	–	–	–	–	–
	WH	–	–	–	–	–	–	–	–	0.182
	MH		–	–	–	–	–	–	0.201	0.239
	Viscera	–	–	–	–	–	–	0.151	0.193	0.726
Cholic acid derivative	Viscera	0.319	0.427	0.043	0.362	0.157	0.066	0.123	–	–

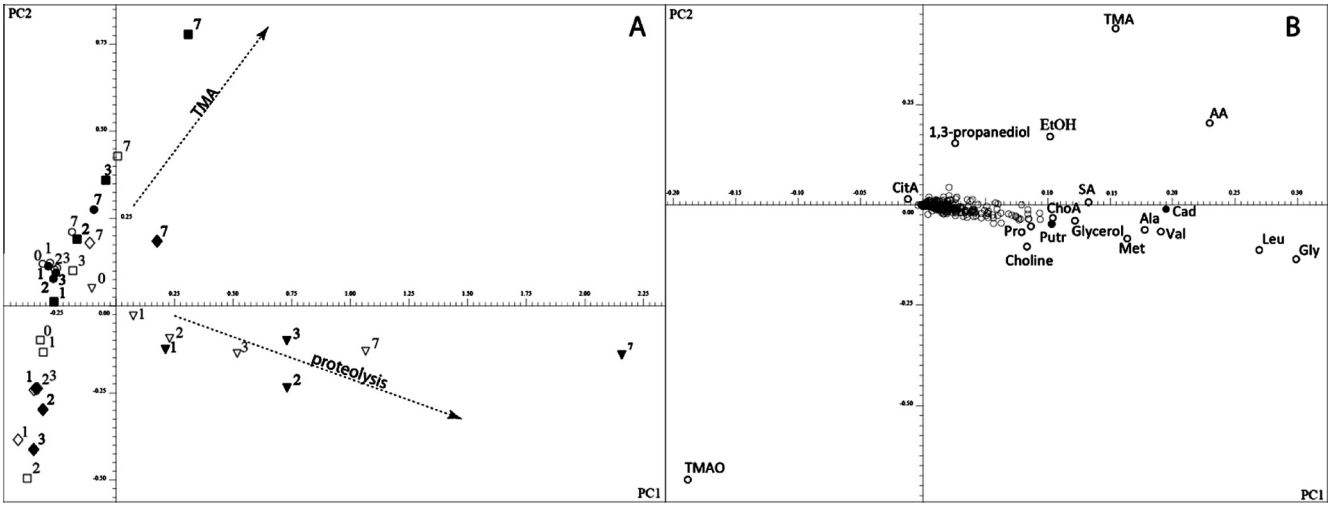

Fig. 2. PCA of 1D ^{1}H NMR spectra of salmon by-products. Panel A – score plot. Backbones at 4 °C – (○), 10 °C – (●); minced heads at 4 °C – (□), 10 °C – (■); whole heads at 4 °C – (◇), 10 °C – (◆); viscera at 4 °C – (△), 10 °C – (▲). Panel B – loading plot. TMA – trimethylamine; TMAO – trimethylamine oxide; EtOH – ethanol; AA – acetic acid; Cad – cadaverine; Putr – putrescine; ChoA – cholic acid derivatives; CitA – citric acid.

one of the major roles in the degradation of sensory attributes of fish products, because TMA has a low odor threshold and is described to have an unpleasant "fishy" odor and a unpalatable taste (Van Waarde, 1988). Despite the fact that many studies positively correlated TMA levels and organoleptic test scores, in some cases the correlation was not found (Hebard et al., 1982). However, a general correlation was proposed between the organoleptic assessment and the TMA concentration for cod and haddock muscle. Fish with TMA levels in the range of 0–4.2 mg/100 g was set to I grade, i.e. prime quality, while fish with TMA between 4.25 and 29.5 mg/100 g to II grade, i.e. acceptable quality. Fish with TMA content higher than 29.5 mg/100 g, was deemed unacceptable for human consumption (Hebard et al., 1982). The data obtained within this investigation show that the initial TMAO concentration

greatly differs in viscera (1.9 mg/100 g), compared to heads (27.9 mg/100 g) and backbones (25.1 mg/100 g) (Table S1). These results agree with literature data (Dyer, 1952). TMA was not detected in any of the fresh salmon by-products. TMA formation was determined to be a temperature dependent process that occurs faster in minced heads, stored at both temperatures and in viscera, stored at 10 °C (Tables 1 and S1). The obtained data indicate that product rejection based on the TMA concentration will occur for: (i) minced heads stored at 10 °C after the second day; (ii) minced heads at 4 °C, viscera at 10 °C and whole heads at 10 °C after the fourth day of storage; (iii) while the other studied products can be stored for more than 7 days. The data point to the fact that mincing by-products as well as elevated storage temperature increases the rate of TMA formation.

As it was mentioned above, TMA can be formed from different trimethylalkylammonium compounds, such as choline, carnitine or betaine (Hebard et al., 1982; Sikorski, 1990; Van Waarde, 1988). The presence of choline in the studied salmon by-products was verified and confirmed by 2D TOCSY and HMBC spectra (cross peaks at 3.50/4.05 ppm and 3.19/70.67 ppm, respectively). Betaine and carnitine were more challenging to assign due to the possible overlapping with some strong anserine and PCr signals. In order to identify possible precursors of TMA, the initial amount of TMAO and choline were compared to the amount of TMA formed after storage. Only in backbones TMAO and choline are completely converted to TMA. In this case, the initial total amount of TMAO and choline – 0.39 mmol/100 g is close to the final amount of TMA – 0.40 mmol/100 g. The final amount of TMA for the other by-products significantly exceeds the initial quantity of TMAO and choline (Table 1) indicating that TMA in this case is produced also from other sources, mentioned above.

3.3.3. Proteolysis

The PCA plot (Fig. 2) shows that proteolysis is an important process during viscera storage and its rate is significantly higher than in the other studied by-products. The free amino acids concentration increases nearly linearly during storage. The rate of the process can roughly be estimated by the slope of the increase in amino acids concentration. For example, the rate of alanine formation is <0.1 mmol/100 g/storage day for heads and backbones and is 0.5 and 0.28 mmol/100 g/storage day for viscera stored at 10 °C and 4 °C, respectively.

3.3.4. Acetic acid, succinic acid, ethanol and 1,3-propanediol

The formation of different organic acids and alcohols such as lactic, acetic, succinic acids, ethanol and 1,3-propanediol was detected by NMR during by-products storage (Tables 1 and S1).

1,3-Propanediol (1,3-PDL) is the product of glycerol anaerobic fermentation (Biebl, Menzel, Zeng, & Deckwer, 1999). Complete glycerol to 1,3-PDL conversion carried out by bacteria results also in the formation of acetate, butyrate, ethanol, butanol, 2,3-butanediol (2,3-BDL), lactate and succinate (Biebl et al., 1999). In our study the quantitative determination of glycerol is difficult due to the strong overlap of its signals. Nevertheless, the presence of this metabolite was detected in salmon heads. The formation of 1,3-PDL and butyrate is detected in head samples after T_3 and at T_7 respectively and it is a temperature dependent process. On the contrary, 2,3-BDL, ethanol and acetic acid are formed in all the studied by-products. Although the quantitative description of glycerol degradation is hampered in our study by NMR signal overlap, all final products of this pathway are well detectable and can be quantified (Tables 1 and S1).

3.3.5. Cholic acid derivatives

Bile acids are synthesized in the liver (Hofmann & Hagey, 2008). The most common primary C24 bile acids are chenodeoxycholic and cholic (ChoA) acids. After their biosynthesis from cholesterol, C24 bile acids can be conjugated either with glycine or taurine (Hofmann & Hagey, 2008). Salmonids produce predominantly cholic acid (James Henderson & Tocher, 1987). It was shown that the rainbow trout bile contained cholate and chenodeoxycholate at 85 and 14%, respectively, with over 92% of them being conjugated with taurine (Denton, Yousef, Yousef, & Kuksis, 1974).

The presence of some cholic acid derivatives was detected in the studied salmon viscera. The 1D ^{1}H NMR spectra of the viscera from the slaughter to the third day of storage contain three characteristic peaks at 0.71, 0.91 and 0.97 ppm. The intensities of the mentioned peaks decrease with storage time in a temperature dependent process (Fig. S1). These peaks were assigned to the methyl groups of cholic acid derivatives. The presence of the characteristic triplet at 3.07 ppm (52.47 ppm, HSQC spectrum) that decreases with storage time with the same rate as the peak at 0.70 ppm allows us to conclude that it tentatively belongs to the taurocholic derivative of cholic acid (Table 1).

3.3.6. Biogenic amines

The storage of all studied salmon by-products is affected by the formation of biogenic amines (BA). BA are usually generated by microbial decarboxylation of free amino acids (Askar & Treptow, 1986; ten Brink, Damink, Joosten, & Huis, 1990). Although BA such as histamine, tyrosine and putrescine are needed for many essential functions in man and animals (ten Brink et al., 1990), consumption of food containing high amounts of these amines can have toxicological effects. BA may exert vasoactive effects (tyramine), psychoactive effects or both (histamine) (ten Brink et al., 1990).

The formation of cadaverine and putrescine is one of the main processes during viscera storage (Fig. 2). Tyramine and histamine were also detected in our study (Tables 1 and S1).

Tyramine is an aromatic amine that is formed from tyrosine. The maximum tolerable levels of tyramine in fish and fishery products was suggested to be 95 mg/100 g (Paulsen, Grossgut, Bauer, & Rauscher-Gabernig, 2012). Our data indicate that tyramine is formed in all the studied by-products and its concentration reaches the maximum tolerable levels only in viscera at the 7th storage day at 10 °C (Tables 1 and S1).

The formation of histamine (a histidine derivative) was detected only in viscera. Two taumeric aromatic protons are easily detected at the low-field region in the NMR spectra at 7.95/7.99 and 7.09/7.14 ppm. The European legislation (EC., 2005) limits permitted histamine levels in fishery products to 10–20 mg/100 g. The amount of histamine in the studied viscera stored at 4 °C is within the limits up to day seven, while upon storage at 10 °C, its concentration exceeds the allowed levels after two days.

Putrescine and cadaverine are pharmaco-/toxicodynamically less active than histamine and tyramine (Rauscher-Gabernig et al., 2012). An important effect of putrescine and cadaverine is the potentiation of the toxicity of other amines, especially histamine (Rauscher-Gabernig et al., 2012). In absence of a relevant EU-wide legislation for the allowed amounts of putrescine and cadaverine in foods, some national limits were established. The maximum tolerable levels of 17 mg/100 g for putrescine and 51 mg/100 g for cadaverine in fish was set in Austria (Rauscher-Gabernig et al., 2012). In the analyzed salmon by-products the level of cadaverine exceeds the maximum tolerable amount in viscera after two days storage at 10 °C and after 3 days storage at 4 °C and in minced/whole heads stored at 10 °C at day 7. Putrescine is above the defined maximum levels in viscera and minced heads at day seven for both temperatures. Therefore, the industrial processing of salmon by-products should consider the possible formation of BA over time and especially histamine.

Table 2
Freshness relevant values of salmon by-products. The assignment of by-products to a certain category of freshness is based on the following data: concentration of TMA in 100 g: I grade product <4.2 mg, II grade <29.5 mg (Hebard, Flick, & Martin, 1982). Rejection limits of biogenic amines in 100 g: tyramine >95 mg, histamine >10–20 mg; putrescine >17 mg and cadaverine >51 mg (Paulsen, Grossgut, Bauer, & Rauscher-Gabernig, 2012; Rauscher-Gabernig et al., 2012). The background colors indicate: light grey – second grade quality, dark grey – rejection. The concentrations are reported in mg/100 g.

Tissues	Temp	Time	*Ki*	*H*	TMA	Tyramine	Histamine	Cadaverine	Putrescine
Backbones	4 °C	0	52	6	–	–	–	–	–
		1	89	11	–	–	–	–	–
		2	93	20	1	–	–	–	–
		3	100	28	2	–	–	–	–
		7	100	72	19	1	–	13	–
	10 °C	1	96	22	–	–	–	–	–
		2	100	19	–	–	–	–	–
		3	100	29	1	–	–	–	–
		7	100	100	24	3	–	27	–
WH	4 °C	0	87	31	–	–	–	–	–
		1	100	47	–	–	–	–	–
		2	100	56	–	–	–	–	–
		3	100	68	0	2	–	–	–
		7	100	80.65	17	3	–	21	–
	10 °C	1	100	48.48	–	–	–	–	–
		2	100	63.89	0	1	–	–	–
		3	100	58.82	2	2	–	–	–
		7	100	100	**40**	7	–	**61**	16
MH	4 °C	0	87.18	30.77	–	–	–	–	–
		1	100	57.58	0	–	–	–	–
		2	100	69.44	1	–	–	–	–
		3	100	85.29	14	3	–	–	–
		7	100	100	**42**	4	–	31	**18**
	10 °C	1	100	64.71	1	–	–	–	–
		2	100	86.49	16	–	–	–	–
		3	100	100	**30**	3	–	21	–
		7	100	100	**56**	5	–	**70**	**21**
V	4 °C	0	100	27.27	–	–	–	–	–
		1	100	58.33	–	–	–	–	–
		2	100	64.29	0	–	11	–	–
		3	100	100	3	–	**22**	–	–
		7	100	100	13	42	**32**	**132**	**17**
	10 °C	1	100	68.42	0	–	12	–	–
		2	100	75	1	–	**25**	–	–
		3	100	100	9	16	**40**	**83**	13
		7	100	100	**41**	**124**	**56**	**340**	**64**

3.4. Freshness considerations

3.4.1. K- and H-values

The relative amount of ATP-degradation products is considered as a good indicator of fish freshness (Saito, Arai, & Matsuyoshi, 1959; Shumilina, Ciampa, Capozzi, Rustad, & Dikiy, 2015). Inosine 5′-monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx) were detected in the studied salmon by-products. In order to connect ATP degradation with other processes occurring during the by-products storage K_i- and *H*-values for all the studied tissues were calculated using the following formula (Table 2):

$$K_i(\%) = \left[\frac{\text{Ino} + \text{Hx}}{\text{IMP} + \text{Ino} + \text{Hx}}\right] * 100 \text{ and } H(\%) = \left[\frac{\text{Hx}}{\text{IMP} + \text{Ino} + \text{Hx}}\right] * 100$$

Due to the fact that IMP was detected only in backbones until the second storage day and in heads at T_0, their K_i-values approach 100% (Table 2). Consequently, it was concluded that the K_i-value is less informative for the evaluation of by-products freshness than the *H*-value. A comparative analysis (Table 2) of the K_i- and *H*-values, biogenic amines and TMA concentrations in all the analyzed samples was conducted and a good correlation between the measured parameters was found. An *H*-value higher than 75% points to a product of II grade quality based on the TMA level (Hebard, Flick, & Martin, 1982). Furthermore, an *H*-value close to 100% correlates with the point of rejection of salmon by-products based on the TMA level.

4. Conclusions

In this investigation it was shown that extracts of salmon heads, backbones and viscera contain important bioactive metabolites. It was established that salmon by-products could be stored for up to seven days at 4 °C without any significant changes in the concentrations of the main metabolites and the formation of harmful compounds. Storage at 10 °C shortens this period until three days due to a faster formation of undesirable degradation compounds, such as biogenic amines. Mincing of heads reduces their storage time (in terms of TMA formation) by 2–3 days. The formation of undesirable metabolites was correlated with the freshness indicators K_i and *H*-values. Our data indicate that the *H*-value can be used as a more suitable indicator of by-products freshness than the K_i. This study further extends the applicability of NMR spectroscopy for fish by-products analysis.

Acknowledgments

The authors would like to thank the Research Council of Norway for providing funds for the ERA-NET SusFood project BioSuck and the European Community's Seventh Framework Programme

FP7/2007-2013 for supporting the APROPOS project (grant agreement No. 289170). We are grateful to Prof. Ingve Simonsen, Ben David Normann (Department of Physics, NTNU) and Nikolay Martyushenko (Department of Biotechnology, NTNU) for the fruitful discussions on error analysis.

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.2016.05.088.

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