Article for JAFPT, category : refrigeration and storage

Title :

Quality of filleted Atlantic mackerel (*Scomber scombrus*) during chilled and frozen storage: changes in lipids, vitamin D, proteins and small metabolites, including biogenic amines

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Abstract

Quality changes of vacuum packed Atlantic mackerel (*Scomber scombrus*) fillets during 12 months frozen storage at -27°C and 9 days chilled storage at +4 °C were evaluated. Freezing at -27 °C preserved the long chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs), vitamin D and the low molecular weight metabolites (LMW) (studied by high resolution nuclear magnetic resonance spectroscopy (HR NMR). Protein oxidation however took place, especially between 1-7 months, decreasing water holding capacity and protein extractability. During chilled storage, no lipid or protein oxidation was observed, but lipolysis increased, several LMW metabolites, relevant for sensory and nutritional quality degraded into non-favorable compounds. The content of biogenic amines was high at day 9 (e.g. 18 mg histamine/100g), jeopardizing safety. Preservation of mackerel fillets by freezing at -27°C is thus a better option compared to prolonged chilled storage at +4 °C; the quality was well preserved up till 12 months frozen storage.

Keywords : fish, NMR, nucleotides, trimethylamine, vitamin D, lipids, proteins, oxidation

Introduction

Pelagic fish is considered healthy food based on the content of long chain n-3 polyunsaturated fatty acids (LC n-3PUFAs), vitamin D, proteins and other compounds that are considered bioactive, such as certain free amino acids and peptides. The LC n-3 PUFAs have shown to reduce the risk of cardiovascular diseases, to have a positive effect on early neurodevelopment, and to improve symptoms of rheumatoid arthritis (Mozzafarian and Wu, 2011; Calder, 2011). while Vitamin D protects against e.g. bone diseases, muscle weakness and certain internal cancers (Grant and Holick, 2005). Vitamin D deficiency causes rickets in infants and children and osteomalacia in adults (Undeland et al., 2009; Holick and Chen, 2008). Marine proteins are like other food proteins important to maintain good bone health, but are also good sources of essential amino acids and bioactive peptides (Jensen and Mæhre, 2016). In addition low molecular weight (LMW) metabolites, like taurine and anserine, have been ascribed antioxidant activities and/or antihypertensive effects (Candlish and Daas, 1996).

Atlantic mackerel (*Scomber scombrus*) is caught in the North Sea by purse seiners or trawlers. The main catch period is September/October, but vessels land Atlantic mackerel at Norwegian processing plants also in January/February. In 2014, Norwegian and foreign vessels landed ca 280 000 and 150 000 metric tonnes of mackerel, respectively (Statistics Norway, 2016). The majority of the landed Atlantic mackerel is exported as whole frozen or gutted frozen to other countries e.g in Asia for further processing. At present only 2-4 % of the mackerel is filleted by the domestic processing industry, but there are several initiatives to increase this share, to increase the profitability of the mackerel industry (e.g. The Norwegian Seafood Research Fund, 2016).

Since mackerel is a highly perishable food, there is a need for appropriate treatment to prevent nutritional and sensory deterioration along the production chain from catch via processing to consumption. Most of the mackerel fillets in Norway are conserved by freezing,

but in the main catch season (Sept/Oct and Jan/Feb) also fresh mackerel is used in processing operations such as smoking and canning.

The healthy LC n-3 PUFA are highly susceptible to oxidation, and during frozen storage, lipid oxidation (rancidity) is the most important cause of quality loss in fatty fish (Larsson and Undeland 2010; Børresen, 2008; Romotowska et al, 2017). Protein oxidation has been much less studied than lipid oxidation, but several studies have implied that proteins from muscle foods are susceptible to oxidation during storage (Lund et al, 2008; Kjærsgård et al., 2006; Eymard et al., 2009 Estévez, 2011; Baron, 2014). As lipid radicals can initiate protein oxidation, fish may be more susceptible to protein oxidation compared to land animals due to the high level of PUFA, however, relatively few studies have been conducted on fish protein oxidation. Protein oxidation can lead to biochemical and structural changes, including loss of essential amino acids and formation of cross-linking leading to changes in functional properties, but the precise mechanisms and potential implication of protein oxidation on muscle food quality have not been fully clarified (Lund et al., 2011). When it comes to the stability of vitamin D in pelagic fish; no studies to date have followed changes during chilled and frozen storage of non-processed fillets, only salted and smoked fish has been investigated (Aro et al., 2005; Aminullah Bhuiyan, et al., 1993).

Water soluble LMW metabolites in fish muscle include free amino acids, peptides, and other small molecules such as nucleotides, nucleotide-derivatives, organic acids and bases, sugars and inorganic constituents. After death, enzymatic and bacterial processes lead to changes in the content and composition of these compounds, influencing both sensory and nutritional qualities and safety (Sampels et al., 2015; Konosu and Yamaguchi, 1982; Huss et al., 1995). Apart from the formation of histamine and other biogenic amines from free amino acids and trimethylamine formation during chilled storage (Bennour et al., 1991; Sanjuás-Rey, 2012),

83 little is today known about changes in the small metabolite pool of mackerel muscle during
84 industrial handling and storage conditions.

85 The shelf life of Atlantic mackerel fillets during cold storage - chilled or frozen - varies
86 according to several factors, where compositional differences (e.g. levels of
87 PUFAs/antioxidants/pro-oxidants) and the temperature history after catch are important
88 (Maestre et al., 2011; Aubourg et al., 2005; Romotowska et al., 2016a; 2017). The
89 recommended frozen storage temperature for fishery products in Europe is at present generally
90 - 25 to -30 °C, which is also what is applied for frozen storage of Atlantic mackerel fillets in
91 processing plants of Norway. Recently, several papers on storage of whole mackerel have
92 shown that a lower frozen storage temperature increased the protection against lipid oxidation
93 and hydrolysis (Romotowska et al, 2016a; 2016b; 2017). Previous reported studies on frozen
94 storage of Atlantic mackerel fillets have however generally employed a temperature of ca -20
95 °C (Aubourg et al., 2005, Aas et al., 2003, Zotos et al., 1995). Studies on minced mackerel
96 stored at -20°C and -30°C showed that oxidation of lipids and proteins increased with storage
97 time and was more pronounced at −20 °C than at −30 °C (Saeed and Howell, 2002). Studies
98 on the shelf-life of mackerel fillets at temperatures relevant for industry are thus missing, and
99 so are studies comprising vacuum packaging, which today is common practice in the mackerel
100 industry. Although fresh fish is often preferred over frozen fish, there are only few studies on
101 the nutritional changes, including changes in the LMW-metabolites, during chilled storage
102 compared to frozen storage of mackerel. Previous studies on non-vacuum packed chilled
103 mackerel have indicated that shelf life is very short (9-10 days) (Bennour et al., 1991), but also
104 that it greatly depend on temperature.

105 The aim of the present study was to examine losses of nutrients (LC n-3 PUFA, vitamin
106 D, LMW compounds like free amino acids), changes in functional properties of proteins, and
107 development of degradation products (free fatty acids (FFA), lipid oxidation products, protein

oxidation products, biogenic amines, ATP-degradation products) of vacuum packed skin-on mackerel fillets during frozen storage (≤ 12 months) at -27°C and during refrigerated storage (≤ 9 days) at +4°C. To better understand differences between muscle types, light and dark muscle were also analyzed separately during the storage.

Material and methods

Catch data

The fish were caught 4 am February 2nd, 2013 (coordinates 52 grad 49 min North, 11 Grad 46 min West). The catch was approximately 570 tonnes, and the fish had an average weight of 380 g. The catch was cooled by refrigerated sea water (RSW) and landed at the processing plant, February 5th at 7.30 am where it was filleted mechanically. Average weight of the resulting skin-on fillets was 80 ± 10 g. The fillets were packed in 10 kg vacuum packs (with some sea water), and divided into two groups for chilled and frozen storage respectively. The vacuum packed fish for chilled storage was transported to SINTEF by car and stored at +4 °C until sample collections. The other part of the vacuum packed fish was frozen in a freeze tunnel at the processing plant (-30° C). Frozen fish was then stored at -30°C before it was transported frozen to SINTEF Sealab by boat where the storage temperature was approximately – 27 °C (according to temperature loggers in the vacuum packs).

Sampling

Sample collections were performed at five different times; at day 4 (i.e. directly upon arrival to the laboratory) and day 9 after catch for the chilled fish, and after 1, 7 and 12 months storage for the frozen fish.

For evaluation of gross chemical composition of whole fillets (fat, water, fatty acid profile), five replicate samples (n=5) from three separate 10 kg vacuum packs were taken at each sampling time. Each sample was prepared by pooling and homogenizing three kilos of skin-on

fish fillets using a Kenwood kitchen machine with food mincer. The frozen fish was first thawed overnight at +4^{0}C. The number of replicates measured analyzed varied between n= 3-5 for the different analyses.

For evaluation of lipid and protein oxidation as well as lipolysis, separate light muscle mince and dark muscle mince were prepared in addition to the mince of whole fillet (the mince of whole fillet was prepared as described above). In the preparation of light and dark muscle mince samples, 15 fillets were collected from three separate 10kg vacuum packs (n= 3 from each storage time point) and the muscle was manually divided into light and dark muscle (without skin) and then minced as described above. The prepared minced samples (whole fillet, light and dark muscle) were vacuumed packed and kept at – 80ºC prior to analysis.

For evaluation of LMW-metabolites, the former approach with homogenizing a large batch was avoided in order to prevent degradation of labile molecules. Muscle samples (1cm^3, light muscle without skin) were collected on right fillets at a position right in front of the dorsal fin. Muscle samples from three fish were regarded as one sample, and six samples were collected for each collection time (chilled samples: day 4 and day 9; frozen: 1 month and 12 months). Samples were stored at -80°C prior to freeze drying and extraction.

Analyses

Chemical composition

Dry matter/moisture/ash. The content of dry matter/moisture in the samples was determined gravimetrically after drying of a portion (2 – 5 g) of the material for 24 h at 105°C. The results are expressed as % of water or dry matter (w/w) as average values ± standard deviation of three replicates. Ash content was estimated by charring the dry material in a crucible at 550°C for 12 hours. The results are expressed in % of ash (w/w) in the wet material and presented as average values ± standard deviation of three replicates.

Total lipid amount. The total amount of lipids was extracted from the fish material by the Bligh & Dyer (B&D) method using chloroform and methanol (Bligh and Dyer, 1959). The extraction was performed twice on each sample. Extracted lipids were stored at -80°C prior to analysis.

Fatty acid composition was analyzed on the total extracted lipids (see above) as described in Kristinova et al. (2014). Methylation was applied prior to fatty acid analyses using gas chromatography (GC) FID. An internal standard 21:0 methyl ester (purity (99%, Nu-Chek. Prep. Inc.) was added to the extracted sample prior methylation. Fatty acid methyl esters were identified by the comparison of their retention times with those of a reference solution (Nu-Chek-Prep, Elysian, MN) analyzed under identical gas chromatographic (GC) conditions. The results are expressed in % (w/w) of total fatty acid amount as a mean value ± standard deviation, and as mg/g lipid.

Lipid oxidation and free fatty acids Lipid oxidation products were determined in the total extracted lipids from mackerel muscle samples (n=3).

Conjugated dienes (CD) were measured as described by Pegg (2001). An aliquot (10-30mg) of extracted lipids was dissolved in 25ml of isooctane. The absorbance was measured in a 10 mm QS quartz cuvette at 233 nm with a UV- visible spectrophotometer (Spectronic Genesys 10 Bio, Thermo Electron Corporation). Amount of conjugated dienes was calculated by using the extinction coefficient (molar absorptivity) of linoleic acid hydroperoxide (2.252 × 104 M-1 cm-1). Absorbance measurement on each extract was performed in duplicates. The results are expressed as mM of CD in the lipids ± standard deviation of 4-6 parallels.

Thiobarbituric acid reactive substances (TBARS) in the extracted lipids was determined as described by Ke and Wooyewoda, 1979. A modification was that 3 ml of 3% BHT solution in ethanol was added to 100mL of working TBARS solution in order to protect against oxidation

during analysis time. As a standard 1.1.3.3- tetraethoxypropane (T 9889) was used. The results are expressed as mM of TBARS in the lipids ± standard deviation of 4-6 parallels.

Free fatty acids (FFA) content in the oils was analysed according to Bernardez et al. (2005). Isooctane was however used as a solvent for lipids, instead of cyclohexane. Standard curve prepared with an oleic acid standard (0 – 20 µmol) was used for calculation of the FFA content. The results are expressed as weight % of FFA (as oleic acid equivalents) of total lipids in the sample ± standard deviation of four parallels.

Vitamin D_3 content For the determination of vitamin D_3, 0.5 g of homogenized and lyophilized mackerel sample (n=2) was mixed with 3 g KOH, 15 ml ethanol:methanol (50/50 v/v) with 0.5 % (w/v) pyrogallol and 0.5 mL of 1 µg/ml Vitamin D2, blanketed with N_2 gas, sealed and shaken in ambient temperature overnight. Toluene, 10 ml, was added, and the sample was treated for an additional 30 min, 5 ml H2O was then added and the upper organic phase transferred to a new test tube. The sample was extracted twice with 7 ml petroleum ether:diethyl ether (80:20 v/v). The removed organic phases were pooled, evaporated to a volume of approximately 6ml and washed with H_2O until neutral pH was obtained. The organic phase was evaporated and dissolved in 2.5 mL 1% 2-propanol in heptane. The extracts were then subjected to solid-phase extraction (TELOS Silica, Kinetics, St Neots, Cambridgeshire, UK) according to the method by Jäpelt et al (2011) and vitamin D_3 analysed by HPLC–MS (Agilent 1200 series system with an Agilent 6120 MSD single quadrupole, Agilent Technologies, Santa Clara, CA, USA). The samples were separated on a C18 column (2 9 3 9 250 mm, Luna 3 l C18(2) 100A, Phenomenex, Torrance, CA, USA) by isocratic elution with water:methanol:formic acid (2:97.9:0.1) at a flow rate of 0.7 ml/min. (Sigma-Aldrich, St. Louis, MO, USA). Quantification was made against an internal standard of vitamin CD2 (Sigma-Aldrich, St Louis, MO, USA) and results are expressed as µg/g ww (average ± standard deviation, n=2).

Protein oxidation

At each time point, protein oxidation was evaluated by measuring carbonyl- and thiol content of sarcoplasmic- and myofibrillar protein fractions of minces from whole fillets, light muscle and dark muscle. As indirect measures of protein oxidation, the protein solubility in 50 mM phosphate buffer (pH 7) and salt (0.6 M KCl), as well as water holding capacity (WHC) were also analyzed.

Fractionation of proteins into sarcoplasmic and myofibrillar proteins as well as determination of protein solubility Sarcoplasmic and myofibrillar proteins were extracted in phosphate buffer and salt, respectively, as described by Anderson and Ravesi (1968) (Anderson et al., 1968) and Licciardello et al. (1982) (n=1). Approximately 4g of minced muscle was homogenized for 30 sec in 80ml of buffer 1 (50 mM KH_2PO_4, pH 7.0) at 4℃ using an Ultra Turrax and then centrifuged (8000 x g, 20min). The supernatant was decanted through glass wool and the volume was made up to 100ml with buffer 1. This was the sarcoplasmic protein (water soluble) fraction. The sediment was re-homogenized as above in 80ml of buffer 2 (50mM KH_2PO_4, pH 7 containing 0.6 M KCl) and re-centrifuged. The supernatant was decanted through glass wool and the volume made up to 100 ml with buffer 2. This was the salt soluble, myofibrillar protein fraction. Protein content in the extracts was determined in triplicate in by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard. Protein solubility was calculated as protein concentration in each supernatant divided by the protein concentration of the homogenate prior to centrifugation, and results are given as % of wet weight of two parallels of each storage time.

Carbonyl groups were determined in the two protein fractions (n=2) by an enzyme-linked immunosorbent (ELISA) assay developed by Buss and coworkers (1997). It is based upon derivatization of carbonyl groups with dinitrophenylhydrazine (DNPH) and probing of protein-bound dinitrophenyl (DNP) with an anti-DNP antibody. The indirect ELISA kit, STA-310

OxiSelect™, was purchased from CELL BIOLABS, INC. Company, city, country. Results are expressed as nmole/mg protein.

Total thiol group content was determined spectrophotometrically after derivatization of each protein fraction (n=2 for each sample type and storage time) by Ellman's reagent (DTNP) (Buss et al., 1997; Riddles et al., 1982). Eight hundred µl of 8 M urea in the reaction buffer ((0.1 M Na phosphate buffer (77.4 ml of 1M Na_2HPO_4 and 22.6 ml 1 M NaH_2PO_4), 1mM EDTA, pH 7.4)), and 100 µl (DTNB) was added to 100 µl of water- and salt soluble samples and blanks. The samples and blanks were mixed with a whirl mixer, incubated at room temperature for 30 minutes and centrifuged for 3 minutes at 13,400 xg at room temperature. The absorbance was measured at 412 nm with the blank as reference and the thiol content was calculated using a molar extinction coefficient of 14,290 M^{-1} cm^{-1}. Results are expressed as nmole/mg protein.

Water holding capacity: Water holding capacity of minced muscles (n=4) was determined by low-speed centrifugation as described by Eide et al. (1982) with the modification that a centrifugal force of 210 g was used instead of 1500g. The water holding capacity is expressed as the percentage of original water retained in the mince (average ± standard deviation).

Extraction of low molecular weight (LMW) metabolites

The three freeze dried muscle cubes of each sample (n=6) were pooled (in cold mortar, ice) and extracted by methanol, chloroform and water as previously described (two-step method by Wu et al., 2008). The water/methanol phase was evaporated in a vacuum centrifuge (30 °C, 1h), freeze dried, and dissolved in 200 µL phosphate buffered saline (PBS, pH 7.4) D2O with 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) at a final concentration of 0.5mM and transferred to 3 mm tubes. Extracted LWM metabolites for the 24 samples were identified and quantified by the software Chenomx NMR suite 7.0 (Chenomx Inc, Canada).

NMR analysis of LMW metabolites NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) at ambient temperature

(25°C) with cryo-probe operating at a ^{1}H frequency of 600.23 MHz. The ^{1}H NMR spectra were obtained using water pre-saturation in the relaxation delay (d1= 4 seconds) followed by a 90º excitation pulse (zgpr). A sweep width of 20 ppm was collected into 64k data points, giving an acquisition time of 2.66 seconds. The number of scans were set to 64, and 4 dummy scans were applied. The raw data were multiplied with a 1 Hz exponential line-broadening factor before Fourier transformation into 64K data points. Chemical shift referencing was performed relative to the methyl groups of TSP at 0.00 ppm. Results are expressed as mg/100 g white muscle.

Statistical analysis

Statistical analysis and data processing were performed using Microsoft Excel 2013. All experiments were performed in at least in duplicate and results are expressed as means ± SD. One way analysis of variance (ANOVA) was used to determine significant differences within the same group or between groups. Whenever the ANOVA test was found to be significant ($p \leq 0.05$), a one-tailed Student's t test was used to determine significant differences between two sets of data. To establish a relationship between protein oxidation products and functional properties of muscle (WHC and protein solubility), Pearson correlations were calculated. Differences were considered significant at a p value of <0.05.

Results and Discussion

Lipid and water content

The mackerel fillets contained 21 ± 0.5% lipids and 62.4 ± 0.7% water (**Table 1**). The content of lipid and water was stable both during chilled (+4 °C) and frozen storage and this is in agreement with previous results where time of frozen storage did not exert a significant effect on water and lipid content in mackerel samples (Aubourg et al (2005). Light- and dark muscle samples had different lipid content, i.e. 13.6 ± 0.8 % and 22.8 ± 1.2%, respectively; which is

279 also in agreement with earlier studies of pelagic fish (e.g. Undeland et al. 1998). The lipid and
280 water content of Atlantic Mackerel varies greatly according to season. The fat content of
281 Atlantic Mackerel landed in Norway is normally highest in September/October, with values up
282 to 30% reported, while the fish caught in January/February is usually somewhat leaner. Recent
283 studies on mackerel caught in Icelandic waters have shown that both catching time (season,
284 year) and fishing area influences the fat- and water content (Romotowska et al., 2016a; 2016b).

285 **Fatty acid composition**

286 The content of EPA and DHA was ca 7% and 10 % (w/w), respectively, of the total fatty acids
287 (**Table 1**) (corresponding to ca 1.26 g EPA and 1.80 g DHA pr 100 gram fillet). No significant
288 changes in DHA nor EPA content was observed during chilled- nor frozen storage. According
289 to the European Food Safety Authorities, the recommended intake of EPA + DHA is 0.25 g/day
290 for the general adult population (EFSA, 2010). The fatty acid C22:1n-11 was the most abundant
291 fatty acid at a level of ca 15% of total fatty acids. The relative fatty acid composition, just as
292 the lipid content, has been shown to vary according to both geographical origin (due to available
293 feed and/or water temperature) and season, and the results presented in this study are within the
294 ranges reported in previous studies (Romotowska et al., 2016a).

295 **Lipid Oxidation**

296 Oxidation of lipids was followed in whole skin-on fillets, as well as in its light and dark muscle.
297 The latter has in several studies been established as more prone to rancidity development (e.g.
298 Undeland et al., 1998)**.** Primary oxidation products were quantified as amount of conjugated
299 dienes in extracted lipids from mackerel muscle samples, and the results are presented in **Figure**
300 **1**.

301 No significant changes in conjugated dienes were observed neither during chilled nor frozen
302 storage, and no significant differences were found between dark and light muscle. The

development of secondary oxidation products was followed as changes in TBARS values of the lipid fraction (**Figure 2**). However, also here the values were very low (below 1mmole MDA equivalents/kg lipids). The TBARS values were relatively constant, but a slight decrease was observed at 12th month of storage which could be due to further reactions of carbonyls e.g. with proteins forming for instance Schiff bases (Zamora and Hidalgo, 2005). With an average of ca 0.5 mmole MDA equivalents/kg lipids, the TBARS values are similar to previous results reported on non-oxidised mackerel oil (0.4 mmole /kg) (Ke and Woyeewoda, 1979). Based on the fat content of the mackerel used in this study, an average TBARS value of 0.5 mmole MDA equivalents/kg lipids corresponds to ca 0.1 mmol/kg muscle or 7 mg/kg muscle. TBARS values measured in frozen mackerel varies between studies, and may also be influenced by the extraction and analysis methods used. Saeed and Howell (2002) observed a small increase in primary (peroxide value) and secondary (TBARS) oxidation products in minced Atlantic mackerel fillet stored at -30°C. In that study TBARS values, when monitored in water-soluble extracts, ranged between 0.1-1 mg MDA equivalents/kg of fish during the storage from 0 to 25 months. However, the mincing operation could lead to decreased stability of the muscle as compared to intact fillets, and no vacuum packing was applied. Aas et al., (2013) also observed an increase in peroxide value and TBARS in mackerel fillets (wrapped in plastic) during storage at -20°C. The constant amount of primary and secondary oxidation products in the present study could potentially be due to better packaging compared to the referenced studies (Aas et al., 2003, Saeed and Howell, 2002). In the present study fillets were vacuum packed in 10 kg blocks that protected lipids against contact with oxygen and thus oxidation.

Lipolysis

Storage at +4°C led to a significant increase in FFA between day 4 and 9 (**Figure 3**), but no significant changes in FFA were found in the frozen samples up till 12 months storage. However, slightly higher amounts of FFA were measured in the frozen samples compared to

328 the 4 days sample and this could be due to some lipolytic enzyme activity during the freezing
329 and the thawing of the frozen sample prior to analysis. In the study of Labuza and Dugan (1971),
330 maximum rate of lipolysis of phospholipids was found to take place at -4 °C (in the temperature
331 range 0 °C to -18 °C The study of Aubourg et al., (2005) observed an increase in FFA during
332 storage of mackerel fillets at – 20°C, but the increase rate also depended on the catching season
333 (the leanest fish showed higher lypolysis), which was also found by Romotowska et al., (2016b)
334 in frozen storage of whole mackerel. The formation of FFA have also been shown to be
335 influenced by temperature fluctuations during transportation. Whole mackerel stored at
336 constant temperature of – 25 °C up till 9 months showed only a modest increase in FFA, from
337 below 1 g/100 g lipids to ca 1.6 g/100 g lipids (Romotowska et al., 2017), while the FFA content
338 was 3 g/100 g lipids when exposed to temperature fluctuations (1 month at -12 °C at the
339 beginning of the storage). Storage at -27°C thus seems to more effectively hinder the enzymatic
340 activity in the fish as no significant increase in FFA in samples stored at -27ºC was observed.
341 Significantly higher FFA was found in the dark muscle compared to light muscle and this in
342 line with a generally higher enzyme activity in the dark muscle than in light muscle (Hwang
343 and Regenstein, 1993).

344 **Vitamin D**

345 The mackerel fillets that had been stored chilled for 4 days (+4 °C) contained in average 0.05µg
346 vitamin D_3/g muscle (**Figure 4**), which is lower than what is reported for skinless mackerel
347 fillets by the Swedish Food Agency (12.8 µg/100g), but within the range reported in the
348 equivalent Danish database (2,1-18,9 µg vitamin D/100g). Slightly higher levels were found in
349 the dark than light muscle; 0.071 vs 0.052 µg/g. Measurements of the whole fillet revealed that
350 no significant ($p>0.05$) changes took place during refrigerated storage for up to 9 days. In fact,
351 the average value was slightly higher at this time point (0.68µg/g), which could have been due

to a batch-to-batch variation. Also, during storage for up to 12 months at -27°C, no significant changes in vitamin D_3 content was documented, showing that this is a very stable vitamin. Only one earlier study reporting storage stability of vitamin D in pelagic fish has been found Aro et al., 2005. In this, vitamin D content did not change during 6 months chilled storage of vacuum packed light-salted gutted herring or herring fillets, and during 12 months storage of pickled herring in glass jars. Levels between 12 and 34 µg/100 g were recorded in this study. High vitamin D stability has also been proven during smoking of mackerel (Aminullah Bhuiyan, 1993). As summarized by Aro et al., (2005) vitamin D concentrations vary within different species and also within the same species caught in different areas; the latter typically being caused by varying dietary factors of fish (Mattila et al., 1997).

Protein oxidation

During chilled storage the carbonyl content was stable both in sarcoplasmic and myofibrillar proteins (**Figure 5A and B**). This is in agreement with the results of Tokur and Polat (2010) who found that carbonyl contents of proteins did not change during refrigerated storage of Gray Mullet fillets for 10 days at 4°C.

No significant changes were observed in the samples stored frozen for 1 month, however, between 1 and 7 months frozen storage both sarcoplasmic and myofibrillar protein carbonyl content increased in all muscle types (**Figure 5 A and B**). This can be explained by the cell disruption during freezing leading to release of pro-oxidants such as H_2O_2, iron, and myoglobin (Mb) creating a highly oxidative environment in the unfrozen phase. That protein oxidation was in fact more visible than lipid oxidation in our study could indicate how proteins can be the first site of oxidative attack from pro-oxidants located in the aqueous phase of the muscle, e.g. the highly reactive hydroxyl radical or ferryl/perferryl-Mb (Hultin, 1994). In the study of Soyer and Hultin (2000), non-enzymatic protein oxidation was more pronounced than non-enzymatic lipid oxidation when using a cod sarcoplasmatic reticulum model system, while the opposite

was true for enzymatic lipid/protein oxidation. Our results are in contrast with those of Baron et al., (2007) who found that the carbonyl content of a rainbow trout (*Oncorhynchus mykiss*) protein homogenate was approximately stable during frozen storage at -30℃ up to 13 months. However, it should be stressed that the content of e.g. pro-oxidative heme-proteins is higher in mackerel muscle than in trout muscle.

The carbonyl content both in sarcoplasmic and myofibrillar protein was slightly higher in dark compared to light muscle at all storage times (**Figure 5a and 5b**). This can be explained by the higher amount of heme proteins, fat and LMW-transition metals, as well as the higher content of oxidative fibers in dark muscle (Lund et al., 2011; Undeland et al., 1998).

The carbonyl content was higher in myofibrillar protein compared to sarcoplasmic protein for all storage times (tenfold higher for samples stored chilled 4 and 9 days, and frozen 1 month; and twentyfold higher for samples stored frozen 7 and 12 months). This is in agreement with the results from chilled storage of thin-lipped mullet (*Liza Ramada*) (Tokur and Polat, 2010). The larger increase of carbonyl groups in myofibrillar proteins compared to sarcoplasmic proteins, are well known, and in accordance with previous studies showing a higher susceptibility of myofibrillar proteins to denature during storage and processing (Duun and Rustad, 2007).

Sarcoplasmic thiol content of all muscle types (light, dark and whole) was stable during chilled storage and frozen storage up till 1 month, but was significantly lower after 7 and 12 month storage ($p < 0.05$) (**Figure 6a**). Similar results have been reported by Benjakul et al. 2003 who found a continued decrease in total thiol groups in lizard fish, croaker, threadfin bream and bigeye snapper during frozen storage for 6 months at -18℃. No significant difference was observed in sarcoplasmic protein thiol content between the muscle types between the different sampling times (**Figure 6a**).

There was a small but significant ($p < 0.05$) decrease in myofibrillar protein thiol content (**Figure 6b**) of light muscle and whole fillet during chilled storage of samples indicating occurrence of protein oxidation. Similar results have been reported by Eymard et al. 2009 who found a decrease in thiol content of a protein homogenate (myofibrillar plus sarcoplasmic proteins) from mackerel mince stored 1 day at 5 ºC.

Myofibrillar proteins generally had significantly lower thiol content compared to sarcoplasmic proteins. This is in agreement with the results from the formation of carbonyl groups. The lower thiol content of myofibrillar proteins could implicate that protein oxidation occur faster and more intensively in myofibrillar protein or that less thiol groups are found in the myofibrillar fraction, as found in Wang and Luo (2013).

Protein extractability

Protein oxidation promoted by frozen storage can result in formation of cross-linkages between polypeptides and proteins, leading to protein aggregation and decreased protein solubility (Zang et al., 2013). During chilled storage, the myofibrillar protein extractability decreased significantly (**Figure 7b**), while the sarcoplasmic protein extractability was more stable (**Figure 7a**). Freezing reduced the extractability of both myofibrillar and sarcoplasmic proteins (**Figure 7a and b**), especially from 1 to 7 months of storage. This is in accordance with several studies reporting a reduction in protein extractability during frozen storage of fish (Saeed and Howell, 2002, Leelapongwattana et al., 2005). A significant negative correlation was found between myofibrillar protein carbonyls and myofibrillar protein extractability ($r = -0.77$, $p<0.05$) during frozen storage. A significant positive correlation was found between myofibrillar protein extractability and myofibrillar protein thiol content ($r = 0.94$, $p<0.05$) during frozen storage.

Water holding capacity (WHC)

WHC was relatively stable during the chilled storage and during 1 month frozen storage (**Figure 8**). Similar results were reported by Mørkøre et al. (2002) who found no significant differences in liquid leakage and consequently WHC during storage of rainbow trout at 4℃ for 7 days. WHC of all mince types was significantly lower after frozen storage for 7 and 12 months compared to 1 month frozen stored samples and chilled samples, and this can be explained by the increasing extent of protein oxidation with storage time as discussed above. Loss of WHC could also be due to other chemical modifications of the amino acid side chains and protein backbones that are triggered during muscle protein oxidation, resulting in decreased ability of muscle proteins to bind and hold water (Poulanne and Halonen, 2010).

There was a significant correlation between the formation of carbonyls groups and loss of WHC during storage, but the correlation was stronger for myofibrillar proteins ($r = -0.89$, $p < 0.05$) than for sarcoplasmic proteins ($r = -0.44$, $p < 0.05$). Also, a significant correlation was found between loss of thiol group and WHC during storage ($r = + 0.89$ and $r = + 0.75$, $p < 0.05$, for sarcoplasmic and myofibrillar proteins, respectively).

Low molecular weight (LMW) metabolites

Table 2 gives an overview of the LMW metabolites quantified in the mackerel extracts.

Amino acids and biogenic amines

Histidine dominated as the overall most abundant free amino acid in the samples analyzed. Active migratory fish species have been shown to have high levels of free histidine (Konosu and Yamaguchi, 1982). During chilled storage (+4°C), the level of histidine was significantly reduced from 133 ± 6 mg/100 g (ww), at day 4 to 106 ± 18 mg/100g at day 9 ($p < 0.05$), while the histamine content increased (see later discussion on biogenic amines). Frozen storage preserved histidine, and actually, a slight increase could be observed after 12 months.

Taurine content in the fresh mackerel at day 4 was 27 ± 3 mg/100 g, and the levels increased somewhat during chilled storage (40 ± 6 mg/100g). The frozen fish had a similar content of taurine as the fresh fish at day 4. Lysine showed a pronounced decrease from 18 ± 4 mg/100g at day 4 to barely detectable levels at 9 days of chilled storage (0.3 ± 0.1 mg/100g), while cadaverine increased correspondingly (see discussion later). Freezing preserved the lysine content (17 ± 4 mg/100g and 20 ± 4 mg/100g after 1 and 12 months, respectively).

The amino acids histidine, lysine, tyrosine, tryptophane, ornithine are well known precursors of biogenic amines such as histamine, cadaverine, tyramine , tryptamine, and putrescine, respectively, responsible for scombroid poisoning caused by spoiled fish (Hungerford, 2010, Tayloer, 1986)). Normal amount of histamine in fresh fish is 0.1-5 mg/100g, and scombroid fish poisoning is generally associated with fish with histamine levels above 5-10 mg/100 g (Lehane and Olley, 2010). The levels of biogenic amines were below the detection limit in the mackerel at day 4, this indicate that the fish had been treated hygienically and that it had not been stored at elevated temperatures during longer periods of time. **Figure 9** shows the 1H NMR region where the formation of histamine (and to a lesser extent tyramine) can be observed. After 9 days at +4 °C, histamine and cadaverine had been formed in significant amounts (11 ± 2 mg/100 g, and 18 ± 3 mg/100 g, respectively), and tyramine was also detected (0.8 ± 0.2 mg/100g)). In comparison, Bennour et al., (1991) found that the histamine content in ice-stored Atlantic mackerel caught outside Morocco with an ice:fish ratio of 1:2 was ca 8mg/100 g at day 9, and that increasing amount of ice (i.e. lower temperature) hindered the histamine formation. The FDA has set a maximum level of histamine in Atlantic mackerel of 5 mg/100 g (FDA 1996), while in Europe the limit is 10 mg/100 g (EC 2003). Studies have proposed that there might be potentiation of histamine toxicity by other biogenic amines, such as cadaverine, putrescine, tyramine (Lehane and Olley, 2010), but general safety limits for these have not been established.

Nucleotide derivatives

Adenosine triphosphate (ATP) predominates in muscle of live animals under normal conditions, but after death a series of enzymatic reactions leads to decomposition of ATP to adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx). ATP, ADP and AMP may be observed in fish muscle directly after killing the fish, by careful sample preparation and extraction (Standal et al., 2007), while fresh fish for consumption usually shows IMP as the main ATP related compound (Martinez et al., 2005). IMP contributes to the desirable taste of fresh fish, while Hx is a contributor to the bitter off-flavour of spoiled fish.

In the present study IMP, followed by inosine, were the dominating ATP related compounds in the fresh (day 4) and frozen stored mackerel. Both IMP and inosine were well preserved during frozen storage up till 12 months. IMP content was measured to be somewhat higher in the frozen stored mackerel compared to the fresh mackerel at day 4, and the reason for this is probably that the time of freezing at the processing plant was a few hours earlier than the time for sample collection of the day 4 samples. After 9 days of chilled storage, the levels of IMP and inosine had decreased, and the levels of Hx had increased. The degree of freshness of fish is often expressed as the K-value (Saito et al., 1959), defined as the ratio of the sum of Inosine and Hx to the sum of all ATP related catabolites. The acceptable K-values differ among fish species- but a general limit for consumption is approximately 80 % (Saito et al., 1959). In the present study, the frozen mackerel (after 1 and 12 months) had a K-value similar to the fresh one sampled at day 4 after catch (20%). The chilled stored fish had a K value of 93% at day 9 (**Figure 10**), thereby exceeding the upper recommended limits for consumption.

Amines (TMA, TMAO and DMA)

Upon death and during ice storage, the osmolyte TMAO is metabolized to TMA by certain bacteria, causing off odours (Huss, 1995). The content of TMAO decreased remarkably during chilled storage (from 61 ± 5 mg/100 g at day 4 to 0.4 ± 0.1mg/100g at day 9) while TMA increased from barely detectable levels at day 4 to 22 ± 4 mg/100 g at day 9. No significant changes were measured in TMAO content during frozen storage. There are no official levels set on the limits of TMA for human consumption, and many different ranges have been proposed for fish as summarized by Barbuzzi et al, (2009), who chose an intermediate limit of 6 mg/100g as a quality limit in their study of formation of TMA in different fish minces (hake, mackerel, sea bass, sea bream). Mackerel was the most perishable species compared to hake, seabass and seabream when it comes to TMA levels (Barbuzzi et al., 2009). However, there were no catch data given in this article (days after purchase from local supermarket reported), so the initial qualities of the fish samples might have varied. Bennour et al., (1991) found that TMA content at day 9 was 5, 10 and 15 mg/100 g in Atlantic Mackerel (with ice:fish ratio of 1:4, 1:3 and 1:2, respectively). During freezing, the endogenous enzyme TMAOase metabolizes TMAO to DMA and formaldehyde, which are proposed to cause toughening of fish muscle during frozen storage (Konosu and Yamaguchi, 1982). However, DMA may also be formed non-enzymatically by e.g. heating processes and drying (Spinelli and Koury, 1981), and has been observed in clip fish samples (Martinez et al., 2005). The level of DMA increased somewhat during both chilled and frozen storage in the present study (from 0.20 ± 0.04 mg/100g at day 4, to 0.7 ± 0.2 mg/100g at day 9 of the chilled storage)), and to 0.5 ± 0.1 mg/100g and 0.6 ± 0.1 mg/100g after 1 and 12 months frozen storage, respectively.

Conclusion

In conclusion, this study showed that freezing at -27 °C under vacuum preserved the LC n-3 PUFAs, vitamin D and the LMW metabolites studied. However, protein oxidation took place, especially between 1-7 months frozen storage. Sarcoplasmic and myofibrillar protein carbonyl

contents increased significantly, and at the same time, the total thiol group contents decreased slightly. These changes occurred concomitantly with a loss of WHC and protein extractability. During chilled storage at +4°C, no lipid nor protein oxidation was observed, but lipolysis increased, and several LMW metabolites relevant for sensory and nutritional quality degraded into non-favorable compounds. For instance, the content of biogenic amines was high at day 9 (e.g. 11 mg histamine/100g), jeopardizing safety. Preservation of mackerel fillets by freezing at -27°C is thus a better option compared to prolonged chilled storage at +4 °C and the quality was well preserved up till 12 months frozen storage.

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The authors gratefully acknowledge the Research Council of Norway for the financial support for carrying out the present research (NFR project No. 222476/E40).

Figures

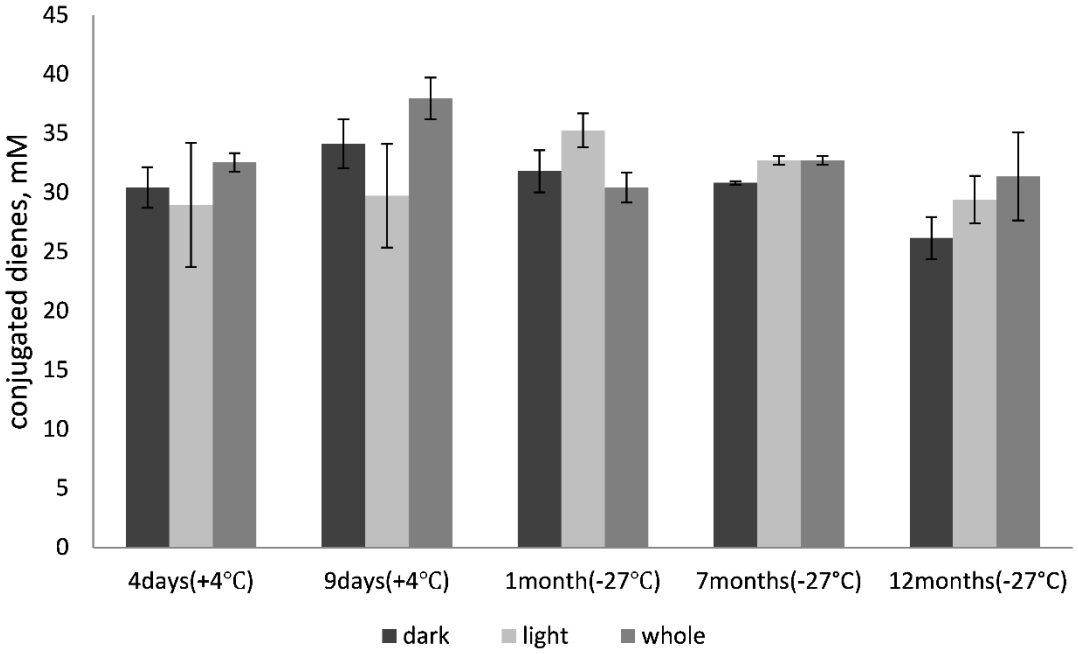

Figure 1 Content of conjugated dienes (mM) in total lipids extracted from mackerel (whole fillet with skin, light and dark muscle) stored at +4°C and -27°C. The values are given as an average of at least three samplings, with standard deviations.

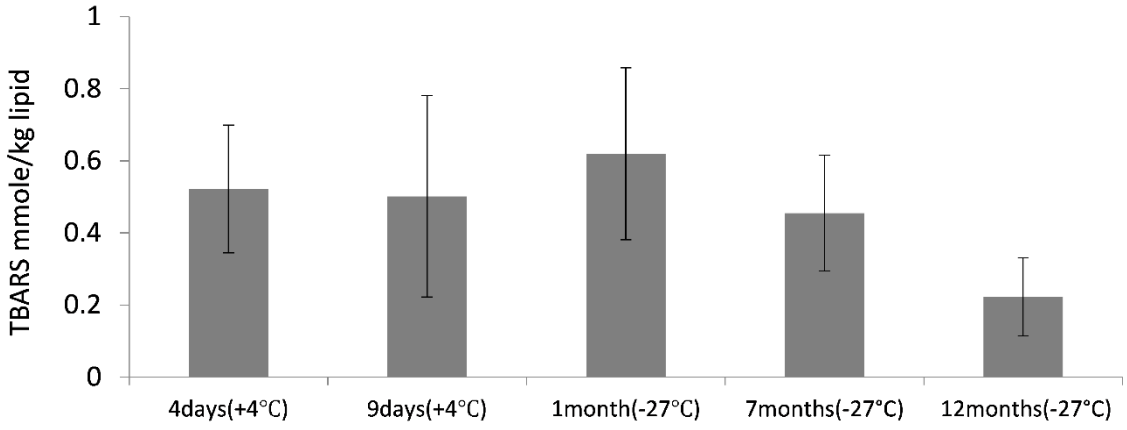

Figure 2 TBARS values in total lipids extracted from whole mackerel fillets with skin (mmole/kg lipid) stored at +4°C and -27°C. The values are given as an average of at least three samplings, with standard deviations.

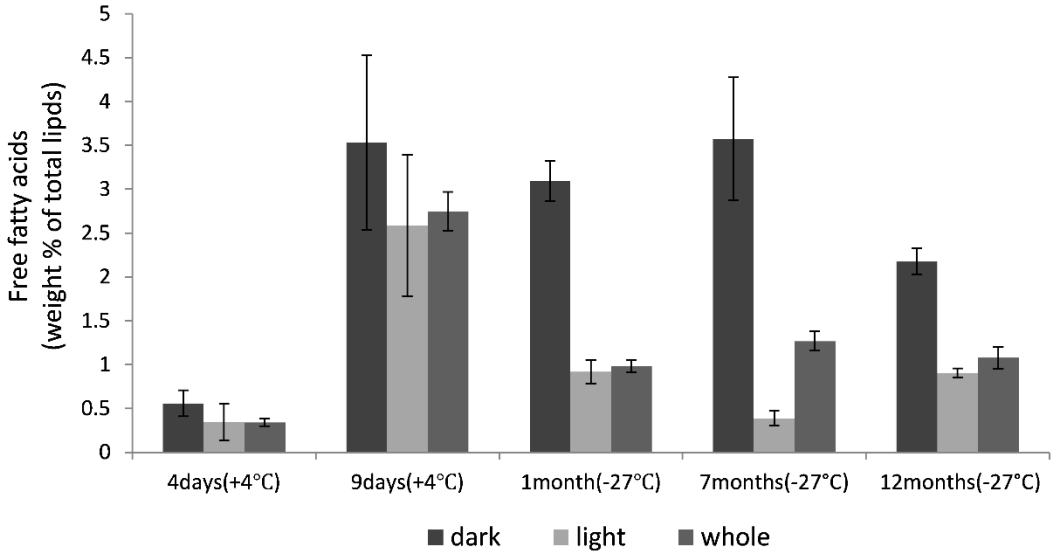

Figure 3 Free fatty acids in total lipids (% of total lipid w/w) extracted from mackerel (whole fillet with skin, light and dark muscle) stored at +4°C and -27°C. The values are given as an average of at least three samplings, with standard deviations.

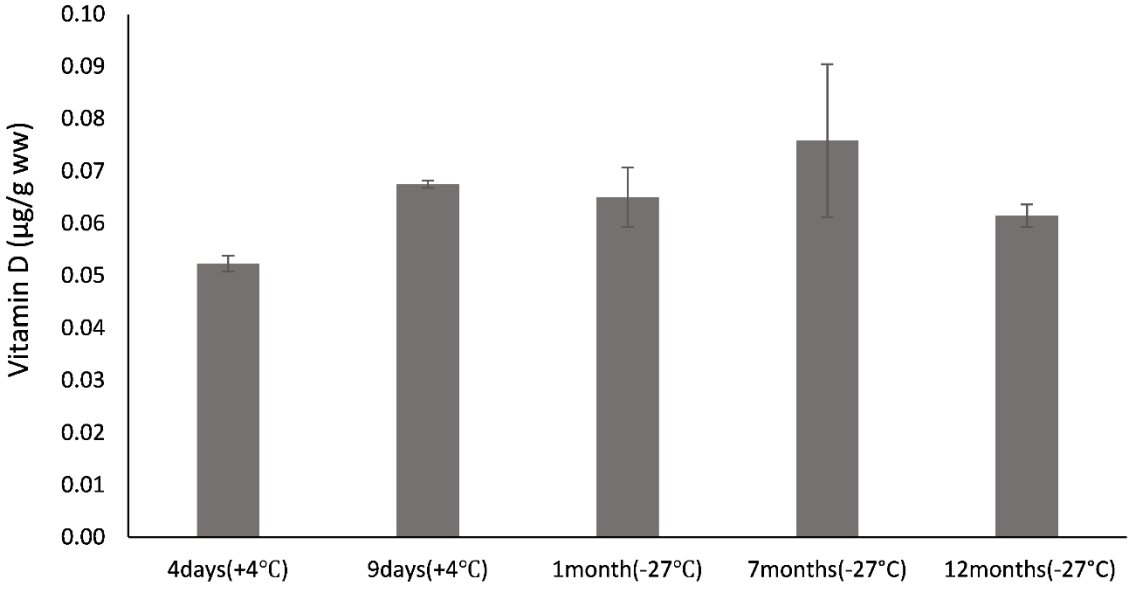

Figure 4 Vitamin D content in mackerel fillets (µg/g ww) stored at +4°C and -27°C. The values are given as an average of at least two samplings.

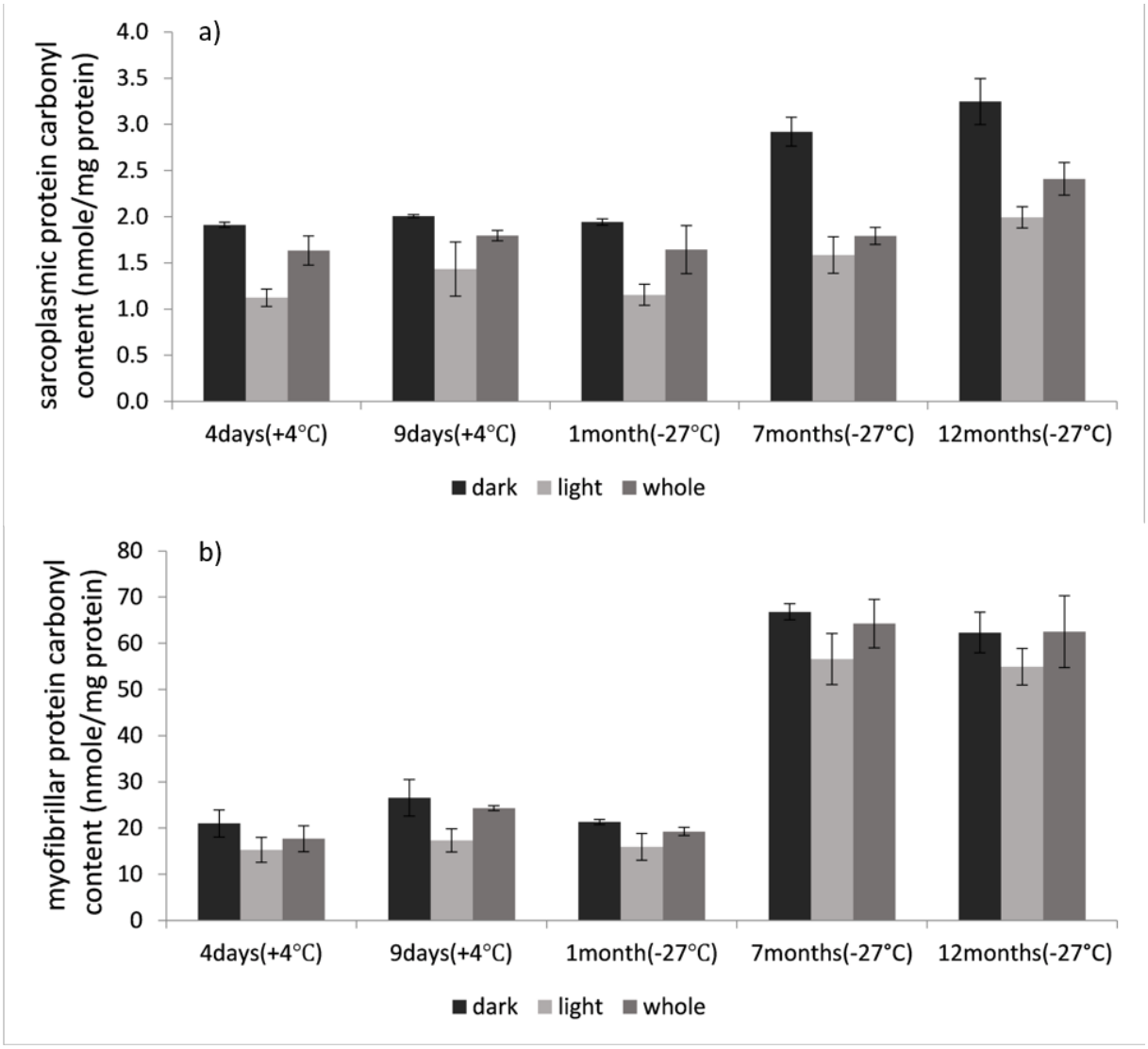

Figure 5 Carbonyl content of proteins (nmole carbonyl/mg protein) for a) sarcoplasmic proteins (upper) and b) myofibrillar proteins (lower) of dark- and light muscle, and whole fillet stored at + 4°C and -27°C for. The values are given as an average of two samplings, with standard deviations.

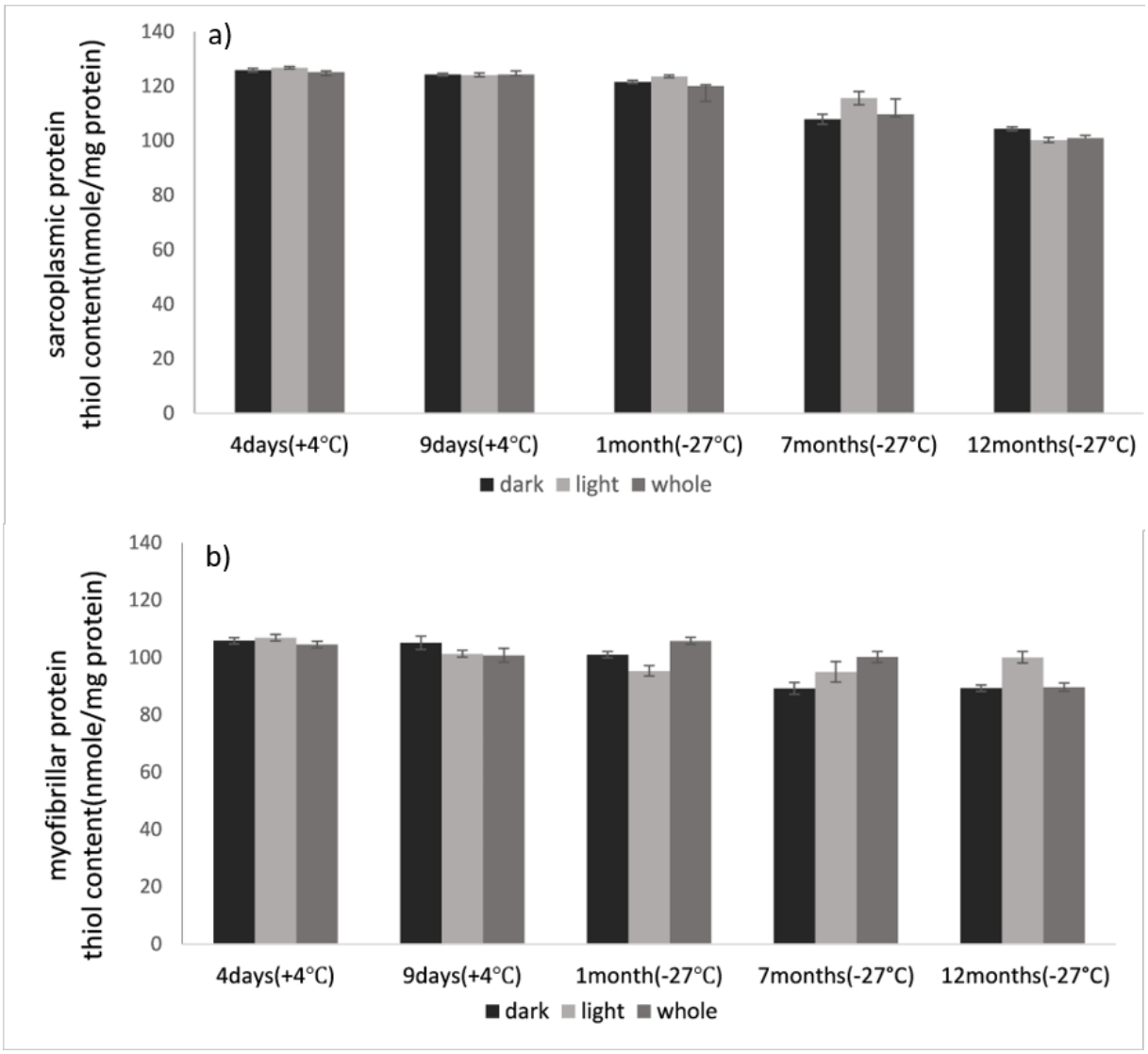

Figure 6 Thiol content of proteins (nmole thiol/mg protein) for a) sarcoplasmic proteins (upper) and b) myofibrillar proteins (lower) of dark- and light muscle, and whole fillet stored at +4°C and -27° C. The values are given as an average of three samplings, with standard deviations.

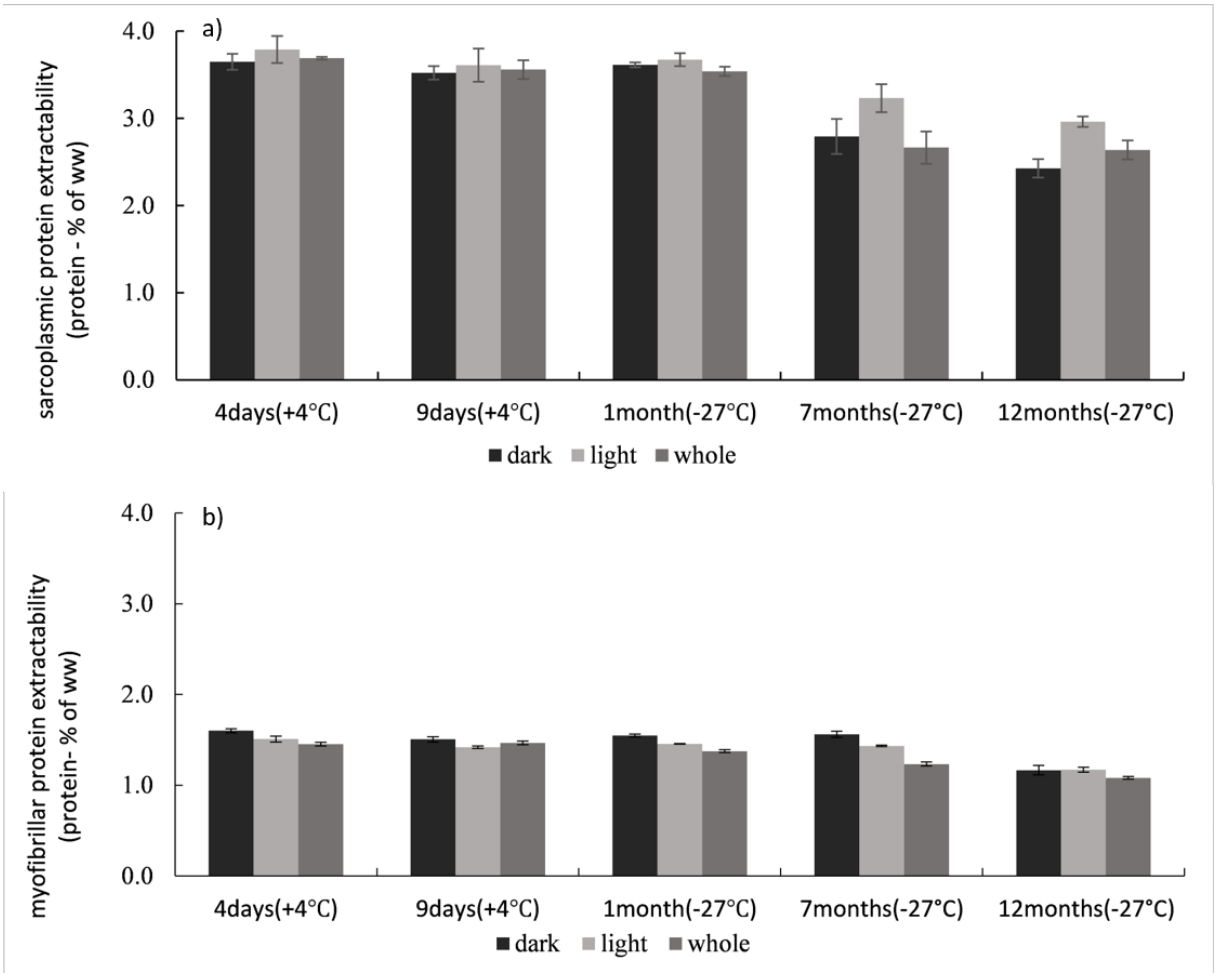

Figure 7 Protein extractability (protein in % of muscle wet weight) for a) Sarcoplasmic proteins (upper) and b) myofibrillar proteins (lower) of dark- and light muscle, and whole fillet stored at + 4°C and -27° C. The values are given as an average of three samplings, with standard deviations.

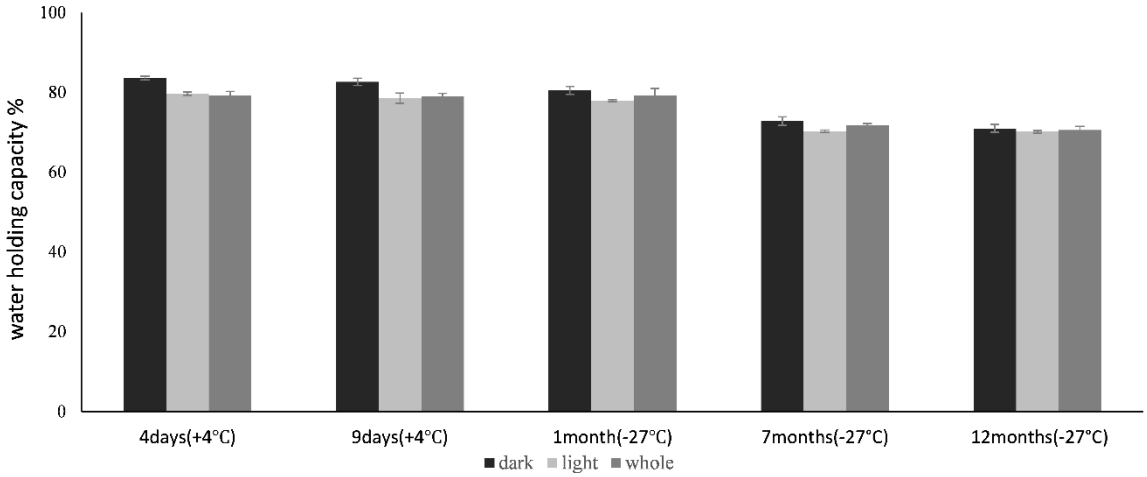

Figure 8 Water holding capacity, WHC (% of original water) of mackerel dark- and light muscle, and whole fillet stored at +4°C and -27 °C. The values are given as an average of four samplings, with standard deviations.

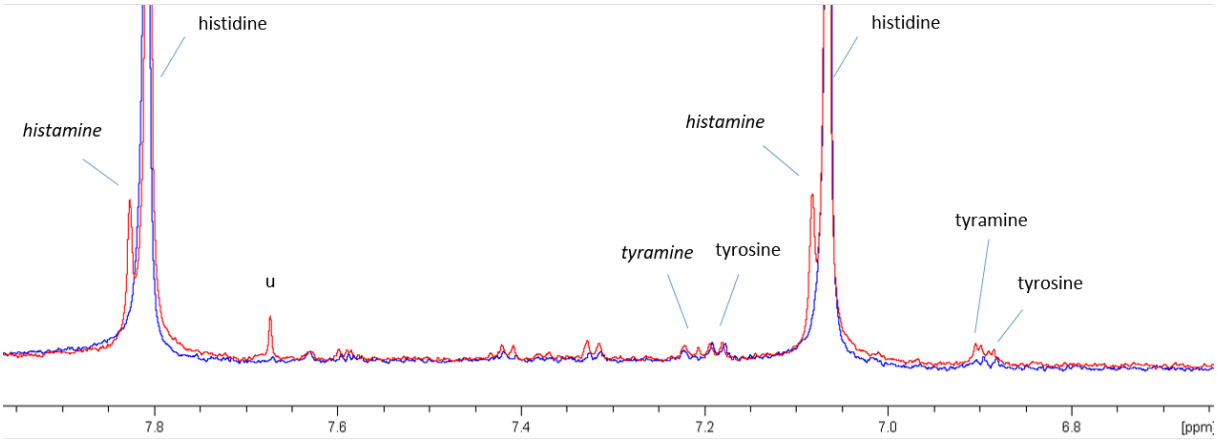

Figure 9 1H NMR region between 6.8- 8 ppm for low molecular weight (LMW) metabolites extracted from mackerel fillets stored at+4 oC at day 4 (blue) and day 9 (red), illustrating the formation of histamine (and tyramine) in the day 9 sample. (u- unassigned peak).

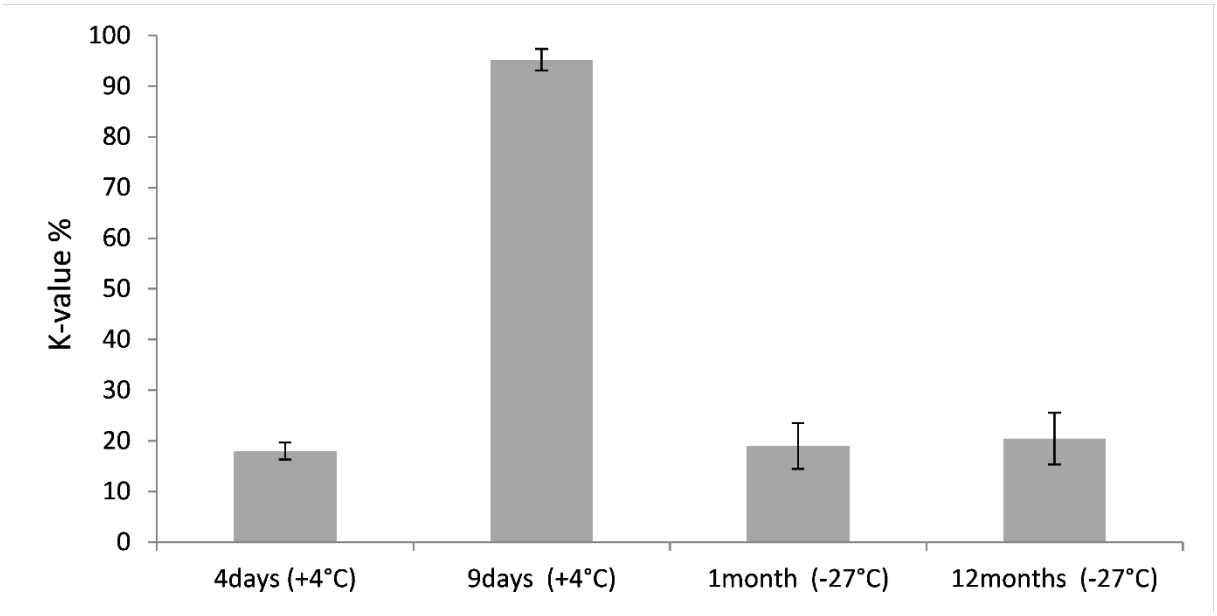

Figure 10 K – values calculated from 1H NMR data on extracted low molecular weight (LMW) metabolites in mackerel fillets at the different storage conditions.

Tables

Table 1.Water and lipid content, in addition to fatty acid composition of the mackerel samples.

	4d (+4 °C) whole fillet	4d (+4 °C) light muscle	4d (+4 °C) dark muscle	9d (+4 °C) whole fillet	1m (-27 °C) whole fillet	7m (-27 °C) whole fillet	12m (-27 °C) whole fillet
Composition							
water	62.4 ± 0.7	68.1 ± 0.6	60.7 ± 0.8	62.1 ± 0.8	61.7 ± 0.6	62 ± 0.9	61.4 ± 0.3
lipid	21 ± 0.5	13.6 ± 0.8	21.3 ± 0.7	21.3 ± 0.7	21.4 ± 0.4	20.6 ± 0.8	21.1 ± 0.4
Fatty acid profile (% of total FA)*							
C14:0	7.4 ± 0.2	7.6 ± 0.1	7.7 ± 0.1	7.5 ± 0.3	7.5 ± 0.2	6.9 ± 0.1	6.5 ± 0.1
C14:1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
C16:0	13.7 ± 0.2	14.1 ± 0.8	13.5 ± 0.1	13.9 ± 0.4	13.8 ± 0.3	12.7 ± 0.2	12.3 ± 0.3
C16:1n9	4.4 ± 0.0	4.3 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	4.3 ± 0.2	0.2 ± 0.0	0.3 ± 0.0
C16:1n7	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	4.0 ± 0.2	3.9 ± 0.1
C18:0	2.4 ± 0.1	2.5 ± 0.3	2.3 ± 0.0	2.5 ± 0.2	2.4 ± 0.1	2.3 ± 0.1	2.1 ± 0.1
C18:1n9	12.1 ± 0.6	13.4 ± 3.1	11.1 ± 0.0	12.0 ± 1.3	11.7 ± 0.3	11.4 ± 0.5	11.3 ± 1.0
C18:1n7	2.2 ± 0.1	2.2 ± 0.2	2.0 ± 0.0	2.2 ± 0.2	2.2 ± 0.1	2.1 ± 0.1	1.7 ± 0.1
C18:2n6	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.0	1.7 ± 0.1	1.7 ± 0.0	1.6 ± 0.1	1.8 ± 0.0
C18:3n6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
C18:3n3	1.6 ± 0.1	1.5 ± 0.0	1.5 ± 0.0	1.6 ± 0.1	1.6 ± 0.0	1.4 ± 0.0	1.4 ± 0.0
C18:4n3	4.3 ± 0.2	4.2 ± 0.1	4.3 ± 0.1	4.2 ± 0.2	4.2 ± 0.1	3.9 ± 0.1	4.0 ± 0.0
C20:0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
C20:1n11	11.6 ± 0.3	11.4 ± 1.6	12.2 ± 0.3	11.3 ± 0.5	11.6 ± 0.2	11.5 ± 0.4	11.7 ± 0.7
C20:1n9	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
C20:2n6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
C20:3n6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C20:4n6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.0	0.5 ± 0.1
C20:3n3	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
C20:4n3	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1
C20:5n3	7.2 ± 0.2	6.8 ± 0.3	6.6 ± 0.1	7.1 ± 0.2	7.0 ± 0.2	6.9 ± 0.3	7.1 ± 0.2
C22:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C22:1n11	15.5 ± 0.3	15.0 ± 2.7	16.7 ± 0.3	15.5 ± 1.1	15.9 ± 0.2	17.4 ± 0.7	17.9 ± 1.4
C22:1n9	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.9 ± 0.0	1.0 ± 0.0
C22:5n3	1.3 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.3 ± 0.0	1.2 ± 0.0	1.4 ± 0.1	1.3 ± 0.0
C24:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C22:6n3	10.0 ± 0.2	9.8 ± 0.0	10.1 ± 0.3	10.1 ± 0.2	10.4 ± 0.2	10.8 ± 0.1	11.0 ± 0.4
C24:1n9	0.8 ± 0.1	0.7 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.2 ± 0.0
Sum sat	23.7 ± 0.3	24.3 ± 1.1	23.7 ± 0.2	24.2 ± 0.3	24.0 ± 0.6	22.5 ± 0.3	21.5 ± 0.3
Sum mono	47.8 ± 0.3	48.0 ± 1.1	48.4 ± 0.5	47.5 ± 0.7	47.6 ± 0.2	45.0 ± 0.6	45.6 ± 1.0
Sum poly	28.2 ± 0.4	27.4 ± 0.1	27.5 ± 0.4	28.0 ± 0.6	28.1 ± 0.4	28.5 ± 0.3	29.0 ± 0.7
sum omega 3	25.7 ± 0.4	24.9 ± 0.2	25.0 ± 0.4	25.6 ± 0.5	25.6 ± 0.4	25.6 ± 0.4	26.1 ± 0.6

Table 2. Low molecular weight (LMW) metabolites quantified in the mackerel fillets stored at different temperatures and times given as mg/100 g wet weight with standard deviations (n=6)

Compound	4d (+4 °C)	9d (+4 °C)	1m (-27 °C)	12m (-27 °C)
Acetate	3.2 ± 2.5	20.2 ± 5.4	1.2 ± 0.3	2.0 ± 0.5
Alanine	23.0 ± 2.0	31.2 ± 5.7	28.0 ± 4.9	29.2 ± 8.2
Cadaverine	0.0 ± 0.0	18.3 ± 7.0	0.0 ± 0.0	0.0 ± 0.0
Creatine	194.8 ± 22.7	193.0 ± 27.2	223.5 ± 33.1	238.2 ± 37.0
Dimethylamine	0.2 ± 0.0	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1
Formate	0.6 ± 0.3	7.9 ± 1.6	0.5 ± 0.1	2.7 ± 0.9
Glutamate	11.2 ± 1.8	11.7 ± 2.7	12.6 ± 1.8	16.1 ± 2.8
Glutamine	2.1 ± 0.4	5.7 ± 2.1	2.5 ± 0.9	4.0 ± 0.7
Glycine	5.9 ± 1.0	5.2 ± 1.3	6.6 ± 1.2	7.9 ± 1.8
Histamine	0.0 ± 0.0	11.4 ± 2.3	0.0 ± 0.0	0.0 ± 0.0
Histidine	132.9 ± 6.2	106.6 ± 17.7	124.4 ± 12.0	166.6 ± 23.4
Hypoxanthine	1.1 ± 0.2	18.3 ± 3.0	0.9 ± 0.3	1.4 ± 0.2
Imidazole	0.2 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.1 ± 0.1
IMP	120.3 ± 11.9	1.1 ± 0.4	160.5 ± 39.9	171.0 ± 35.8
Inosine	25.1 ± 1.7	7.8 ± 3.3	30.1 ± 4.9	41.2 ± 6.9
Isoleucine	1.0 ± 0.2	1.0 ± 0.2	1.5 ± 0.2	1.9 ± 0.4
Lactate	149.0 ± 13.8	155.4 ± 19.8	190.0 ± 27.6	238.0 ± 41.0
Leucine	1.8 ± 0.2	2.7 ± 0.7	2.9 ± 0.4	3.2 ± 0.8
Lysine	18.1 ± 4.0	0.3 ± 0.1	16.9 ± 3.6	19.8 ± 4.2
Methionine	1.5 ± 0.3	1.3 ± 0.3	1.4 ± 0.3	1.9 ± 0.4
Nicotinurate	1.5 ± 0.4	2.2 ± 0.4	2.5 ± 0.3	3.8 ± 0.8
O-Acetylcholine	0.6 ± 0.7	0.2 ± 0.1	1.9 ± 0.4	0.6 ± 0.8
O-Phosphocholine	0.3 ± 0.1	10.2 ± 2.9	0.2 ± 0.1	1.1 ± 0.2
Phenylalanine	0.7 ± 0.1	1.5 ± 0.2	1.8 ± 0.2	2.4 ± 0.4
Propylene glycol	0.0 ± 0.0	3.8 ± 1.0	0.1 ± 0.0	0.0 ± 0.0
Sarcosine	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Succinate	1.3 ± 0.3	5.4 ± 0.6	2.1 ± 0.4	2.8 ± 1.0
Taurine	26.8 ± 2.6	39.9 ± 6.1	33.8 ± 3.7	35.0 ± 4.1
Threonine	1.6 ± 0.3	1.4 ± 0.3	1.3 ± 0.3	1.0 ± 0.2
Trimethylamine	0.1 ± 0.2	22.4 ± 4.4	0.6 ± 0.2	0.4 ± 0.1
Trimethylamine N-oxide	61.4 ± 5.0	0.4 ± 0.1	69.1 ± 9.7	76.8 ± 14.1
Tyramine	0.0 ± 0.0	0.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Tyrosine	1.8 ± 0.1	1.6 ± 0.4	2.6 ± 0.2	3.2 ± 0.3
Valine	1.9 ± 0.1	3.1 ± 0.4	2.9 ± 0.4	3.4 ± 0.6