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Philosophiae Doctor (PhD) Thesis 2011:07

The effect of pancreas disease and salting conditions on the quality of raw and cold-smoked Atlantic salmon (*Salmo salar* L.)

Effekt av pankreas sykdom og ulike saltebetingelser på kvaliteten til rå og kaldrøkt Atlantisk laks (*Salmo salar* L.)

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ABBREVIATIONS

AA	Arachidonic acid
*a**	reddish when $a^* > 0$
ADI	acceptable daily intake
ATP	adenosin triphosphate
A_w	water activity
*b**	yellowish when $b^* > 0$
CV	coefficient of variance
CF	condition factor
DHA	*all-cis*-docosa-4,7,10,13,16,19-hexa-enoic acid
ΔE	the colour difference
EFSA	European food safety authority
EPA	*all-cis* -eicosa-5,8,11,14,17-pentenoic acid
FA	fatty acid
GAG	glycosaminoglycans
H°_{ab}	red hue when $H^{\circ}_{ab} = 0$, yellowish when $H^{\circ}_{ab} = 90$
HPLC	high performance liquid chromatography
HYP	hydroxyproline
JECFA	Joint FAO/WHO expert committee on food additives
*L**	lightness / translucence (0-100; 0 = black, 100 = diffuse white)
Nitrite salt	nutrient quality with 0.6% sodium nitrite, $NaNO_3$
PD	pancreas disease
PL	phospholipid
SAV	salmonid alpha virus
SCF	European commission's scientific committee for food
Fine refined salt	nutritional quality, >99.8% sodium chloride, NaCl
TAG	triacylglycerol
TMAO	trimethylamine *N*-oxide
VIS	visible light

SUMMARY

Fillet quality of farmed Atlantic salmon (*Salmo salar* L.) is affected by a broad range of pre- and post-mortem factors. This thesis focuses mainly on the impact of pancreas disease (PD), salt composition and salting method on pigment concentration and colour of raw and cold-smoked salmon fillets, but fillet composition, texture and drip losses are as well considered.

The fish material used to study effects of PD on fillet quality was sampled from commercial farming locations to obtain a broad overview and identify potential common properties of PD affected salmon (Paper I). The farming locations participated in a cohort study reported by Jansen et al. (2010), hence the salmon were sampled from populations with a well documented disease history. Salmon from seven farms were diagnosed with PD (diagnosed 0-12 months prior to slaughtering); salmon from one farm were infected with salmonid alpha virus (SAV) without an outbreak of PD, whereas salmon from two farms had no records of PD diagnosis and worked as control farms. To obtain knowledge on underlying causes to quality deviations of PD affected salmon, individuals with different PD pathology within a population diagnosed with PD at time of slaughtering was investigated (Paper II). Anecdotal information indicates that PD may cause poor general muscle quality and in particular pale and irregular colouration. Salmon diagnosed with PD at slaughter, or six months prior to slaughter confirmed this. Changes in quality in the order of their appearance were decreased CF, depleted muscle glycogen, increased drip loss of raw muscle, paler colour, depleted protein and finally harder texture in smoked salmon fillets. Salmon infected with salmonid alphavirus (SAV) without a PD outbreak and salmon diagnosed one year before slaughtering, had similar quality as unaffected fish, although paler colour might occur even after one year recovery. Within a population of farmed Atlantic salmon diagnosed with PD at time of slaughtering, a large variation was observed among individuals in pathological profile, gene expression profile in heart tissue, and fillet quality characteristics. Deteriorated quality and dysfunction of the digestive system was most pronounced in fish with severe loss of pancreas.

Dry salting significantly reduced the stability of carotenoids in the fillet surface of cold-smoked salmon fillets as compared to injection salting (Paper IV). The salt content in pre rigor injection salted fillets was similar as those salted post rigor (2.4% versus 2.6%, respectively), whereas the fillet colour was more translucence. However, injection salting of pre rigor fillets can be recommended. Moreover, state of rigor mortis at time of salting had no effect on the carotenoid stability. Low correlations between colorimetric parameters and

content of carotenoids in the fillet surface, illustrated that additional factors affects the visual appearance of cold smoked salmon.

Nitrite salt curing (Paper III) improved the colour of cold-smoked salmon fillets (more reddish, darker and less yellowish). However, only slight higher carotenoid stability during processing was observed. The improved colour was most probably related to formation of nitrosomyoglobin. Salmon fillets treated with nitrite (fine refined salt added 0.6% sodium nitrite) had a relatively high amount of residual nitrite, however higher contents of *N*-nitrosoamines were not observed.

It is concluded that salmon diagnosed with PD at slaughter, or six months prior to slaughter showed deteriorated fillet quality, whereas salmon infected with salmonid alphavirus (SAV) without a PD outbreak and salmon diagnosed one year before slaughtering, had similar quality as unaffected fish. Moreover it is concluded that nitrite salt improved the appearance of cold-smoked salmon fillets and dry salting reduced the stability of carotenoids in the fillet surface as compared to injection salting.

NORSK SAMMENDRAG (NORWEGIAN SUMMARY)

Filetkvalitet hos oppdrettslaks (Atlantisk laks, *Salmo salar* L.) blir påvirket av ulike faktorer pre og post mortem. I denne avhandlingen er hovedfokuset rettet mot betydningen av pankreas sykdom (PD), saltets sammensetning og saltemetode i forhold til pigmentkonsentrasjon og farge hos rå og kaldrøkte laksefileter. I tillegg er filetsammensetning, tekstur og væsketap tatt i betraktning.

For å studere effekten av PD på filetkvalitet hos laks, ble det hentet fisk fra kommersielle oppdrettsanlegg. Dette ble gjort for å få en bred oversikt og for å identifisere potensielle fellestrekk hos PD affisert laks (Artikkel I). Oppdrettsanleggene som ble benyttet var også med i en kohortstudie beskrevet i Jansen et al. (2010), noe som ga fisken en veldokumentert sykdomshistorie. Laks fra syv anlegg var diagnostisert med PD (diagnostisert 0-12 måneder før slakting) og ett anlegg var infisert med SAV uten at PD utbrudd var registrert. Laks fra to andre anlegg var uten tegn til PD og fungerte som kontrollanlegg. For å finne underliggende årsaker til kvalitetsavvik hos PD diagnostisert fisk, ble individer fra samme populasjon (diagnostisert med PD under slakting), men med ulik PD patologi undersøkt (Artikkel II). Anekdotisk informasjon indikerer at PD kan forårsake generelt dårlig muskelkvalitet, og da spesielt lys og ujevn farge. Laks diagnostisert med PD under slakting, eller seks måneder før slakting bekrefter dette. Endringene i kvalitet i forhold til når de inntreffer var; redusert kondisjonsfaktor, forbruk av glykogen, økt væsketap i rå muskel, blassere farge, forbruk av protein og til slutt hardere tekstur i kaldrøkte fileter. Laks infisert med SAV uten utbrudd av PD og laks diagnostisert ett år før slakting, hadde lik kvalitet som upåvirket fisk, selv om laks med blassere farge kan eksistere selv ett år etter sykdom. I en populasjon av Atlantisk laks diagnostisert med PD under slakting, ble det observert stor variasjon mellom ulike individer. Variasjonen viste seg i patologisk profil, genekspresjonsprofil i hjertemuskel og i filetegenskaper. Forringet kvalitet og nedsatt fordøyelsessystem var mest synlig i fisk med kraftig redusert pankreas.

Sammenlignet med injeksjonssalting reduserer tørrsalting stabiliteten til karotenoider i filetoverflaten hos kaldrøkt Atlantisk laks (Artikkel IV). Saltkonsentrasjonen i fileter injisert med salt pre rigor var forholdsvis lik de respektive filetene saltet post rigor (2,4 versus 2,6 %). Pre rigor injiserte fileter var samtidig lysere. Likevel kan injeksjonssalting av pre rigor laksefileter anbefales. Rigorstatus ved saltetidspunktet hadde ingen effekt på stabiliteten til karotenoidene. En lav korrelasjon mellom fargeparametre og innhold av karotenoider i kaldrøkt laks, viser at andre faktorer også påvirker det visuelle uttrykket av fileten.

Nitrittsalting (Artikkel III) forbedrer fargen hos kaldrøkte laksefileter (filetene blir rødere, mørkere og mindre gule), selv om stabiliteten til karotenoidene kun øker marginalt. Forbedret filetfarge skyldes høyst sannsynlig dannelse av nitrosomyoglobin. Laksefileter behandlet med nitritt (fint raffinert salt tilsatt 0,6 % natrium nitritt) hadde et relativt høyt innhold av resterende nitritt, uten at det ble funnet høyere verdier av *N*-nitrosoaminer.

Det er konkludert med at laks diagnostisert med PD under slakting, eller 6 måneder før slakting, viser redusert filetkvalitet. Laks infisert med SAV uten utbrudd av PD og laks diagnostisert med PD ett år før utbrudd, viser lik kvalitet som upåvirket fisk. Videre er det konkludert med at nitrittsalt gir bedre farge hos kaldrøkte laksefileter og tørrsalting reduserer stabiliteten til karotenoider sammenlignet med injeksjonssalting.

LIST OF PAPERS

I. Lerfall, J., Larsson, T., Birkeland, S., Taksdal, T., Dalgaard, P., Afanasyev, S., Bjerke, M.T., Mørkøre, T.
Effect of Pancreas disease (PD) on quality attributes of raw and smoked fillets of Atlantic salmon (*Salmo salar* L.).
Submitted manuscript

II. Larsson, T., Krasnov, A., Lerfall, J., Taksdal, T., Pedersen, M., Mørkøre, T.
Fillet quality and gene transcriptome profiling of heart tissue of Atlantic salmon with pancreas disease (PD)
Submitted manuscript

III. Lerfall, J., Østerlie, M.
Use of sodium nitrite in salt-curing of Atlantic salmon (*Salmo salar* L.) – Impact on product quality
Food Chemistry, 2011; 124: 759-766.

IV. Lerfall, J., Akse, L., Østerlie, M., Birkeland, S.
Salting method affects the retention of carotenoids in the fillet surface of cold-smoked Atlantic salmon (*Salmo salar* L.)
International Journal of Food Science and Technology, 2011; doi:10.1111/j.1365-2621.2011.02723.x

1 GENERAL INTRODUCTION

Commercial production of farmed Atlantic salmon (*Salmo salar* L.) started in small scale early in the 70ties and is today a high technological industry, based on knowledge obtained through decades of research and development on feed, production management throughout the life-cycle and processing and logistics of the final products. The Norwegian export of salmon fillets increased from 14% of the total amount exported in 2008 to 19% in 2010, whereas the total export of Norwegian Atlantic salmon increased in the same period from 0.76 to 0.95 million tonnes (EFF, 2010, 2011). The main determinant would be implementation of new technology and increased focus on pre rigor filleting of Atlantic salmon. This trend is expected to continue, due to increased focus on environmental (Winther et al., 2009) and economical benefits of exporting fillets compared to gutted whole Atlantic salmon.

The term "product quality" is wide and is frequently divided into five different subjects. Those subjects are; sensory, nutritional, microbiological, technological and ethical quality (Nortvedt et al., 2007). In this PhD-Thesis the main focus will be on the nutritional, sensory and technological qualities of raw and smoked salmon fillets, with specific focus on pigments and colour characteristics.

The fillet quality of farmed Atlantic salmon is generally acceptable, but deviations may occur. Currently, buyers of farmed salmon are concerned about insufficient and irregular fillet colour and soft texture. The underlying causes for such quality problems are often complex, but the impact of pancreas disease (PD) has been pointed out by the processing industry as an important cause for quality deviations of raw and smoked fillets. PD is a serious viral disease that causes large economic losses, mainly due to mortality and failure to thrive (Ruane et al., 2005). However, salmon that survive a PD outbreak may perform well and have normal exterior appearance. Hence fish from PD affected populations can be sold on the international market. However, the quality of raw and smoked fillets of salmon from PD affected populations has not been documented scientifically. The salting and smoking procedures applied are as well of significant importance for the appearance and other quality related characteristics of smoked salmon fillets. Therefore, to improve the quality of smoked fillets, it is important to understand the underlying factors influencing on the final smoked product, including the impact of salt composition and salting method and interaction with raw material properties.

2 AIMS OF THE STUDY

- Elucidate the impact of pancreas disease (PD) on quality related characteristics of raw and cold-smoked fillets of adult Atlantic salmon (*Salmo salar* L.), with particular focus on carotenoids, colour and texture of PD affected salmon from different populations diagnosed with PD from 0 to 12 months prior to harvest (**Paper I**). Moreover, the aim has been to study differences in fillet quality properties and gene expression profile of hearth tissue of Atlantic salmon within a population diagnosed with PD at time of slaughtering (**Paper II**).

- Examine the impact of nitrite salt curing on carotenoids, colour, salt uptake, residual nitrite and formation of unhealthy chemical compounds like *N*-nitrosoamines in cold-smoked Atlantic salmon (*Salmo salar* L.) (**Paper III**).

- Study carotenoid retention and colour in the fillet surface layer throughout processing and 14 days vacuum storage of cold-smoked Atlantic salmon (*Salmo salar* L.) using dry or injection salting and raw material of different rigor status at time of salting (**Paper IV**).

3 THEORETICAL BACKGROUND

Farmed Atlantic salmon (*Salmo salar* L.) is an excellent raw material for the processing industry due to good availability and even quality. Today's generation of farmed Atlantic salmon is a descendant of wild Atlantic salmon collected in early 70ties (Gjedrem et al., 1991) and reared through decades with focus on growth, limited lipid deposition, intense fillet colour and resistance against diseases.

The quality of cold-smoked fillets of Atlantic salmon is complex, and affected both by raw material characteristics and processing conditions (Cardinal et al., 2001; Espe et al., 2001; Skjervold et al., 2001a,b; Espe et al., 2002; Bjerkeng, 2004a; Rørå et al. 2004a; Birkeland and Bjerkeng, 2005; Hoel et al., 2007). Different markets have different criteria when it comes to quality preferences of salmon fillets (Torrissen et al., 1995). On smoked fillets, specific criteria regarding content of salt also exist. Due to this, producers have to customize specific products to each market to satisfy consumers world-wide.

3.1 The salmon muscle

The salmon muscle consists of two kinds, a white muscle with anaerobic metabolism and a dark aerobic muscle used for continuous swimming. The white muscle dominates and the dark muscle is located as a stripe just under the skin on both sides of the body running beneath the lateral line. Nutritional composition differs between white and dark muscle, where the dark muscle consists of more lipids and certain vitamins than the white muscle (Aursand et al., 1994; Murray and Burt, 2001).

Muscles in salmon are layered instead of bundled as in the other vertebrates (Figure 3.1). Each sheet of muscles, which consist of horizontally muscle fibres, is called a myotome and is separated from each other by a sheet of connective tissue (Junqueira et al., 1989). This connective tissue consists of collagen, elastine, reticulin and blood vessels, and is called myosepta. Collagen, which is a left-handed helix evolved to provide strength, is stabilised by covalent cross-links and a high content of hydroxyproline (HYP) (Nelson and Cox, 2005). Connective tissue is also located along the vertical midline of the body separating the left and right parts and along the lateral line separating the dorsal and abdomen muscles.

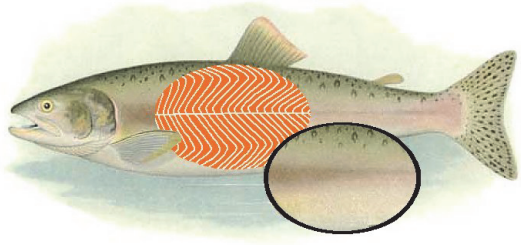

Figure 3.1 Salmon muscle structure

http://www.earthlife.net/fish/muscles.html - last access 30.06.2010

The myotoms are folded into a 3D-shape and are angled against the line of the body, with the innermost edge nearer the front of the body. Each myotome consist of myofibrils located horizontally, witch range from myosepta to myosepta. These myofibrils consist of at least four main proteins, which compose the striated filaments: actin, tropomyosin, troponin, and myosin. Together with actin and myosin (55% of total protein), these striated filaments include α-actinin and desmin, which are believed to tie adjacent sarcomeres together (Junqueira et al., 1989).

Atlantic salmon may be classified among the fatty fish species, where the proximate composition of slaughter size salmon flesh is water (60-65%), followed by protein (18-20%) and lipids, 16-19% (Shearer et al., 1994; Einen et al., 1998; Mørkøre and Rørvik, 2001). In general, the content of lipids varies individually and between seasons (Shearer, 1994; Shearer et al., 1994; Mørkøre and Rørvik, 2001) and is strongly related to the content of water and vice versa (Shearer, 1994; Katikou, et al., 2001; Quinton et al., 2005). Moreover, the distribution of lipids within salmon fillets is varying, where the anterior and abdomen part of the fillet consists of more lipids as compared to the posterior and dorsal parts (Aursand et al., 1994; Einen et al., 1998; Katikou, et al., 2001). The protein content in healthy slaughter sized Atlantic salmon is relatively stable (Shearer et al., 1994). In addition, together with glycogen, < 0.2% (Einen and Thomassen, 1998), the salmon muscle contains of certain amount of important vitamins such as vitamin A and E.

3.1.1 Lipids

Fish lipids occur as two main groups (Love, 1997). The first group, consists of triacylglycerols (TAG), and is the main source of stored energy in the fish. In salmon, these lipids are mostly stored in the myosepta between the myotoms and under the skin (Zhou et al., 1995). The second group, mostly consisting of phospholipids (PL) and cholesterol, are

essential components of the cell walls, mitochondria and other sub-cellular structures. Lipids in fish tissue are important for the fish quality in different ways (Love, 1997). Fish lipids influence on the mouth feeling and are notably beneficial to the consumer's health, because of high concentrations of highly unsaturated (*n*-3) fatty acids like *all-cis* -eicosa-5,8,11,14,17-pentenoic acid (EPA) and *all-cis*-docosa-4,7,10,13,16,19-hexa-enoic acid (DHA) (Sergent et al., 2002). Highly unsaturated fatty acids like EPA and DHA are normally easily oxidised when exposed to air, light and high temperatures. However, in salmonids, EPA and DHA are probably protected against oxidising by antioxidants like astaxanthin and vitamin E (Palozza and Krinsky, 1992a,b; Nakano et al., 1995; 1999; Østerlie, 2000).

3.1.2 Proteins

Fish proteins are grouped into three groups, based on solubility properties (Haard, 1992). These are water-soluble sarcoplasmic proteins (18-20%, mainly enzymes; myogen, myoalbumin, globulin), salt soluble myofibril proteins (65-80%, contractile network; actin, myosin, tropomyosin, troponin) and insoluble stroma proteins (3-5%, connective tissue; collagen, elastin, reticulum) (Hall and Ahmad, 1997). The distribution between groups of protein implies that the myofibril proteins are considerably important for the textural and water-holding properties of the fish muscle.

3.1.3 Pigments

The reddish colour of farmed Atlantic salmon is mainly due to the carotenoid, astaxanthin (3,3`-dihydroxy-β,β-carotene-4,4`dione), which are the most commonly used carotenoid for muscle pigmentation of salmonid fish (Skrede and Storebakken, 1986a,b; Christensen et al., 1995; Bjerkeng, 2000). In EU, canthaxanthin (β,β-carotene-4,4`dione) is allowed to be used as an additive in salmonid feed. However, in Norway it is forbidden due to possible formation of crystalline deposits in the human retina (Arden and Barker, 1991; Köpcke et al., 1995). Although, an European food safety authority (EFSA) panel (EFSA, 2010) have concluded that for both adults and children, total anticipated combined exposure to canthaxanthin from application as food and feed additive is unlikely to exceed the acceptable daily intake (ADI) of 0.30 mg kg bw^{-1} day^{-1}. Introduction of vegetable oils and alternative microbiological sources of carotenoids in the feed, has during the last years introduced traces of other carotenoids like lutein (3*R*,3`*R*,6`*R* -β,ε-carotene-3,3`diol), zeaxanthin (β,β-carotene-3,3'-diol), adonirubin (3,3`-dihydroxy-4-oxo-β,β-carotene) and β-carotene (*β,β*-carotene) in the salmon flesh. In production of organic salmon, natural sources of astaxanthin is required. Today, astaxanthin produced by the yeast *xanthophyllomyces dendrorhous* (previously *phaffia*

rhodozyma) and the bacteria *paracoccus carotinifaciens* (Panaferd-AX) are most commonly used.

Within the fish, astaxanthin is present in the muscle in its unesterified form (Henmi et al., 1987) where the geometrical all-*trans* isomer is predominating (>90% of total astaxanthin; Henmi et al., 1990a; Bjerkeng et al., 1997). Astaxanthin was believed to bind weakly to hydrophobic sites of actomyosin in salmon flesh (Henmi et al., 1987, 1989, 1990b, 1991). However, recent research has indicated astaxanthin to be associated to other muscle proteins as well as actomyosin (Birkeland and Bjerkeng, 2004; Saha et al., 2005) and it has been concluded that the major astaxanthin binding protein in salmon muscle is α-actinin (Matthews et al., 2006). The nutritional function of astaxanthin has not been clearly established, but it has been proposed that carotenoids might play a role as an antioxidant (Haila, 1999; Martin et al., 1999; Østerlie, 2000), and in salmonid growth and health (Christiansen et al., 1995). Antioxidant properties of astaxanthin is mainly due to physical quenching of singlet oxygen ($^1O_2$), where energy absorbed from $^1O_2$ to produce triplet oxygen ($^3O_2$) is converted to rotary and vibratory energy by the chromophore system of the carotenoid (Stahl and Sies, 1993). Furthermore, the accumulation of astaxanthin in the muscle may function as a depot for carotenoids needed at the time of spawning when the male develops a strong red colour in the skin and the female transport carotenoids into the eggs (Anderson, 2000; Bjerkeng, 2008). For proper development after fertilization the latter seems to depend heavily on the amount of carotenoids. It is clearly seen that the muscle colour of salmonids fades at the time of spawning (Bjerkeng et al., 1992). In addition, amounts of idoxanthin (3,3`,4`-trihydroxy-β,β-carotene-4-one), which is a metabolite of astaxanthin (Bjerkeng et al., 2000), is decreasing during growth (Schiedt et al., 1989).

In nature, more than 600 carotenoids exist (Britton et al., 1995), where 5-6 of them are relevant today in carotenoid analyses of salmon flesh. In addition to the wide variety of carotenoids in nature, carotenoids occur in different stereo isomers (*cis* and *trans*) and optical isomers (*R* and *S*). Extraction of carotenoids from muscle tissue is quite challenging. These components are rather labile and can easily decompose or be isomerised when exposed to high temperatures, light and chemicals, and be oxidised when exposed to oxygen or acids (Schiedt and Liaaen-Jensen, 1995). It is possible to increase the stability of carotenoids by adding an antioxidant or a piece of solid CO_2 into the sample (Schiedt and Liaaen-Jensen, 1995). Another critical point is the lipids which are extracted together with the carotenoids. To stabilise the samples before analysis it is possible to add Butylated Hydroxytoluene (BHT)

and/or rinse the samples on a solid phase extraction (SPE) column as described by Schierle and Frey (2005). In addition, to obtain good results it is important to protect the samples from heating and cover them with a dark cloth during the homogenisation and extraction procedures.

Chromatographic analyses of carotenoids are complex, and the choice of method relevant to a particular study depends on the information at hand. Astaxanthin is often analysed by a method after Vecchi et al. (1987) on a $H_3PO_4^-$ modified silica gel HPLC column (For example; Lichrosorb SI60-5, 125×4.0 mm, 5 µm, Hichrom, Reading, UK) and detected at 470 nm with hexane:acetone (86:14) as mobile phase (isocratic, flow 1.0 ml min^{-1}). This system separates *cis* and *trans* isomers of astaxanthin and other actual carotenoids like canthaxanthin, zeaxanthin and lutein. One of the limitations to this system is that it is difficult to detect and impossible to separate *cis* and *trans* isomers of idoxanthin. *Cis* and *trans* isomers of idoxanthin can be analysed by a method after Aas et al. (1997) using a nitrile HPLC column (for example; Luna CN 100A, 250×4.6 mm, 5 µm, Phenomenex®, USA) with hexane:acetone (80:20) as mobile phase (isocratic, flow 1.5 ml min^{-1}). In addition, total amounts of other carotenoids like astaxanthin and lutein can be quantified. A new method for homogenising, extracting and quantifying astaxanthin, canthaxanthin, lutein and zeaxanthin from fish flesh has been developed in 2010 by a scientific group put together by the Norwegian Seafood Federation, FHL (not published, Marianne Østerlie, personal communication). This method is based on Schierle and Frey (2005) and Vecchi et al. (1987) and may present itself as valid in future analyses.

For specific information on stereoisomerism (except for astaxanthin) and optical isomerism, methods adapted to the specific topic of exploration must be chosen.

3.1.4 Vitamins

Salmon flesh contains several vitamins, which are of nutritional importance to the salmon itself and for humans. Vitamin A has at least two main functions and is important for normal growth and health. First, vitamin A functions as a signalling molecule of many cellular pathways, which include regulation of many gene transcriptions. Second, vitamin A has important functions in the human eye, where 11-*cis*-retinal functions directly in the vision by undergoing photoisomerization and to obtain normal morphology and function of the various cells in the cornea (Ross, 1999). Carotenoids, which have at least one unsubstituted β-end group function as pro-vitamin A carotenoids in humans (Britton, 2008). Astaxanthin belong to the xanthophylls and have two substituted β-end groups and can not directly function as pro-

vitamin A in humans. On the other hand, salmonids can convert astaxanthin and other xanthophylls to vitamin A when the fish has insufficient vitamin A status (Schiedt, 1998). Another important vitamin found in salmonids, is Vitamin E, which is a group of molecules, consisting of four tochoperols (α, β, γ, δ) and four tochotrienols (α, β, γ, δ). α-Tocopherol is the most abundant form in nature, which has the highest biological activity based on fetal resorption assays, and reverse vitamin E deficiency symptoms in humans (cf. Brigelius-Flohe and Traber, 1999). Vitamin E mainly functions as radical scavenging antioxidants in both hydrophilic and hydrophobic environments and is preventive against diseases caused by oxidative stress (Cynchi et al., 1995; Huang et al., 1995). In addition, it is reported that β-carotene and vitamin E show a synergistic antioxidative action (Palozza and Krinsky, 1992a), whereas this action was not observed with astaxanthin and canthaxanthin (Palozza and Krinsky, 1992b).

3.2 Biological variations

Among commercial farmed Atlantic salmon there are biological variations. Several studies have shown effects of genetic variations in feed intake, growth and feed utilization, meat quality, disease resistance and age at sexual maturation (Gjerde et al. 1994; Thodesen et al. 2001a,b; Kolstad et al., 2004; 2005; Vieira, et al., 2007; Wetten et al., 2007; Powell et al., 2008). Other important factors witch influences on the biological variation are; seasonal variations (Shearer, 1994; Shearer et al., 1994; Mørkøre and Rørvik, 2001) and geographic and local environments such as temperature, light, water quality and local current conditions (Brett, 1979; Austreng et al., 1987; Saunders and Harmon, 1988; Smith et al., 1993; cf. Shearer, 1994; Boef and Le Bail, 1999; Poli, 2009). Regarding geographical variations in Norway, there is a north-south gradient caused by differences in light and water temperature. Effects of feed on biological variations in commercial farming are relatively small in comparison due to similarity of feed managements amongst the farmers (Einen and Roem, 1997). Inclusion level of plant oils in salmon feeds vary according to price and availability. Hence, there may be significant variations in fatty acid (FA) composition of salmon fillets as the FA composition in the fish is reflected by the FA composition of the feed (Torstensen et al., 2005). In addition, diseases may reduce the flesh quality of Atlantic salmon (Whitaker et al., 1991; Moran et al., 1999; Dawson-Coates et al., 2003; Hoel et al., 2007).

3.2.1 Pancreas disease (PD)

Today, one of the most serious diseases of Atlantic salmon is Pancreas disease (PD). In Norway, PD is caused by the salmonid alphavirus subtype 3 (SAV 3) that is related to the

salmonid alphavirus subtype 1 (SAV 1) which causes PD in Ireland and Scotland (McLoughlin and Graham, 2007). PD is notifiable to the Norwegian Fish Health Service. The disease behaviour of infected populations is developing differently and the mortality in affected pens is reported by Jansen et al. (2010) to vary between 1-27%. Failure to thrive is a further consequence of the disease, resulting in poor condition and thin fish that are susceptible to parasitism and secondary bacterial diseases (Ruane et al., 2005). Stress plays a key role in the development of PD, with several examples of PD occurring after handling fish (Raynard et al., 1992; Brun et al., 2006), and associations between amounts of salmon lice (*L. salmonis*) burden and outbreaks of PD has also been registered (Ruane et al., 2005).

Hoel et al. (2007) reported that quality downgrading was contributing to the economical losses upon PD outbreaks, and anecdotal information indicates that PD may cause poor general muscle quality associated with severe discoloration (Bjerkeng, 2004a; Hoel et al., 2007). There is, however need for more objective information on the impact of PD on fillet quality of salmon, besides from observations of gray shadows of melanin on fillets after recurrent PD outbreaks (McLoughlin, 2005). Economic losses due to PD have been estimated to reach approximately one billion NOK per year (Torrissen, 2008), and according to Aunsmo et al. (2010), a single PD outbreak on a fish farm with 500.000 smolts can results in a total loss of 14.4 million NOK.

3.3 Fillet processing of Atlantic salmon

Fillet processing is in this thesis related to filleting of gutted fish, and salting, drying and smoking of salmon fillets. Fillet yield is defined as the ratio of the trimmed fillets (without bones and head) to the gutted body weight multiplied with 100%. The fillet yield is a function of both size and condition factor, where large fish with high condition factor (≥1.3) could give yields up to 69% (Mørkøre et al., 2001; Rørå et al., 2001). The smoking yield is the ratio of raw fillet to the smoked fillet weight multiplied with 100%, where the smoking yield is strongly affected by the salting, drying and smoking methods applied (Rørå et al., 1998; Cardinal et al., 2001; Birkeland et al., 2003; Birkeland et al., 2004a). In addition, both filleting and smoking yields are of grate economical importance for the processing industry.

3.3.1 Filleting

Filleting of Atlantic salmon has traditionally been performed 3-5 days post mortem in order to avoid handling and processing of the fish in an in-rigor state (Skjervold et al., 2001a, 2002; Birkeland et al., 2007). Rigor mortis is observed when the glycolysis stops to produce

Adenosin triphosphate (ATP) (FAO, 2005). The rigor process starts with an initial contraction phase (Tornberg et al., 2000), followed by a second stiff phase due to permanent cross-bridges between the contractile proteins myosin and actin (Kiessling et al., 2006). New improved slaughtering procedures and knowledge about how temperature, starvation and stress affect the onset of rigor mortis (Bell et al., 1991; Roth et al., 2002; Skjervold, 2002; Kiessling et al., 2004a; Kiessling et al., 2006; Mørkøre et al., 2008; Roth et al., 2009), have together with improved filleting technology emerged a potential of pre rigor filleting of Atlantic salmon. Today, an increased part of the processing industry in Norway is changing from post to pre rigor filleting. Pre rigor filleting give rise to economical benefits due to increased processing of salmon in Norway, improved logistics at the processing plant and lowered costs related to transportation of head, bones and excess of ice (Kiessling et al., 2007; Mørkøre et al., 2008). No differences regarding yield have been reported between pre and post rigor filleting technologies (Skjervold et al., 2001a). Furthermore, pre rigor fillets are ticker, firmer, have less gaping and show improved colour as compared to post rigor fillets (Skjervold et al., 2001a,b; Einen et al., 2002; Skjervold, 2002). The improved quality of pre rigor fillets could be associated with the absence of the normal 3-5 days of cold storage before filleting and that the muscle fibres are allowed to contract freely from the vertebrae (Einen et al., 2002). Moreover, pre rigor fillets reach the market in a fresher state. Pre rigor filleting gives the industry opportunities to handle super fresh by-products, which could be used in production of high quality *n*-3 oils and different hydrolysed products used for human applications (RUBIN, 2007). At last, environmental benefits are obtained due to decreased emission of carbon dioxide (CO_2) related to less transportation of ice and by-products (Kiessling et al., 2007).

3.3.2 Salting

Traditionally, salting of fish was performed to lowering water activity and extending the shelf-life (Horner, 1997). Nowadays cold-smoked Atlantic salmon appears as a lightly preserved product with sodium chloride (NaCl) content in the range of 2.0-3.9% in the water phase (Truelstrup Hansen et al., 1995, 1996, 1998; Bannerman and Horne, 2001; Huang et al., 2002). Salt is usually added to the fillet by dry salting, brine salting or injection salting (Birkeland and Bjerkeng, 2004). Dry salting leads to a salting-out process due to loss of water (Horner, 1997; Gallart-Jornet et al., 2007), while brine salting will results in a salting-inn process when NaCl concentrations lower than 20% is used (Thorarinsdottir et al., 2004; Gallart-Journet et al., 2007). Both dry salting and brine salting are driven by diffusion to

approach equilibrium in which the driving force is presented as differences between the ambient and the fish brine concentration (Dyer, 1942; Wang et al., 2000). Another minor driving force is pressure gradients, which promote the bulk transport of solution (water and solutes) by hydrodynamic mechanisms (Barat et al., 2003). Basic studies by Sen and Aitken (1965) demonstrated that the phenomenon of salt penetration in the fish muscle follows the ordinary Fick`s law (cf. Cussler, 1997). A number of mathematical models based on Fick`s law have been made to explain and calculate salt diffusion in tissue of fish in general (Zugarramurdi and Lupin, 1980) and in Atlantic salmon (Wang et al., 1998a, 2000).

Several factors affect the diffusivity of salt in fish tissue. Skin, scales and hypodermic fat works as barriers to diffusion of water and solutes (Zugarramurdi and Lupin, 1980), while high contents of lipids incorporated in muscle tissue will decrease salt diffusion because of diffusion barriers made by the lipids (Wang et al., 2000; Gallart-Jornet et al., 2007). The main content of lipids in salmon muscle is stored in the connective tissue (myosepta) instead of in the muscle fibres (Zhou et al., 1995; Nanton et al., 2007). Therefore, lipids will not decrease the diffusivity as much as expected in salmon fillets where the orientation of the myosepta is in the same direction as the main diffusion fluxes (Wang et al., 2000). Rigor mortis is another important factor that affects the salt diffusivity. Lower salt diffusion and distribution has been observed in pre rigor as compared to in- and post rigor fillets (Wang et al., 1998b, 2000; Rørå et al., 2004a). Wang et al. (2000) explained this phenomenon with the intact muscle structure in those fillets and that significant amount of ATP was still present in the muscle tissue. The ATP-driven ionic pumps are able to maintain concentration gradients across membranes; which might lead to higher resistant to salt diffusion and distribution (Wang et al., 2000). For salting pre rigor fillets, injection salting is found to be suitable fitted salting method gaining salt concentrations between 1.7-6.1% depending on the salting parameters used (Birkeland et al., 2007; Akse et al., 2008).

To obtain satisfactory fillet quality, fine refined salt with minimum 99.8% NaCl is normally used in curing of Atlantic salmon. Other types of salt like solar and rock salt have varying purity and higher content of minerals such as Calcium (Ca^{2+}), Magnesium (Mg^{2+}) and traces of Copper (Cu^{2+}) and Iron (Fe^{2+} and Fe^{3+}). Such ions might work as prooxidants and accelerate the rancidity of lipids (Akse, 1995; Lauritzsen, 2004). Furthermore, Ca^{2+} and Mg^{2+} bind more readily to negative sites of the protein as compared to Na^{+} (Horner, 1997), which are resulting in muscle swelling and reduced NaCl diffusivity. Salt noticeably affect the water binding by proteins due to electrostatic interactions (Damodaran and Kinsella, 1982). The

effects of salts vary with the cationic and anionic species involved related to the hydrated radii of that specific ion (Hofmeister series) (cf. Lauritzsen, 2004) or the polarising power (charge divided by ion radius) of the hydrated ion (Reid and Fennema, 2008). The cations (Ca^{2+}>Mg^{2+}>Na^{+}) tend to enhance the solubility of proteins when added together with Cl^{-}, while, with Na^{+}, the anions (NO_3^{-} >Cl^{-}) have a similar effect (cf. Lauritzsen, 2004). The effect of the anions on water-holding capacity and diffusivity is most prominent at high salt concentrations (0.1-1 M) whereas the cations contribute most at lower salt concentrations (<0.1 M) (Sarkar, 1950). Nitrite salt (nutrient quality with 0.6% sodium nitrite, $NaNO_3$) is commonly used in meat curing and is allowed in curing of salmonids in the USA (CFR, 2009). When added to meat, nitrite salt has several functions. Nitrite gives the products a stable red colour (nitrosomyoglobin), act as an antioxidant and give increased growth of lactic acid bacterial (Nilsson et al., 1999) and prevent growth of the spoilage- and pathogenic bacterial flora (Woods et al., 1990; Pelroy et al., 1994; Honikel, 2008). Nitrite is unstable at acidic pH and can disproportionate into nitrous acid, which again decomposes to nitrous oxide (NO). NO might react with secondary amines to form volatile *N*-nitrosoamines (Yurchenko and Mölder, 2006; Reinik et al., 2008). However, the low content of trimethylamine *N*-oxide (TMAO) in farmed Atlantic salmon and the fact that salmonid fishes have relatively low contents of secondary amines (Belatti and Parolari, 1982) indicates that residual nitrite in the smoked fillet might be the most serious health consideration due to nitrite-curing of Atlantic salmon.

A high intake of nitrite by humans is related to many health problems such as methemoglobinemia, cancer, cardiac and vascular diseases, circulatory shock, inflammatory etc. (Pacher et al., 2007). Therefore, in EU and Norway, the use of nitrite salt in salt-curing of fish is forbidden. However, due to the positive benefits of nitrite salt against pathogenic bacterial and the zero-level tolerance of *Listeria monocytogenes*, USA have not prohibited nitrite salt-curing of salmonids (CFR, 2009). The complex chemistry of nitrite, nitric oxide and related compounds makes it difficult to establish a level associated with health risk (Pacher et al., 2007). However, acceptable daily intake (ADI) given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and European Commission's Scientific Committee for Food (SCF) for a 70-kg person is set to be 4.9 mg day^{-1}. Environmental Protection Agency is more liberal and has set ADI for a 70-kg person to be 7 mg kg^{-1} body weight (Erkekoglu et al., 2009).

3.3.3 Drying and smoking

Cold-smoked Atlantic salmon is a lightly preserved product with water activity (A_w) between 0.93-0.98 (Shimasaki, et al., 1994; Alzamora et al., 2003), so the effect of reduced bacterial growth is minor. A broad range of wood chippings used for smoke generation is found on the market. Normally, chips from hardwoods like oak, hickory, cherry, apple and beech are used, which give pleasant taste, colour and aroma to the product (Horner, 1997). Therefore, substantial variations occur in smoking parameters between different smokehouses, and often each smokehouse has their own in-house receipt (Bjerkeng, 2004a,b). This is reflected by the high variation in quality of smoked salmon products available on the market (Cardinal et al., 2004; Espe et al., 2004a). Furthermore, different smoking parameters is found to affect the product quality (Cardinal et al., 2001; Espe et al., 2002; Birkeland et al., 2004a,b; Rørå et al., 2005).

The smoke generated in a smoking cabinet consist of more than 400 components, which contains acids, alcohols, carbonyls, esters, furans, lactones, phenols and other components (Maga, 1987). These components distribute in an emulsion of droplets in a continuous phase of air and vapours stabilised by electronic charges on the droplets (Horner, 1997). For flavouring, colouring and microbiological purposes, the vapours are of greatest importance in smoking. Modern technology has raised the opportunity to combine drying and smoking in different pulses to optimise absorption of both hydrophilic and hydrophobic components from the smoke. A dehydrated fillet surface favours absorption of hydrophobic components with good taste from the smoke and avoids absorption of acetic acid and other short-chained organic acids, which would give the product a sour taste (Lynum, 2005). It is generally considered that phenols play an important role in the desirable characteristic flavour of smoked fish (Horner, 1997). Colour of fillets is changing during smoking. Increased yellowness and decreased redness are observed, which could be explained by colour imparted from the smoke due to carbonyl-amino reactions of the Maillard type (Horner, 1997) and structural changes on the fillet surface. Birkeland (2004) reported that depigmentation due to decomposition of carotenoids during the smoking step has little influence on colour changes caused by smoking. In salmon, Maillard browning predominates at high A_w, and protein-lipid browning is the major reaction mechanism at low A_w (Sikorski et al., 1998). The main chemical effect on bacteria is generally attributed to the combined action of formaldehyde and phenolic components in the vapour phase of the smoke (Doe et al., 1998). Phenolic components also function as antioxidants and influencing on the chemical stability and the

shelf-life of smoked products. Cold-smoking of Atlantic salmon is performed in the temperature range 20-30 °C to avoid heat coagulation of muscle proteins and altered sensory characteristics.

The choice of temperature in the smoke generator affects the components of the smoke. FAO/WHO (1983) recommended that the temperature in the smoke-generator should be between 250 °C and 350 °C to avoid production of carcinogenic components like polycyclic aromatic hydrocarbons (PAH).

3.4 Fillet quality

3.4.1 Colour and appearance

Colour is a key attribute of food items (Francis, 1995) and an important decision maker for consumers when purchasing raw (Anderson, 2000) and smoked salmon products (Gormley, 1992; Rørå et al., 2004b). It is not only the colour intensity which is important, but colour evenness of the whole fillet is also of great importance (Bjerkeng, 2004a,b). The colour of salmon fillets is mainly due to the carotenoid concentration in the muscle tissue (Skrede and Storebakken, 1986a,b; cf. Bjerkeng, 2000). However, Atlantic salmon is not capable to endogenously synthesise carotenoids; therefore, it must be supplemented in the fish's ration (Anderson, 2000). Today, the normal range of astaxanthin in commercial feed is between 20-50 mg kg^{-1} for growing Atlantic salmon in the seawater phase. When astaxanthin concentrations in salmon muscle exceed 6-8 ppm, the human eye becomes saturated leading to inaccurate perceptions of colour (Foss et al., 1984, 1987). This leads to a plateau in perception even with an increase in pigment concentration (Johnsen and Wathne, 1990; Bjerkeng, 2000). Colour of salmon flesh is affected by many different parameters, among others; composition and amounts of carotenoids in the feed (Bjerkeng, 2000; cf. Bjerkeng, 2008), genetic background (Torrissen and Naevdal, 1988), seasonal variations (Mørkøre and Rørvik, 2001), starvation and stress prior to slaughtering (Einen and Thomassen, 1998; Erikson and Misimi, 2008; Mørkøre et al., 2008), slaughtering procedures (Kiessling et al., 2004a; Roth et al., 2010), ice chilling and temperature during frozen storage (Espe et al., 2004b), muscle fibre density (Johnston et al., 2000), and salting and smoking procedures (Cardinal et al., 2001; Birkeland et al., 2004a). The colour of the flesh is also affected by the health status of the fish where anecdotal information indicates that PD may cause severe discolorations (Bjerkeng, 2004a).

Colorimetric analyses are in the later years improved with introducing of digital image analysis to assess colour characteristics of salmon fillets (Stien et al., 2006; Folkestad et al., 2008). Benefits of digital imaging against traditionally methods such as Minolta Chroma meter are the possibility of multi-point measurements, where average colorimetric values of a defined area are used to describe the colour. In addition, it is easy to predict colour variations and evenness on the fillet surface with analysis of coefficient of variance (CV) between different defined areas of the fillet surface. Limitations of a Minolta Chroma meter are that only single-point measurements are possible, where the colour intensity could be influenced by the fat layer and the myosepta between the myotoms as well as the red colour of the myotoms. In addition, colorimetric variations and evenness on the fillet surface, as equally difficult to predict.

Dark and brown spots in the belly, organs or in the fillets of Atlantic salmon are normally classified as melanin spots. These spots occur in 10-15% of farmed Atlantic salmon fillets and causes downgrading of quality (Mørkøre, 2008). Melanin spots in fillets have been connected to vaccination (Midtlyng, 1997; Koppang et al., 2005; Gelderen et al., 2009), but occur also in unvaccinated fish which means that other mechanisms as well are reasonable. Melanin spots are often found in connection with inflammatory responses and Gelderen et al. (2009) found a link between infections and increased content of melanin in the flesh of Atlantic salmon.

3.4.2 Texture

Firmness is a critical parameter that determines the acceptability of the seafood product (Veland and Torrissen, 1999), where too soft flesh leads to reduced acceptability by the consumers (Hatae et al., 1985; Ando, 1999; Veland and Torrissen, 1999). Textural properties such as shear force, firmness and breaking strength of smoked Atlantic salmon fillets are inversely related to the water content (Indrasena et al., 2000; Jittinandana et al., 2002) and amounts of myofibril-to-myofibril attachments (Tayler et al., 2002). Moreover, fillet firmness depends on the number of pyridinoline (PYD) cross-links (Johnston et al., 2006) and HYP is found related to firmer texture of fish muscle (Hatae et al., 1986; Sato et al., 1986). The content of HYP is today used as an indicator of the amount collagen present in muscle tissue. To understand the complexity of factors influencing textural properties, it is important to understand the impact of pre and post mortem handling of fish. Factors that affect textural properties of fish are seasonal variations (Mørkøre and Rørvik, 2001), starvation and stress prior to slaughtering (Mørkøre et al., 2008), slaughtering procedures (Kiessling et al., 2004a), ice chilling and temperature during frozen storage (Espe et al., 2004b; Hultmann and Rustad,

2004), muscle fibre density (Hatae et al., 1984; Johnston et al., 2000), and salting and smoking procedures (Birkeland et al., 2004a).

3.4.3 Gaping

Gaping is ruptures in the salmon fillets and depends on the strength, composition and amounts of connective tissue between the myotome filaments (Kiessling et al., 2004a). Bjørnevik et al. (2004) suggested a connection between collagen composition and gaping, which was confirmed by Espe et al. (2004b) who found a plausible effect of collagen composition on gaping, considering that ruptures in the collagen matrix is an underlying cause. Furthermore, gaping increases in the summer month's simultaneously as pH falls (Lavèty et al., 1988).

Gaping cause's severe problems to the processing industry, and leads to downgrading of salmon fillets (Mitchie, 2001), and decreased storage stability caused by increased surface area. Gaping increases during ice-storage (Espe et al., 2004b), and a study by Skjervold et al. (2001a) showed less gaping of pre rigor as compared to post rigor fillets.

3.4.4 Drip loss

The main drip loss of raw salmon is water, but some loss of lipids, proteins and carotenoids may as well happen under storage of smoked fillets. The liquid-holding capacity in flesh is due to swelling and shrinking of myofibrils which is influenced by both pH and the ionic strength (Hamm, 1961; Offer and Trinick, 1983; Ofstad et al., 1995). Drip loss of raw salmon is affected by factors such as starvation (Mørkøre et al., 2008) and stress prior to slaughtering (Roth et al., 2006). During production of cold-smoked salmon fillets, salting influences the ionic strength of the muscle and thus affects the drip loss (Ofstad et al., 1995).

3.5 Molecular profiling of Atlantic salmon

Molecular profiling of salmon tissues is a new interesting tool which in the future probably can be used to predict and explain quality differences and abnormality within salmon species. Molecular profiling of Atlantic salmon can be performed on mRNA isolated from different tissues using the PureLink RNA Mini Kit (Invitrogen Corporation, Carlsbad, CA). Furthermore, the individual level of gene expression might be analysed by the two-colour, 4 × 44000 DNA MicroArray system from Agilent (Agilent Technologies, Santa Clara, CA), developed for Atlantic salmon by Krasnov et al. (2010). Using molecular profiling to explain quality differences in fish flesh may provide novel knowledge, and bring new insights regarding underlying factors determining fillet quality variations.

4 MAIN RESULTS AND DISCUSSION

4.1 Fillet composition

The fillet composition of adult Atlantic salmon is affected by the nutritional and health status of the fish (Paper I and II). In addition, substantial variation exists between individuals within the same population (Paper II).

The protein content was significantly lower of all pancreas disease (PD) affected fish groups as compared to the control group, except for the group infected with salmonid alphavirus (SAV) without a PD outbreak (Figure 4.1A). In addition, a higher variation between individuals was observed in PD affected groups as compared to the control group and salmon infected with SAV without a PD outbreak. Significantly lowest protein content was found in fish from a population diagnosed with PD at slaughter (Paper I). In this population, large variation in nutritional and health status between the individuals was observed (Paper II). Within this population (Figure 4.1B), salmon with severe loss of pancreas (PD (+)) differed from those infected with SAV only (SAV (+)), by having a significantly lower protein content in the fillet (16% versus 20%), coinciding with a thinner body shape (low condition factor, CF 0.6 versus 1.0). Severe protein depletion of group PD (+) is comparable with decreased protein content (up to 60%) in feed deprived Pacific salmon (*Oncorhynchus nerka*) during spawning migration were the muscle proteins most likely were used as fuel (Kiessling et al., 2004b). In addition to lower content of protein in salmon with severe loss of pancreas (Paper II), different amino acid profile was observed. Shortage in fuel supply and the need to reduce energy expenditure probably explain reduced levels of essential amino acids; cysteine, aspartic acid, threonine, valine, isoleucine, phenylalanine and histidine in white muscle, along with the suppression of amino acid metabolism in heart (determined by transcriptome analyses). Higher content of hydroxyproline (HYP) reflects higher content of collagen (scar tissue).

The protein content in healthy adult salmon is relatively stable, while the lipids and water (80%) may show considerable variations (Shearer et al., 1994; Einen and Roem, 1997). Hence, the lower protein content in PD affected salmon is considered to be a primary or secondary consequence of nutritional disturbances induced by pancreatic insufficiency.

Significantly higher contents of water were observed in fish diagnosed with PD at slaughter as compared to the control group (Paper I).

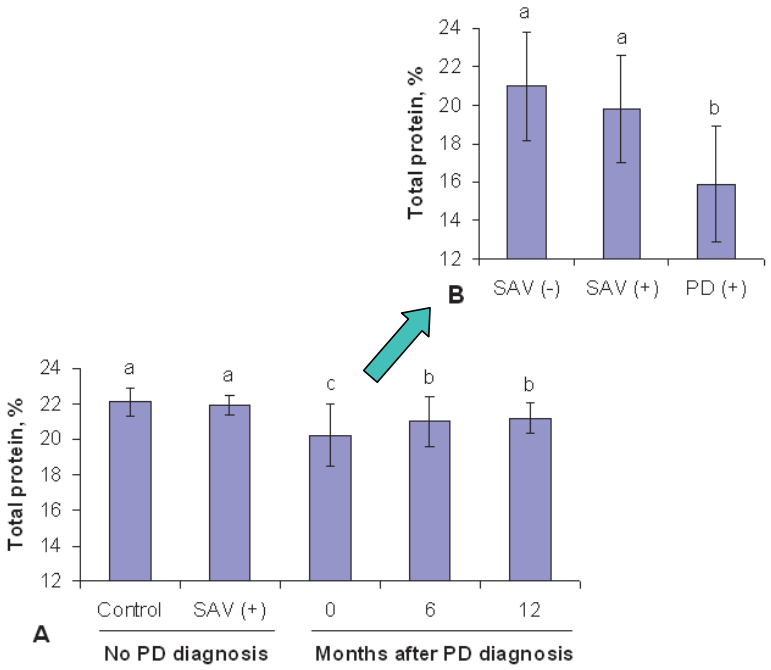

Figure 4.1 A: Protein contents in groups of Atlantic salmon harvested 0 (n=19), six (n=50) and 12 months (n=65) after PD diagnosis as compared to unaffected salmon (Control, n=60) and a group infected with salmonid alphavirus (SAV, n=30) without a PD outbreak. **B:** Average protein contents in groups of individuals with similar health status within the population diagnosed with PD at slaughter. SAV(-): SAV negative (n=4), SAV(+): SAV positive without a PD outbreak (n=4) and PD(+): SAV positive with severe loss of pancreas (n=4). All results are shown as mean ± SD and different small letters indicate significant variation (P = 0.05) between groups.

The content of lipids (7.7-9.5%) did not vary significantly between the salmon groups (Paper I). However, the quantitative amount of lipids in fish with a low condition factor is lower due to the reduced muscle mass. Atlantic salmon is considered as a fat fish species where the lipid content ranges from 8-24% with an average of 17% (Mørkøre et al., 2001). The distribution of lipids within salmon fillets is varying (Aursand et al., 1994; Einen et al., 1998; Katikou et al., 2001; Nanton et al., 2007). In this study with PD affected salmon, the fat content was analysed in the centre of the dorsal cutlet just anterior to the dorsal fin (Paper I). This part of the fillet is relatively lean compared with other parts. Hence it is possible that the tendency to

lower fat content in salmon diagnosed with PD at slaughter (Paper I) would have been clearer if the fat content was analysed in fattier parts of the fillet. However, higher amounts of DHA (22:6 *n*-3) and Arachidonic acid (C20:4 *n*-6) of the PL fraction of salmon with severe loss of pancreas (Paper II) indicate selective preservation of important membrane lipids.

Higher pH of PD affected salmon as compared with unaffected salmon indicates decreased glycogen stores in the living fish upon PD outbreaks, even after one year (Paper I and II). The muscle pH is normally inversely related to the drip loss (Ofstad et al., 1995). However, higher drip loss observed in salmon diagnosed with SAV without a PD outbreak (SAV) and in a population with recurrent PD outbreaks (PDchronic) (5.8-5.8%) as compared to the other groups (range 4.2-4.8%) indicates that drip loss is related to other factors than pH *per se*. For example PD pathology, that differs between SAV and PDchronic.

4.2 Salt

The salt (NaCl) uptake in Atlantic salmon fillets was affected by the nutritional and health status (Paper I), rigor status at point of salting (Paper IV), salting procedure (Paper III and IV) and duration and salt composition (Paper III). The average salt content of the cold-smoked fillets in the different studies ranged from 1.5 - 4.7% (Paper I, II, III and IV).

Salmon from populations which were diagnosed with PD six months prior to slaughter or salmon from a population with recurrent PD outbreaks showed higher salt uptake as compared to unaffected fish, although the differences were relatively small (0.8-1.0 percentage points) (Paper I). Salt uptake in salmon fillets is normally affected by the lipid and water content (Jason, 1965; Horner, 1997). However, in Paper I, it was not possible to explain higher salt uptake with higher contents of water or lipids. Hence, higher salt uptake may be related to a higher fillet surface relative to the fillet thickness in fish with a low CF.

In paper IV, the salt content in the surface layer of salmon fillets (5mm) is reported, however the fillet salt content was as well measured. Dry salted pre rigor fillets contained significantly lower salt content after smoking (1.5 ± 0.3%) as compared to dry salted post rigor fillets (2.6 ± 0.5%) (Results not reported in paper IV), as also reported by (Rørå et al., 2004a). However, for injected fillets, no significant difference was observed between pre and post rigor fillets (2.4-2.6%). Therefore, salt injection is a fitted salting method of pre rigor salmon fillets which was also recommended by Birkeland et al. (2007) and Akse et al. (2008).

Injection salting resulted in lower salt content in the fillet surface layer as compared to dry salting, in particular for fillets processed in the pre rigor state (Paper IV). After smoking, the

salt content in the fillet surface layer continued to be significantly different between the groups, except from pre and post rigor injection salted fillets (Paper IV). However, after 14 days storage, the surface salt content reflected the salt content of the whole filet (not reported in paper IV). This indicated a diffusion of salt from the surface layer to interior parts of the fillet (Dyer, 1942; Sen and Aitken, 1965; Wang et al., 1998a, 2000). Salt uptake by injection is mainly driven by mechanical forces, explaining lower and more equal salt content in the surface layer after salting and after smoking of injected as compared to dry salted fillets.

It is generally accepted that diffusion of salt throughout the muscle structure is a time consuming process (Lautenschlager, 1985) explaining more similar salt content between the protocols after 14 days storage as compared to after the smoking step. In addition, the water content was significantly lower in the fillet surface of dry salted as compared to injection salted cold-smoked fillets. This variation pattern was preserved through 14 days storage. Loss of solutes from the fillet surface during processing is caused by different mechanisms related to that specific salting method applied. Dry salting caused loss of solutes during processing and storage due to salting-out effects (Gallart-Jornet et al., 2007) whereas decreased water content of injected fillets after smoking and storage is caused by physical ablution of solutes from the fillet surface.

In paper III, salmon fillets were dry salted with fine refined salt (minimum 99.8% NaCl) and nitrite salt (NaCl, nutrient quality with 0.6% sodium nitrite). Fillets salted with nitrite or fine refined salt showed no significant differences in salt uptake after 15 (on average 2.8%) or 35 (on average 3.5%) hours salting. However, after 60 hours salting, significant higher salt uptake was found in nitrite salted fillets as compared to fillets salted with fine refined salt (4.4% versus 3.5%). Increased diffusion of NaCl in nitrite salted fillets might be explained by faster diffusion of $NaNO_2$ into the matrix (Hofmeister series, cf. Lauritzsen, 2004), followed by more rapid protein denaturation and higher degradation of the cell structure.

As a consequence of nitrite salting, relatively high contents of residual nitrite were observed in cold-smoked fillets (averaged 78.0 mg kg^{-1}) as compared to fillets salted with fine refined salt (averaged 1.2 mg kg^{-1}) (Paper III). Plausible adverse effects of nitrite salting of salmon fillets are considerations about increased contents of residual nitrite and formation of *N*-nitrosoamines in the cold-smoked product. However, no increased amounts of *N*-nitrosoamines were found when nitrite salting was applied although the content of residual nitrite was relatively high (cf. Erkekoglu et al., 2009). Hence, the most prominent adverse

effect of nitrite salting of Atlantic salmon is due to health considerations about high intake of nitrite (reviewed by Pacher et al., 2007).

4.3 Carotenoids and fillet appearance

Pigment feeding is regarded as the most important management practice for marketing of farmed salmon (Moe, 1990), but differences in colour intensity between fillets may be related to additional factors such the health status of the fish (Paper I and II), state of rigor mortis (Paper IV) and processing conditions (Paper III and IV).

Because of the lack of scientific documentation of the impact of PD on fillet quality of raw and smoked salmon fillets, a broad range of fish were collected from commercial farming locations to obtain a broad overview and identify potential common properties of PD affected salmon. The fish examined had similar age, and they were reared by the same farming company within a limited geographic area. The farming locations participated in a cohort study reported by Jansen et al. (2010), hence the salmon were sampled from populations with a well documented disease history, with different time from PD diagnosis to slaughter. In this screening study (Paper I) a relatively high number of fish (250 individuals) were sampled from ten farming location, as a preliminary project indicated high variation in quality properties between individuals within the same population (Mørkøre et al., 2011). To obtain knowledge on underlying causes to quality deviations of PD affected salmon, individuals with different PD pathology within a population diagnosed with PD at time of slaughtering was investigated (Paper II).

4.3.1 Raw fillets

Significantly lower content of astaxanthin (16 percentage points) was found in salmon from a population diagnosed with PD at slaughter compared with unaffected salmon (Paper I). Within this group, significantly lower astaxanthin content was found in salmon with severe loss of pancreas as compared to those with SAV only (Paper II). Moreover, significantly lower contents of idoxanthin (19-62 percentage points) were found in fish within all PD affected groups (Paper I).

Astaxanthin is mainly bound specific to α-actinin in skeletal muscles of Atlantic salmon (Matthews et al., 2006). Hence, decreased pigment deposition in PD affected salmon might be associated with muscle lesions following decomposition of proteins or lower amount of binding cites due to accumulation of collagenous scar tissue. PD may as well cause oxidative stress in salmon due to lesions in pancreas, heart and other organs, and skeletal muscle

(reviewed by McLoughlin and Graham, 2007). Hence, it is possible that astaxanthin was used as an antioxidant in interactions with vitamin E (Stahl and Sies, 1993; Mortensen and Skibsted, 1997; Haila, 1999; Martin et al., 1999; Østerlie, 2000) or as a precursor for vitamin A (Schiedt, 1998). Moreover, stress might cause imbalances in the metabolic pathways of astaxanthin which could affect idoxanthin deposition in muscle tissue (Schiedt et al., 1989; Ytrestøyl et al., 2005). Nutritional and health status apparently also affected the deposition of 13-*cis* isomer of astaxanthin (not reported in Paper II). Salmon with severe loss of pancreas showed higher content of 13-*cis* astaxanthin as compared to those with SAV only (6.7% versus 3.5%, respectively; $P = 0.024$). Hence, the nutritional and health status of the fish might disturb the absorption of astaxanthin or the astaxanthin metabolism.

Degeneration of muscle tissue caused by PD and failure to recover resulted in uneven colour of seriously injured individuals (Paper I and II). This was shown with higher coefficient of variance (CV) of colorimetric parameters within raw fillets of PD affected salmon as compared to the control group (Paper I). In addition, in Paper II, salmon with severe loss of pancreas was more translucence (higher L^* value) and less red (lower a^* value) as compared to those with SAV only. Fish diagnosed with PD had lower a^* and higher L^* (Paper II), which in accordance with Skrede and Storebakken (1986b) and Christiansen et al. (1995) coincided with lower levels of astaxanthin.

Changes in colorimetric parameters on the fillet surface of PD affected salmon, even of salmon diagnosed with PD 12 months prior to slaughter (higher L^*, lower a^* and lower b^* values) as compared to the control group; indicate that visual colour perception is complex (Paper I). Hence, consistently higher translucence (higher L^* value) of PD affected salmon fillets is believed to be a consequence of disease related factors not associated with pigment deposition. For example, PD related changes in muscle structure and composition might have affected the light scattering of the muscle.

The carotenoid content in raw salmon fillets was not affected by the state of rigor mortis (Paper IV). However, colorimetric analyses of the raw material showed that pre and post rigor fillets had significantly different colour characteristics (L^* and a^* values). Time of filleting is earlier found to affect the colour characteristics of salmon fillets due to changes to a more compact surface structure which affect the light scattering surfaces to be fewer in pre as compared to post rigor fillets (Skjervold et al., 2001a,b). However, lower a^* value of pre rigor fillets was not in accordance to a previous study by Skjervold et al. (2001b), who found higher a^* values in pre as compared to post rigor fillets. The a^* value is previously found to

be the colorimetric parameter showing the most significant correlation to the carotenoid content (Skrede and Storebakken, 1986a,b; Christensen et al., 1995). However, in Paper IV, no such significant correlation was found which was probably due to the limited number of fish studied (r = 0.180-0.392, P = 0.262-0.642).

Fillet appearance might be disturbed by dark and brown spots normally classified as melanin. Anecdotal information indicates that PD affected fish may have increased extent of melanin spots. However, higher contents of melanin were only observed in salmon diagnosed with PD 12 months prior to slaughter (Paper I). Therefore, increased content of melanin might be considered as long-term distant after-affect of PD.

4.3.2 Cold-smoked fillets

The carotenoid stability (retention) and colour characteristics of cold-smoked Atlantic salmon fillets were affected by the health status (Paper I), rigor status at time of salting (Paper IV), salting procedure (Paper III and IV) and salt composition (Paper III).

The astaxanthin content in cold-smoked fillets did not differ significantly between PD affected and unaffected salmon (Paper I). However, a more pronounced average reduction of astaxanthin between raw and smoked fillets were observed for PD affected salmon, except for fillets of salmon from a population diagnosed with PD at slaughter. Furthermore, PD affected cold-smoked fillets showed higher variations in astaxanthin content between individuals as compared to the control group. Contents of idoxanthin were significantly lower (40-50%) in cold-smoked fillets of salmon diagnosed with PD six months or less prior to slaughter as compared to the control fish (Paper I). Cold-smoking of salmon fillets may induce decreased stability of astaxanthin due to smoke induced alterations in the quantitative protein and peptide composition (Latscha, 1990; Lund and Nielsen, 2001). Hence, a more prominent loss of astaxanthin and idoxanthin in the smoking process of PD affected salmon might be a result of disturbances in the carotenoid-protein complex induced by pancreatic insufficiency.

Cold-smoke processing of salmon diagnosed with PD six months or less prior to slaughter resulted in significantly higher L^* (1.2-2.8 colour units) and lower b^* (1.3-3.1 colour units) as compared to fillets of salmon diagnosed with PD 12 months prior to slaughter and the control group. Furthermore, significantly lowest L^*, a^* and b^* values were found in cold-smoked fillets of salmon with recurrent PD outbreaks. Interestingly, salmon diagnosed with PD six months prior to slaughter or with recurrent PD outbreaks showed higher reduction of all colorimetric parameters during smoking compared with that of unaffected salmon. When

comparing colorimetric changes during processing of all groups, the nutritional and health status of the fish affects visualisation of colour differently. This resulted in similar colorimetric characteristics for all fillets after smoking, except for fillets of salmon diagnosed with PD at slaughter and salmon with recurrent PD outbreaks. Cold-smoked fillets of salmon from a population with recurrent PD outbreaks were darker (lower L^*) and, together with fillets of salmon diagnosed with PD at slaughter, less intensely coloured (less red and yellow). In addition, salting and smoking of PD affected salmon fillets resulted in significantly higher reduction of L^* (ΔL, %) from raw to smoked fillets (25.9-9.7%) as compared to the control group (1.5%).

Retention of astaxanthin and idoxanthin (corrected for weight changes) in the fillet surface of cold-smoked salmon was significantly influenced by the salting procedure (Paper IV). In the fillet surface, significantly lower retention of idoxanthin and an indication ($P = 0.074$) of lower retention of astaxanthin was found in dry salted as compared to injected salted fillets. Moreover, 14 days storage strengthened the effect of the salting method applied and resulted in significantly lower retention of both astaxanthin and idoxanthin in dry salted fillets. Thus, the effect of salting method on retention of carotenoids in the fillet surface of cold-smoked salmon fillets seems somewhat time-dependent. Lower retention of astaxanthin and idoxanthin observed in fillets treated with dry as compared to injection salting may be a result of differences in surface water activity during the smoking step due to differences in salt content which, in turn, may affect the absorption of smoke components on the fillet surface (Sikorski et al., 1998) and the carotenoid stability (Latscha, 1990; Schiedt and Liaaen-Jensen, 1995).

Rigor status at time of salting did not significantly affect the retention of astaxanthin or lutein. Regarding retention of idoxanthin, significantly lower retention was found in the surface layer of fillets salted pre rigor as compared to their post rigor counterparts (paper IV). Due to changes in weight during processing the fillets constituents also change. It is therefore important to correct for weight changes when the stability of carotenoids is investigated (Birkeland, 2004). Not correcting for fillet weight changes following the various processing steps would cause errors of 10% applying dry salting and 2% in average retention of astaxanthin for injection salting, which was in accordance to Birkeland et al. (2004b). In addition, not correcting the retention for fillet weight changes during processing would give significantly effects of rigor mortis on the retention of astaxanthin due to different fillet

weight changes during processing caused by lower salt uptake in pre as compared to post rigor fillets (Wang et al., 2000).

Injection salting resulted in increased lightness, redness and yellowness (higher $L^*a^*b^*$ values) of cold-smoked fillets as compared to dry salting (Paper IV). This was most pronounced after 14 days vacuum storage (4 °C), which is probably due to the effect of dry salting on the light scattering in the fillet surface. Colour characteristics initiated by rigor status at time of filleting still dominated the products immediately after smoking, while after 14 days storage those differences became less dominant (Paper IV). In general, pre rigor salted fillets showed larger differences in colour characteristics between salting method applied as compared to fillets salted post rigor. This might be explained by different properties of pre and post rigor muscle tissue (Rørå et al., 2004a).

The smoking step was found to be the main contributor to loss of astaxanthin and idoxanthin in the fillet surface (Paper IV). Moreover, the total retention after 14 days storage was significantly different from the initial level and the level found after the salting step. No significant loss of lutein was observed following each processing steps and storage. Hence, lutein seems to be more stable throughout processing and storage as compared to astaxanthin and idoxanthin. Retention of astaxanthin in smoked fillets correlated negatively both with the salt content after salting (r = -0.488) and after smoking (r = -0.524), but after 14 days storage this relationship was less clear (r = -0.417 and -0.431, $P = 0.058$ and 0.067, respectively). Correlations between the salt content and retention of astaxanthin was probably due to alterations in fillet surface properties during smoking and changes in the protein structure which may influence on the physical ablution and the stability of carotenoids throughout processing and storage (Latscha, 1990; Schiedt and Liaaen-Jensen, 1995; Horner 1997; Lund and Nielsen, 2001).

During processing and storage, dry salting showed highest reduction in translucence (L^*), redness (a^*) and yellowness (b^*) (Paper IV). Moreover, ΔL^* correlated significantly with retention of astaxanthin in the final product (r = -0.540), indicating lower translucence of fillets with the most prominent loss of astaxanthin. In addition, the low and insignificant correlation (r = -0.354, $P = 0.163$) observed between retention of astaxanthin and Δa^* support that colour stability might be affected by other factors.

The colour difference, ΔE (CIE, 1994) for all protocols following each processing step and total ΔE between raw ($L_1a_1b_1$) and smoked fillets stored for 14 days ($L_2a_2b_2$) were

significantly different (Table 4.1, not reported in paper IV). In general, ΔE correlated significantly with the surface salt content measured both after the salting and smoking step (r = 0.51 - 0.74). Moreover, ΔE correlated even better with the dry matter content (r = 0.62 - 0.86), whereas no significant correlations were found between ΔE and any retention of astaxanthin. These observations supported that colour differences obtained throughout processing and storage of cold-smoked salmon fillets was due to conditions related to the salt content and content of water in the fillet surface (Sikorski et al., 1998), whereas the effect of reduced astaxanthin concentrations was minor. The surface colour is an important decision maker for consumers when purchasing smoked salmon products (Gormley, 1992; Rørå et al., 2004b), and ΔE values higher then 4 are normally visible to the human eye, while those of 2 and lower may be visible to an experienced observer (Cruse, without year). Therefore, it is important to consider ΔE values when elaborating different processing protocols used for production of smoked salmon fillets.

Table 4.1 The colour difference (ΔE) for all protocols following each processing step and total ΔE between raw and smoked fillets stored for 14 days (not reported in paper IV)

Parameter	Processing step	Protocol				*Effect of protocol*[1]
		PRE-I	PRE-D	POST-I	POST-D	
ΔE						
	Salting	2.2 ± 0.6^{ab}	1.9 ± 0.7^{ab}	1.3 ± 0.5^{b}	3.0 ± 0.7^{a}	$P = 0.027$
	Smoking	3.7 ± 1.4^{b}	5.5 ± 0.3^{ab}	4.0 ± 1.1^{ab}	6.4 ± 0.7^{a}	$P = 0.023$
	Storage	5.5 ± 1.0^{ab}	9.5 ± 4.0^{a}	3.4 ± 1.6^{b}	8.8 ± 3.3^{a}	$P = 0.020$
	All steps	4.4 ± 1.8^{c}	14.1 ± 2.7^{a}	5.8 ± 1.3^{c}	9.8 ± 1.4^{b}	$P < 0.001$

[1] Different lower case superscripts within each row indicate significant differences between processing protocols by one-way ANOVA and Tukeys pairwise comparison test

The most prominent effect of nitrite salting (Paper III) was significantly improved colour (more reddish, darker and less yellowish) on cold-smoked fillets, although only slightly higher astaxanthin content was observed (7 percentage points; 5.6 versus 5.2 mg kg^{-1}, P = 0.02). Adjusting the astaxanthin content for different fillet weight changes obtained during processing, differences in astaxanthin content between fillets treated with nitrite and fine refined salt became less and not significant (P = 0.27). However, the contents of 9-*cis* and 13-*cis* astaxanthin were significantly higher in nitrite salted cold-smoked salmon fillets (results not reported in Paper III, P < 0,001) as compared to fillets treated with fine refined salt.

Average distributions of the isomers were: nitrite salted; All-*trans* = 86.6 ± 1.6%, 9-*cis* = 1.9 ± 0.3%, 13-*cis* = 9.1 ± 0.9% and regular salted (fine refined salt); All-*trans* = 93.1 ±1.2%, 9-*cis* = 0%, 13-*cis* = 7.0 ± 1.4%. These results indicate that nitrite has an isomerising effect on astaxanthin during processing. *cis*-Isomers of carotenoids shows a hypsochromic shift (displacement of λ_{max} to shorter wavelength) of 2-6 nm for mono-*cis* isomers which indicate a slight more yellowish appearance as compared to All-*trans* (Britton, 1995). There are indications that the improved colour of nitrite salted fillets was related to formation of nitrosomyoglobin (Fox, 1966), which showed increased intensity in the visible spectra (VIS) between 420 nm and 490 nm as compared to myoglobin.

Formation of nitrosomyoglobin potentially cover a low intensity of astaxanthin (VIS, λ_{max} = 470 nm), resulting in increased colour evenness and intensity of redness on the fillet surface. Nitrite salting resulted in significantly lowest reduction of a^* and H°_{ab} from raw to smoked fillets, *i.e.* better colour stability in nitrite-treated fillets as compared to respective fillets treated with fine refined salt during processing. However, no significant differences (P = 0.078) in retention of astaxanthin were observed between cold-smoked fillets treated with nitrite salt and respective fillets treated with fine refined salt (89.6 ± 2.1% and 84.4 ± 5.4%, respectively; not reported in paper III). Significantly higher redness observed in an additional experiment with fillets of Cod (*Gadus murhua*) and herring (*Clupea harengus*) treated with nitrite, confirmed the formation of nitrosomyoglobin when nitrite is used in salt curing of fish. The fish muscle has low levels of myoglobin and other heme-compounds (Olsen et al., 2006), but clearly sufficient levels to produce nitrosomyoglobin (pink colour) and increase the colour quality of cold-smoked salmon fillets (Paper III).

4.4 Texture

PD significantly affected the fillet firmness, especially in salmon slaughtered shortly after PD diagnosis (Paper I and II). Only small differences in firmness were found in raw salmon fillets due to variations in health status (Paper I). However, higher variation was observed in fillet firmness among individuals in PD affected groups as compared to unaffected salmon, especially for salmon diagnosed with PD at slaughter. In this population salmon with severe loss of pancreas showed significantly higher fillet firmness as compared to those with SAV only (Paper II).

The fillet firmness increased during processing (Paper I), resulting in significantly higher firmness in smoked fillets from PD affected groups, except for salmon diagnosed with PD 12

months prior to slaughter as compared to unaffected salmon. Firmer texture of smoked fillets of salmon diagnosed with PD six months, or less prior to slaughter was probably related to increased deposition of collagen (HYP) and sulphated GAGs due to substitution of damaged muscle with scar tissue (Paper II, Hatae et al., 1986; Sato et al., 1986). In addition, dehydration during smoking probably makes the effects of muscle lesions on textural properties even stronger.

No significant texture differences were observed between salmon fillets salted with refined salt (NaCl) or a refined salt added sodium nitrite (0.6%) (Paper III).

4.5 Summary of results

The main impact of nutritional and health status (Paper I and II), state of rigor mortis (Paper IV), salting procedure (Paper IV) and salt composition (Paper III) is shown in Table 4.2. Colour was found to be the quality trait which was affected by all investigated parameters.

Table 4.2 Summary of the investigated factors on quality traits of raw and cold-smoked Atlantic salmon (*Salmo salar* L.)

		Investigated factors					
		Raw fillets		Cold-smoked fillets			
		Pancreas disease[1]	Rigor mortis[2]	Pancreas disease[1]	Rigor mortis[2]	Salting procedure[3]	Salt composition[4]
Quality parameter	**Papers**	*I, II*	*IV*	*I, II*	*IV*	*IV*	*III*
Fillet composition	*I, II, III, IV*	Protein ↓, water ↑, essential AA ↓, HYP ↑	-	-	-	Injection: water ↑	-
Carotenoids	*I, II, III, IV*	Ax ↓, Ix ↓	-	Ix ↓	-	Dry: ↓	Nitrite: Ax (↑)
Colour	*I, II, III, IV*	a^* ↓, L^* ↑	Yes	b^* ↓, L^* ↑	Pre: a^*↓, L^* ↓	Injection: L^* ↑, a^*↑ b^* ↑	Nitrite: L^* ↓, a^* ↑,
Melanin	*I*	distant after-affect ↑	nm	nm	nm	nm	nm
Texture	*I, II, III*	Firmness ↑	nm	Firmness ↑	nm	nm	-
Salt uptake	*I, III, IV*	Related to CF ↑	nm	Related to CF ↑	Pre: ↓	Yes	Nitrite: ↑
Unhealthy effects	*III*	nm	nm	nm	nm	nm	Nitrite: ↑

[1] Effects on quality traits are related to the time between diagnoses and slaughtering. Deteriorated quality is mostly seen in thin fish with low condition factor slaughtered close the time of diagnoses. However, abnormal appearance might be seen even after one year recovery. [2] Pre versus post rigor. [3] Dry versus injection salting. [4] Nitrite versus fine refined salt.

nm: not measured

DM: dry matter, AA: Amino acid, HYP: hypoxanthine, Ax: Astaxanthin, Ix: Idoxanthin, CF: condition factor

5 CONCLUSIVE REMARKS

- Salmon diagnosed with PD at slaughter, or six months prior to slaughter showed deteriorated general and fillet quality. Changes in quality in the order of their appearance were decreased CF, depleted muscle glycogen, increased drip loss of raw muscle, paler colour, depleted protein and finally harder texture in smoked salmon fillets. Salmon infected with salmonid alphavirus (SAV) without a PD outbreak and salmon diagnosed one year before slaughtering, had similar quality as unaffected fish. Deteriorated quality was most pronounced in fish with severe loss of pancreas and paler colour might exist even after one year.

- Use of nitrite salt improved the appearance of cold-smoked salmon fillets. The level of residual nitrite in one meal (50 g) exceed the level recommended by the European Commission's Scientific Committee for Food (SCF), but was lower than limits set by the US Environmental Protection Agency (EPA). Dry salting reduced the stability of carotenoids in the fillet surface of cold-smoked salmon as compared to injection salting, whereas no effects of state of rigor mortis at time of salting were observed. Low correlations between colorimetric parameters and content of carotenoids in the fillet surface, illustrated that additional factors affects the visual appearance of cold smoked salmon.

Based on the results obtained in this thesis it is recommend that PD affected salmon with low condition factor should be excluded from the market chain. In the critical period after a PD outbreak (up to six months), fish should be handled with particular care to avoid stress due to high vulnerability. Moreover, attention should be paid to accelerate muscle recovery after a PD outbreak, e.g. through optimised diets.

Improved retention of astaxanthin throughout processing and during storage requires better knowledge of the binding of astaxanthin in salmon muscle and mechanisms involved in decomposition of astaxanthin.

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I

Paper I

Effect of Pancreas disease (PD) on quality attributes of raw and smoked fillets of Atlantic salmon (*Salmo salar* L.).

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Effect of Pancreas disease (PD) on quality attributes of raw and smoked fillets of Atlantic salmon (*Salmo salar* L.)

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Keywords: Atlantic salmon, Pancreas disease, muscle quality, colour, smoked salmon fillets

Abstract

The impact of pancreas disease (PD) on fillet quality of raw and cold-smoked Atlantic salmon was investigated. Commercially reared fish were sorted into six groups; [1]Control (healthy fish), [2]SAV (infection with salmonid alphavirus, without PD outbreak), [3]PD0 (PD diagnosis at slaughter), [4]PD6 and [5]PD12 (diagnosed 5-7 and 11-12 months before slaughter, respectively) and [6]PDchronic (repeated PD outbreaks). The condition factor (CF) and fillet protein content were significantly higher for the control group (1.13 and 22.1%, respectively). The CF was lowest for PDchronic (0.92), whereas the fillet protein content was lowest in PD0 (20.2%). Fillet fat content did not vary significantly between the groups, but the muscle pH was 0.2 units higher in PD12 as compared to Control. Astaxanthin (Ax) and idoxanthin (Ix) content were significantly lowest for PD0. Ax recovered six months after the outbreak, but the Ix content remained lower in the PD affected salmon. The Ax level after smoking was similar of all groups, but Ix showed a similar pattern as that of raw fillets. Results of the colorimetric analyses (L^*,a^*,b^*) indicated darkest colour for the control group and palest colour for PD0, whereas PDchronic showed highest differences between raw and smoked fillets. Firmness of raw fillets was lowest in PDchronic, but after smoking a significantly higher firmness was found in PDchronic, PD0 and PD6 (16.7-19.7 N) compared with that of Control and PD12 (14.1 N). Changes in fillet quality in the order of their appearance were decreased CF, depleted muscle glycogen, increased drip loss of raw muscle, paler colour, depleted protein and finally harder texture in smoked salmon. It is concluded that the fillet quality deteriorated after a PD outbreak, but the quality may to a large extent recover.

1. Introduction

In Norway, pancreas disease (PD) is caused by the salmonid alphavirus subtype 3 (SAV 3) that is related to the salmonid alphavirus subtype 1 (SAV 1) which causes PD in Ireland and Scotland (McLoughlin and Graham, 2007). Currently, PD in Norway is endemic along the west coast south of the 63^{o} latitude (Hustadvika). Fish stress plays a key role in the development of PD, with several examples of PD occurring after handling of fish (Raynard, 1992; Brun et al., 2006), and associations between the salmon lice (*L. salmonis*) burden and outbreaks of PD has also been reported (Ruane et al., 2005). Fish mortalities can reach 40% in PD affected pens and subsequent failure to grow is a further consequence of the disease resulting in poor condition and thin fish that are susceptible to parasitism and secondary bacterial diseases (Ruane et al., 2005). Economic losses due to PD have been estimated to reach approximately one billion NOK per year (Torrissen, 2008), and according to Aunsmo et al. (2010), a single PD outbreak on a fish farm with 500.000 smolts can results in a total loss of 14.4 million NOK.

The colour intensity is one of the most important quality parameters of Atlantic salmon fillets (Anderson, 2000). In addition, it is important that the variation among fillets from the same populations is low, and that the colour shows low variation between sections of the same fillet. Moreover, lack of dark spots or melanin is important. Firmness is another critical parameter that determines the acceptability of seafood products (Veland and Torrissen, 1999), where soft flesh leads to reduced acceptability by consumers (Hatae et al., 1985; Ando, 1999; Veland and Torrissen, 1999). Aunsmo et al. (2010) reported that quality downgrading contributes to the economic losses upon PD outbreaks, and anecdotal information indicates that PD may cause poor general muscle quality associated with severe discoloration (Bjerkeng, 2004). However, to our knowledge, there is no objective information available on the impact of PD on attributes of salmon fillet quality, apart from observations of

gray shadows of melanin on fillets in the chronic phase of PD (McLoughlin, 2005), and decreased levels of vitamin E (Taksdal et al., 1995). Therefore the present study was undertaken to elucidate the impact of PD on quality related characteristics of commercial slaughter sized salmon. Analyses were performed on raw and cold-smoked fillets of salmon which were diagnosed with PD from 0 to 12 months prior to harvest. This is the first study of two, which is screening quality parameters of separate populations with a PD history. The second paper describes the fillet quality on an individual level based on pathological profile and gene transcriptome profiling (Larson et al., in prep).

2. Material and methods

2.1. Fish material and experimental design

Slaughter ready Atlantic salmon (*Salmo salar* L.) were sampled from ten commercial fish farms, whereof nine farms participated in an epidemiological cohort study of pancreas disease (PD) in Norway, reported by Jansen et al. (2010). Fish in that cohort study were sampled for analyses with regard to salmonid alphavirus (SAV), specific antibodies and histopathological changes two and eight months following transfer to seawater and at the time of harvesting.

Salmon from seven farms were diagnosed with PD; salmon from one farm were infected with SAV without an outbreak of PD, whereas salmon from two farms had no records of PD diagnosis and worked as control farms (Table 1). These 10 farms were sorted into six different groups according to PD and SAV diagnosis; Control (number of fish (n)=60, two farms), SAV (n=30, one farm), PD0 (n=19, one farm, diagnosed with PD at slaughter), PD6 (n=50, two farms, diagnosed 5-7 months before slaughter), PD12 (n=65, three farms, early diagnosis 11-12 months before slaughter) and PDchronic (n=30, one farm, repeated PD outbreaks during the seawater phase). The relatively high number of fish sampled from each location was based on results from a preliminary project, where a high variation in quality

properties was found between individuals within the same populations (Mørkøre et al., 2011). The fish subjected to fillet quality analyses were selected randomly from each group, except that fish below 2 kg and above 5 kg were omitted. The body weight of the fish sampled for analyses averaged 3.7 kg (range 2.5-4.9 kg).

All fish had the same age, were reared in net pens in seawater with relatively similar farming environments and they were fed commercial extruded diets. The fish were sampled randomly from the net pens for analyses, percussive killed, bled, gutted and thereafter stored on ice until sampling of tissues for virus and histopathological examination. Filleting was performed 4-6 days after slaughter. The right fillets were salted immediately after filleting, cold-smoked, vacuum packed, stored at 3 °C for three weeks and analysed for physical and chemical quality attributes. The left fillet was kept raw and subjected to quality analyses the day after filleting and muscle was sampled for subsequent chemical analyses. Raw and smoked fillets were analyzed and sampled using the same procedures. The analyses included: texture, drip loss, pH (only raw fillets), gaping and colour (image analysis). Sectioning of the fillets for the various analyses is illustrated in Fig. 1. Section A (frequently termed the Norwegian Quality Cut, NQC) was stored at -20 °C, whereas section B was stored at -80 °C until chemical analyses. In addition, cold-smoked fillets of salmon originating from farm no. 1 and farm no. 10 (Table 1) were studied in separate storage trials were microbiological and chemical changes were analysed over 5 weeks at 7-8 °C (See 2.9).

2.2. PD and SAV diagnosis

Independent of the present study, all the fish farms were regularly monitored by private fish health services. When disease outbreak was suspected due to enhanced mortality and/or aberrant behaviour, standard diagnostic procedures were followed, including autopsy and submission of samples for laboratory examinations by Norwegian Veterinary Institute. PD

was diagnosed when histopathological examination revealed changes characteristic for PD in Norway (Taksdal et al., 2007) combined with identification of SAV in the same individual. In short, PD pathology included significant loss of exocrine pancreatic tissue and inflammation in heart and skeletal muscle. SAV was diagnosed by real time RT-PCR using primers located in a conserved part of the genome coding for the E1 glycoprotein (Jansen et al., 2010).

For three of the farms, examinations of slaughter samples collected for the research projects confirmed PD and/or SAV infection (Table 1) although this had not previously been confirmed or identified by the diagnostic routines. In one of these farms, PD was already suspected based on histological examinations of tissues submitted by the fish health service 6 ½ month prior to slaughter. When both SAV and specific antibodies against SAV were detected at slaughter, this farm (Table 1, farm 5) was classified as PD affected for the present study. The second farm (Table 1, farm 4) was classified as PD0 because PD was diagnosed based upon the slaughter samples. The third farm (Table 1, farm 3) was diagnosed only as infected with SAV. This farm had probably been infected with SAV shortly prior to slaughter as neither tissue changes characteristic for PD nor specific antibodies against SAV was detected at that time.

For the research project by Jansen et al. (2010), fish samples (tissues for SAV detection, tissues for histology and blood for detection of antibodies against SAV) had been collected two and eight months after sea water transfer and at slaughter (Table 1).

2.3. Dry salting procedure

Salmon fillets were dry salted on grids at 4 ºC using refined NaCl (Akzo Nobel, Fint Raffinert Salt, minimum 99.8% NaCl, Dansk Salt A/S, Mariager, Denmark). After 18 hours, excess salt was removed by careful rinsing of the fillets with water. Before smoking the fillets were rested at 14–15 ºC for approximately 30 min.

2.4. Cold smoking procedure

A Bastramat C1500 smoking cabinet equipped with a MC700 Microprocessor and a Bastra FR 100 smoke generator with automatic ignition and dosing (Bayha Strackbein GmbH, Arnsberg, Germany) was used for the smoke processing of the fillets. Reho Räucher Gold HBK 750/2000 chips (J. Rettenmaier & Söhne GmbH, Rosenberg, Germany) were moistened (200 ml water/kg chips) and used for smoke generation by combustion of the chips. The fillets were dried for 60 minutes followed by four circles of 50 min smoking and 10 min drying. This resulted in a total processing time of 300 min with a total of 100 min drying and 200 min smoking. During this processing temperature was 26±3 ºC, relative humidity 70-80%, and air velocity 1.5 m s^{-1}. After smoking the fillets were stored at 14–15 ºC for 30 min and then vacuum packed.

2.5. Chemical analyses

Dry matter of raw and cold-smoked fillets was estimated gravimetrically after drying at 105 °C for 24 hours (ISO 6496 1983). Total fat was extracted and calculated by the method of Bligh and Dyer (1959) with slight modifications. Nitrogen content was measured on a Tecator Kjeltec system (Model 2020 Digestor and 1026 Distilling unit, Tecator, Höganäs, Sweden) (NCFA, 2003). Protein content was calculated from nitrogen measurements using the formula: %protein = %nitrogen*6.25. Astaxanthin and idoxanthin in tissue were extracted (Bligh and Dyer, 1959) and analysed by HPLC using an Agilent1100 liquid chromatograph (Agilent Technologies, Paolo Alto, CA, USA connected to an Agilent photodiode array UV-VIS detector). Astaxanthin was analysed by the method of Vecchi et al. (1987) using a Lichrosorb SI60-5, 125*4.0 mm, 5 µm, Hichrom, Reading, UK, HPLC column modified with ortophosphoric acid (0.1% in CH_3OH). Idoxanthin was analysed by the method of Aas et al.

(1997) using a Luna 5µ CN 100A, 250* 4.6 mm, Phenomenex®, USA, HPLC column. Astaxanthin and idoxanthin were quantified by response factors (RF-values). Idoxanthin standards were prepared from all-*E*-astaxanthin (AcrosOrganics, 328612500) and 3´,4´-*cis* and 3´,4´-*trans* isomers of idoxanthin were reduced from astaxanthin by a method after Aas et al. (1997) with use of absolute ethanol instead of dry ether as solvent. Differences in carotenoid content between raw and smoked fillets were calculated according to dry matter values of astaxanthin and idoxanthin. The content of NaCl in muscle tissue was determined conductivimetrically after extraction with deionised water by a method after Engdahl and Kolar (1993). The content of NaCl in plasma was measured by a Chloride Analyser (Model 926 Sherwood Scientific Ltd.). Plasma samples (0.3 ml) were added to hot deionised water (80 ˚C, 3 ml), stirred (10 sec.) on an Vortex mixer, type: MECB1719(EU), Merck Eurolab, Germany and heated in a water bath (100 ºC, 10 min), cooled to room temperature and diluted to 10 ml in a volumetric flask before analyses.

All chemical parameters are expressed as % of wet weight except astaxanthin and idoxanthin which are expressed as mg kg^{-1} protein.

2.6. Photometric analyses

Colorimetric analysis and quantification of visual melanin were assessed by photographing the fillets in a light-proof aluminium box with standardised illumination as described by Folkestad et al. (2008). The camera used was a SinarCam 2 (Sinar AG, Feuerthalen, Switzerland), equipped with a Nikon AF Nikkor 35mm 1:2 D lens, connected to a PowerBook G4 (Apple, Cupertino, CA, USA). The software Sinar CaptureShop (version 4.0.1 Sinar AG) was used to capture images which were analysed using software provided by PhotoFish AS (Ås, Norway). *L*a*b**-values (calculated from R, G and B values obtained from the images) and hue (H^0_{ab}) were used to describe fillet colour. *L** describes the lightness of the sample, *a**

intensity in red ($a^* > 0$), b^* intensity in yellow ($b^* > 0$) and the hue angel H^0_{ab}, where $H^0_{ab} = 0$ for red hue and $H^0_{ab} = 90°$ for yellowish hue. Colorimetric parameters were analysed on seven areas placed manually on the fillet, avoiding dark spots and melanin (Fig. 1a), to enable calculation of variations in colour between areas within fillets (CV%). Differences in lightness (ΔL^*), redness (Δa^*), yellowness (Δb^*) and hue (ΔH_{ab}) between raw fillet ($L_1a_1b_1$) and the smoked fillet ($L_2a_2b_2$) was calculated. Areas of visual melanin on the fillet surface were quantified (mm^2) by using the free medical imaging software ImageJ (National Institute of Mental Health, Bethesda, Maryland, USA).

2.7. Texture, gaping and pH analyses

Instrumental textural analyses were performed using a Texture Analyser TA-XT2 (SMS Ltd., Surrey, England) equipped with a 30 kg load cell. A flat-ended cylinder probe (12.5 mm diameter, type P/0.5) was used for both raw and smoked fillets. The force-time graph was recorded by a computer equipped with the Texture Expert software for windows (version 1.15, SMS), which was also used to analyze the data. Analyses were performed in duplicates per fillet (area C and E, Fig. 1b) and the average was used for data analysis. The resistance force (N) was recorded with a constant speed (1 mm s^{-1}), and the force required to press the cylinder down to 60% of the fillet height (termed F60%) was used to describe firmness.

Gaping score was assessed by visual inspection using a scale from 0-5 where 0 indicated no gaping and 5 extreme gaping (Andersen et al., 1994).

The pH was measured in area D (Fig. 1) using a pH-meter 330i SET (Wissenchaftlich-Techniche-Werkstätten GmbH &Co. KG WTW, Weilheim, Germany) connected to a muscle electrode (Scott pH-electrode, Blueline 21 pH, WTW, Weilheim, Germany) and a temperature probe (TFK 325, WTW, Weilheim, Germany).

2.8. Drip loss

A 12 g slice of white dorsal muscle (area D, Fig. 1b) was placed on a thin-bedded honeycombed pad in a sealed polyethylene bag for five days at 3 °C (Mørkøre et al., 2007). Drip loss was calculated as: 100*weight increase of the pad (g)*initial muscle weight $(g)^{-1}$.

2.9. Microbiological changes

Cold-smoked fillets of salmon originating from farm no. 1 and farm no. 10 (Table 1) were divided into skin-on pieces of 75±5 g. These pieces were vacuum-packed and stored at 7-8 °C during 35 days. At regular intervals during the storage period four pieces from each of the two farms were analysed by microbiological and chemical methods. Changes in concentrations of aerobic plate counts (APC), lactic acid bacteria (LAB), H_2S-producing bacteria, *Enterobacteriaceae*, luminous bacteria and *Photobacterium phosphoreum* were determined. Chemical characterization of samples included pH, water phase lactic acid, water phase salt, smoke intensity measured as phenol and nitrite. The storage temperature was recorded by data loggers. Analyses were carried out as previously described in studies of raw and cold-smoked salmon from Norway (Emborg et al., 2002; Ginénez and Dalgaard, 2004; Lakshmanan and Dalgaard, 2004).

2.10. Statistical analyses

Data were analysed by one-way ANOVA and correlation (Pearson's correlation coefficient) analyses using the SAS program (Version 9.1; SAS Institute Inc., Cary, USA). The coefficient of variation (CV) was calculated as the ratio of the standard deviation to the mean. The alpha level was set to 5% ($P < 0.05$). Forward stepwise procedure of linear discriminant analysis was used to search for the most informative parameters that separated the groups. Of those

parameters a multivariate analysis was performed where a classification matrix was constructed for quantitative assessment of differences between the groups.

3. Results

3.1. Condition factor

The condition factor (CF) of the salmon sampled for analyses averaged 0.99 (range 0.92-1.13) (Table 2). CF was highest of the control group (CF 1.13) and lowest for PDchronic (CF 0.92). CF was similar for SAV, PD0 and PD6 (CF 0.96). The overall correlation between gutted body weight and CF was 0.50 ($P < 0.001$).

3.2. Chemical parameters and muscle pH

Fillet dry matter (DM) and protein content in raw fillets showed an overall average of 29.8% and 21.3%, respectively (Table 2). Both DM and protein content were lowest for PD0, but DM of the other PD affected groups did not differ significantly from the control group. The protein content was significantly lower of all PD affected groups (1-2 percentage points) compared with the control group, except from SAV. The protein content showed lower variation between individuals within the control and SAV (CV 3%) as compared with the other groups (CV 5-6.5%). A similar trend was seen for the DM content. The fat content in raw fillets showed no significant variations between fish groups (7.7-9.5%), although the fat content of PD0 (7.7%) tended to be lower compared with that in Control, PD12, and PDchronic (9.2-9.5%, $P = 0.11$). The variation in fat content between individuals was highest for PD0, with a CV of 48% compared with an average CV of 31% for the other groups. The muscle pH was significantly lowest in the control group (6.10) and highest in PD12 (6.29).

The NaCl content (%) in plasma was significantly higher in SAV (1.07) compared with that of Control (0.94) and PD6 (0.89) (Table 3). The NaCl content (%) in smoked fillets

was significantly higher in PD6 (4.7) and PDchronic (4.5) compared with that in Control (3.7). Otherwise the NaCl content showed no significant difference between the fish groups.

3.3. Astaxanthin and idoxanthin content

Astaxanthin content in raw fillets was significantly lower in PD0 (21.8 mg kg^{-1}) and higher in PD12 (27.9 mg kg^{-1}) compared with that in the control group (25.7 mg kg^{-1}) (Fig. 2.). The variation between individuals was highest for PD0, with a CV of 25.7% compared with a range of average CV of 13-20.7% for the other groups. The astaxanthin content in smoked fillets showed no significant variations between the fish groups (Fig. 2.). The variation between individuals was highest for all PD groups with an average CV of 22.7% compared with an average CV of 14.1% for Control and SAV. The reduction (%) of astaxanthin content from raw to smoked fillets was significant for all groups (11.7-22.5%), except for PD0.

Idoxanthin content in raw fillets was significantly lower in PD0 and PDchronic (0.5-0.8 mg kg^{-1}) compared with that in Control and SAV (1.2-1.3 mg kg^{-1}) (Fig. 2.). Significantly higher idoxanthin content was found in PD12 (1.0 mg kg^{-1}) compared with that in PDchronic (0.5 mg kg^{-1}). The variation between individuals was highest in PD0, with a CV of 90.3% compared with an average CV of 57.9% for the other groups. The idoxanthin content in smoked fillets was significantly lower in PD0, PD6 and PDchronic (0.3-0.4 mg kg^{-1}) compared with that in the control group and SAV (0.7-0.8 mg kg^{-1}). The variation between individuals was higher in Control (CV 73%) compared with the other groups (CV 57.7% on average).

3.4. Photometric analyses

Lightness (L^*) in raw fillets was significantly lowest in the control group (44.6), whereas hue (H^0_{ab}), redness (a^*) and yellowness (b^*) were lowest in PD0 (43.8, 25.3 and 24.2,

respectively). The colour parameters $L^*a^*b^*$ were highest in PDchronic (51.9, 32.1 and 32.0, respectively) (Table 4). The only colour parameter which where correlated to the content of astaxanthin was a^* ($r = 0.33$, $P < 0.001$).

The variation (CV%) in $L^*a^*b^*$ values between sections (Fig. 1a) was 4-10% for raw fillets and 5-19% for smoked fillets. PDchronic had the highest CV of L^* and b^* of raw fillets and a* of smoked fillets (7-10%), whereas the CV of a^* in raw fillets was highest for PD0 and PD6 (6-7%). The CV of L^* and b^* of smoked fillets were highest in the control group (9.5 and 19%, respectively).

The reduction of L^* (ΔL, %) from raw to smoked fillets was significantly highest in PDchronic (25.9%) followed by PD6, PD0, SAV and PD12 (15.5-9.7%), and significantly lowest in the control group (1.5%). The reduction of a^* (Δa, %) and b^* (Δb, %) were likewise highest in PDchronic (45 and 35%, respectively), but for these parameters the change from raw to smoked fillets was similar or higher for the control group as compared with the other groups (SAV, PD0, PD6 and PD12).

After smoking, no significant differences in L^* values was observed (range 42.7-45.5), except for PDchronic (38.4). For a^* and b^* values, significant differences were observed for PD0 (a^*: 20.9 and b^*: 23.7) and PDchronic (a^*: 17.7 and b^*: 20.8) compared with that of the other groups (a^*: range 22.4-23.3 and b^*: range 25.0-26.8).

Fillet melanin content was significantly higher in PD12 (50.9 mm^2) compared with that in Control (8.3) and PDchronic (8.3-9.1 mm^2) (Table 2).

3.5. Fillet texture and gaping

Firmness (N) in raw fillets was significantly lowest in PDchronic (8.8 N), the only group differing significantly from the control group (9.8 N) (Fig. 3.). In smoked fillets, significantly higher firmness (N) was found in PD6, PDchronic and PD0 (16.7-19.7 N) compared with that

in Control and PD12 (14.1 N). Firmness of both raw and smoked fillets showed lower variation between individuals within the SAV and control group (CV 11-18%) as compared with the PD affected groups (CV 20-27%)

Gaping score of raw fillets was significantly higher in PD6 (2.3) compared with that in the control group, PD12 and PDchronic (1.1-1.6) (Table 3). In smoked fillets, no significant variations between fish groups were found.

3.6. Drip loss

Significantly higher drip loss (%) was found in raw fillets of SAV and PDchronic (5.7-5.8%) compared with that in the other groups (range 4.2-4.8%) (Table 3). In smoked fillets, significantly higher drip loss (%) was found in PDchronic (2.9%) and significantly lower in SAV (2.0%) compared with that of the other groups (2.4-2.6%).

3.7. Microbiological changes

No significant growth of any of the studied groups of microorganisms was determined during 35 days at the average storage temperature of 7.5 °C. Low concentrations of APC, LAB, luminous bacteria and *Photobacterium phosphoreum* were detected in several samples from both farms. The concentration of microorganisms was not significantly different ($P > 0.05$) for samples from farm no. 1 (without PD) and farm no. 10 with repeated PD outbreaks (Results not shown). Nitrite was not detected in any of the samples and product characteristic did not differ significantly between the two farms ($P > 0.05$). The ranges of the average values were: pH (6.18-6.19), water phase lactic acid (7920-8708 ppm), water phase salt (4.42-4.53%) and phenol (6.07-6.31 mg kg^{-1}). With these product characteristic and a storage temperature of 7.5 °C the absence of microbial growth is surprising and deserves further study. The absence of microbial growth may be related to limited microbial contamination

during cold-smoking in pilot scale processing at Nofima Norconserv as substantial microbial growth has been observed previously for commercial cold-smoked salmon with similar product characteristic (Leroi et al., 2001, Mejlholm and Dalgaard, 2007).

3.8. Multivariate analysis

Forward stepwise procedure of discriminant analyses identified six parameters (*L*, a*, b**, and fillet content of fat, protein and astaxanthin) and defined linear functions that described the differences between the experimental and control groups. Cumulative proportion of first two canonical variables was 91% (Fig. 4a). Clustering of the relative positions of centroids (single linkage) combined (i) PD6 and Chronic, (ii) PD0, PD12 and SAV and (iii) Control (Fig. 4b). The clusters were well separated although differences between the groups within clusters were small. The proportions of correct assignments to clusters were 92.8%, 91.6% and 94.7% respectively. However, within the clusters the accuracy of classifications was lower due to overlap between the groups (Table 5). Overall, the discriminant functions classified correctly 78% of the samples.

4. Discussion

All the fish analysed were slaughtered within a short period from September-November 2007, except from one farm (Farm 5, PD6) that was slaughtered early January 2008. Hence, it is unlikely that inherent seasonal changes (Mørkøre and Rørvik, 2001; Roth et al., 2005) caused the significant condition factor (CF) variations between the fish groups. The lower CF of the PD affected salmon is considered as a negative consequence of the disease, as low CF leads to reduced fillet yields and value (Bosworth et al., 1998; Einen et al., 1999). The problem with the slim and varying body shape was also found six months after the PD outbreak, while increased CF after 12 months may indicate rebuilding of skeletal muscle.

Loss of pancreatic tissue is one of the main features of PD. Individual fish that survive with no or severely reduced amount of pancreatic tissue will have a reduced ability to absorb and metabolize nutrients from the feed. This may explain the lower condition factor of individual fish from the PD-affected fish farms. The present study indicates better CF and slaughter quality between 6 and 12 months after the diagnosis. This could be caused by either regeneration of pancreatic tissue in survivors or by the death of starving fish that gradually will die off because of their inability to utilize the feed.

It might, however, be difficult to detect early acute stage PD because of the complex spectrum of clinical signs and lesions associated with the disease (reviewed by McLoughlin and Graham, 2007). Furthermore the severity of an outbreak of PD will depend not only on the presence of virus, but also on factors such as stressful environmental conditions and nutritional imbalances (Rodger et al. 1991). The SAV group showed no PD pathology, but the slimmer body shape and stable fat content correspond well with observations made in feed deprived salmon (Einen et al., 1998; 1999). Therefore, the results indicate that the SAV group may have had decreased feed intake or impaired nutritional utilisation during a period prior to the virus detection at harvesting. Moreover, elevated Cl^- levels in plasma sampled at slaughter indicate osmotic regulation problems (Table 3). However, the cause may be unrelated to PD as no specific antibodies were detected in the blood plasma sampled at slaughter. This strongly suggests a recent infection with SAV.

The protein content in healthy adult salmon is relatively stable, while the fat and water may show considerable variations (Shearer et al., 1994; Einen and Roem, 1997). Hence, the lower protein content in the PD affected salmon is considered to be a primary or secondary consequence of nutritional disturbances induced by pancreatic insufficiency. The reduction in protein was most prominent in the fish that was diagnosed with PD at slaughter (PD0), while there appeared to be higher protein content in salmon slaughtered six months or more after a

PD outbreak. The average fat content in the muscle showed no significant variation although exocrine pancreatic insufficiency is a condition that has been associated with fat malabsorption (Layer, 1999; Sikkens et al., 2010). On the other hand, the relative amount of fat showed a higher variation between individuals of the PD affected salmon compared with the control group. The muscle pH, on fish meat without added salt, reflects the conversion of glycogen to lactic acid. Therefore, the higher pH of the PD affected salmon indicates decreased glycogen stores upon PD outbreak with no recovering, even after one year. Drip loss is normally inversely related to the muscle pH (Ofstad et al., 1995). Higher drip loss in the SAV and the PDchronic groups indicate that drip loss is related to other factors than pH *per se*, i.e. PD pathology.

Visual colour of salmon fillets is strongly related to amounts of astaxanthin and other carotenoids (Schiedt et al., 1981; Bjerkeng, 2000; Birkeland and Bjerkeng, 2005; Ytrestøyl et al., 2006). Astaxanthin is bound no specifically to actomyosin in skeletal muscle of salmon (Henmi et al., 1990; 1991), and it is possible that decomposition of proteins may reduce astaxanthin deposition. Astaxanthin functions as an antioxidant in interactions with vitamin E (Mortensen and Skibsted, 1997), hence PD induced oxidative stress may be another cause for reduced astaxanthin content in PD affected salmon, particularly in PD0. Stress might also cause imbalances in idoxanthin (a metabolite of astaxanthin) deposition (Schiedt et al., 1989; Ytrestøyl et al., 2005).

The L^* value was consistently higher in salmon with a PD history despite that the astaxanthin level was similar or higher 6 and 12 months after a PD outbreak. The translucence (higher L^* value) of PD affected salmon is believed to be a consequence of disease related factors not associated with pigment deposition. For example, PD related changes in muscle structure and composition might have affected light scattering of the muscle.

Smoking of salmon induce colour changes due to Maillard browning (Sikorski et al., 1998) and decreased stability of astaxanthin due to smoke induced alterations in the quantitative protein and peptide composition (Latscha, 1990; Lund and Nielsen, 2001). Hence, a more prominent loss of astaxanthin in the smoking process of PD affected salmon may be a result of disturbances in binding strengths of the astaxanthin-protein complex induced by pancreatic insufficiency. However, salmon diagnosed with PD at slaughter (PD0) showed no loss of astaxanthin during smoking. On the other hand, recurrent PD outbreaks (PDchronic) gave the highest change in colorimetric parameters during smoking. Therefore, the colour of smoked salmon seems to depend on the PD history, in particularly several PD outbreaks seems to alter the colour development during smoke processing. Moreover, scars on regenerated muscle fibres probably weaken the binding strength between actomyosin and post-PD bonded astaxanthin, yielding astaxanthin to be more exposed to physical ablution and decomposition during salting and smoking. Processing conditions affect textural changes during smoking (Sigurgisladottir et al., 2000; Hultman et al., 2004). However, firmer texture and larger textural differences between raw and smoked fillets of salmon diagnosed with PD at slaughter (PD0), PD6 and PDchronic, compared with that of Control and PD12, could not be explained by different processing conditions in this study. Hence, these differences may be related to increased amounts of connective tissue and skeletal muscle lesions caused by PD. In addition, dehydration during smoking probably makes the effects of muscle lesions on textural properties stronger.

An interesting phenomenon related to colour, occurred in salmon slaughtered 6 months or more after the PD outbreak. These groups showed higher reduction of all colorimetric parameters during smoking compared with the control group. When comparing colorimetric changes during processing of all groups, PD affects visualisation of colour differently. This resulted in similar colorimetric characteristics for all groups after smoking,

except for PDchronic and PD0, where PDchronic was darker (lower L^*) and, together with PD0, less intensely coloured (less red and yellow). Furthermore, one year after the PD-outbreak, average quality is normalised but some individuals may still show pale colour. Therefore special care and control has to be considered when raw material with a PD history is used in cold-smoke processing.

Quality characteristics such as colour (Bjerkeng, 2000), fat content and textural properties (Mørkøre and Rørvik, 2001) show high variation between farms and also within the same population. The present study showed that all the before mentioned properties were affected by PD. To obtain a general overview of the results, multivariate analyses were carried out, taking into account the different parameters analysed. Discriminant analysis identified colorimetric parameters and pigment, fat and protein content as those describing differences between the experimental and the control groups. From the multivariate analyses it is concluded that salmon harvested ≤6 months after a PD outbreak and salmon exposed to recurrent PD outbreaks show the most deviated quality, whereas newly infected salmon and salmon slaughtered one year after a PD outbreak show similar quality as uninfected fish (Control).

5. Conclusion

It is concluded that PD significantly can alter quality attributes in salmon but that the quality to a large extent can recover. This confirms earlier anecdotal evidences. Changes in fillet quality in the order of their appearance were decreased CF, depleted muscle glycogen, increased drip loss of raw muscle, paler colour, depleted protein and finally harder texture in smoked salmon.

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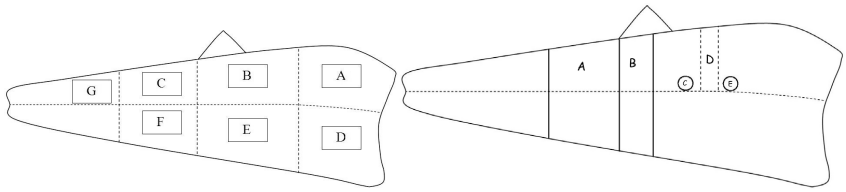


a) **b)**

Fig. 1. **a)** The rectangles A-G illustrate the fillet surfaces analysed photometrically for colour of raw and smoked fillets. **b)** Schematic illustration showing the areas upon the fillet from which the analyses were conducted. A: NaCl in smoked fillets, B: dry matter, fat, protein in raw fillets, and astaxanthin and idoxanthin in raw and smoked fillets, C and E: instrumental texture analyses of raw and smoked fillets, D: pH in raw fillets and drip loss in raw and smoked fillets.

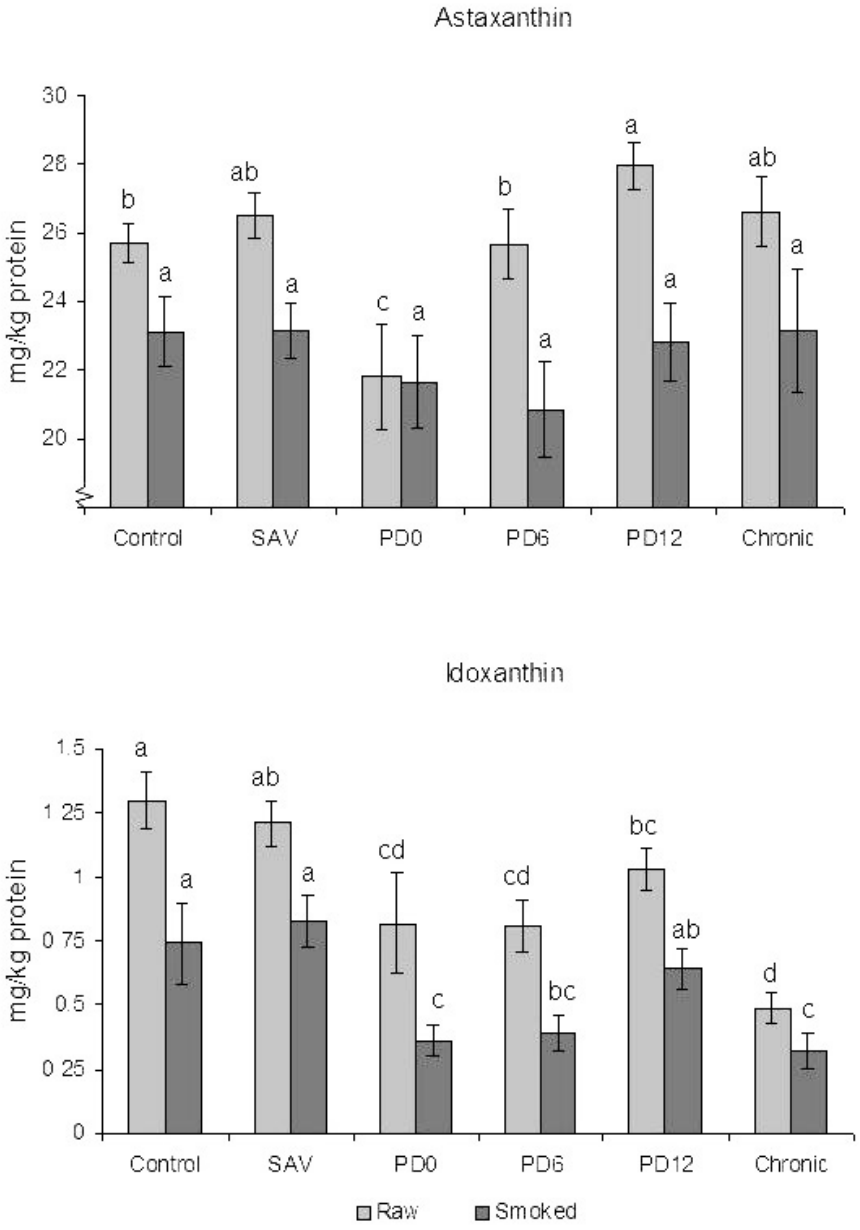


Fig. 2. Astaxanthin and idoxanthin content (LSMeans±SE) of raw and smoked salmon fillets. Different letters indicate significant variation ($P<0.05$) between groups within raw and smoked salmon, respectively.

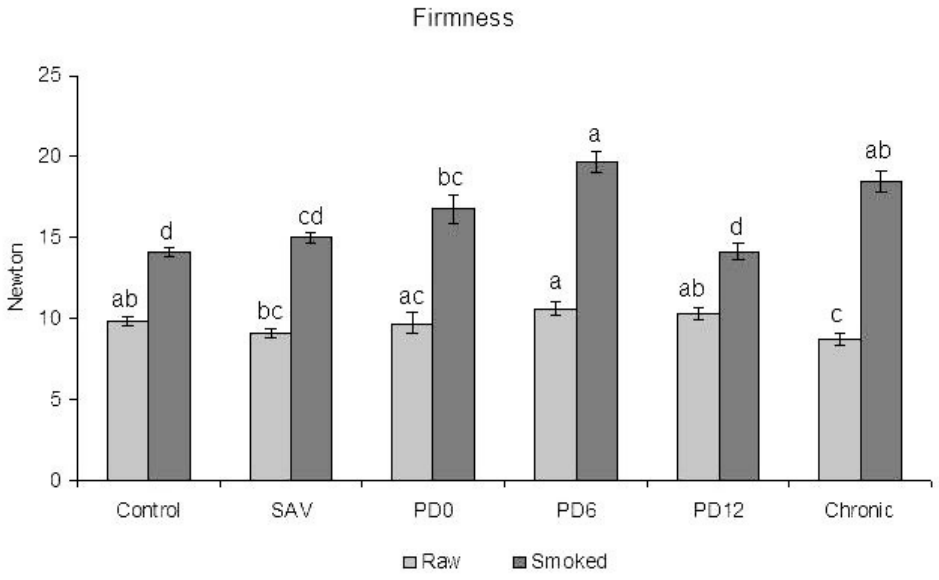
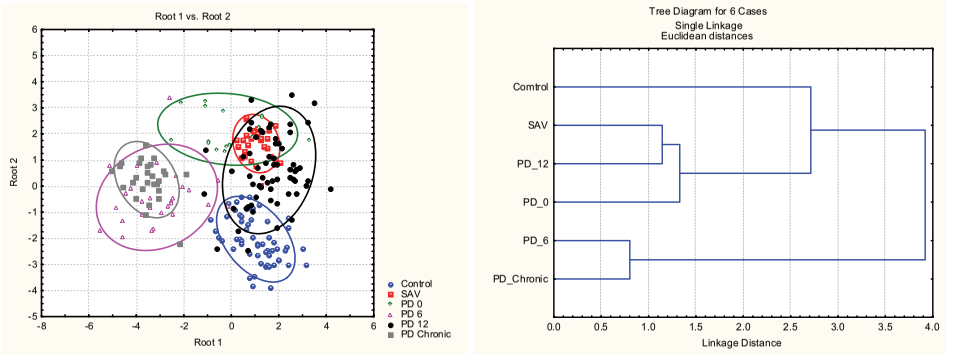


Fig. 3. Firmness (LSMeans±SE) of raw and smoked salmon fillets determined instrumentally as the force at 60% compression of the fillet height. Different letters indicate significant variation ($P<0.05$) between groups within raw and smoked salmon, respectively.



a) **b)**

Fig. 4. **a)** Discriminant analysis of raw salmon samples. Root 1 = -0.43(L*)-0.5(a*)-0.11(b*)+0.21(fat)+0.59(protein)+0.11(astaxanthin)+21.19. Root 2 = 0.21(L*)+0.31(a*)-0.61(b*)+0.02(fat)+0.07(protein)+0.11(astaxanthin)-5.91. Eigenvalue for root 1 = 4.11 (cum.prop = 61%), for root 2 = 1.95 (cum.prop. root1+root2 = 91%). Confidence interval for all ellipses is 0.8. **b)**: hierarchical clustering of the relative positions of centroids.

Table 1

Groups of Atlantic salmon (*Salmo salar* L) sampled at ten different locations in Norway, number of individuals from each group, gutted body weight (mean and SD), pancreas disease (PD) diagnosis, detection of salmonid alphavirus (SAV) at different time points after sea transfer, specific antibodies to SAV determined at slaughter, and time between PD outbreaks and slaughter (months before slaughtering)

Group	Farm	n	Gutted weight, kg	PD diagnosis [1]	SAV				Time between PD outbreak and slaughter [3]
					2 months [2]	8 months [2]	Slaughter	Antibody	
Control	1	30	4.90 (1.00)	No	No	No	No	No	
	2	30	4.40 (1.06)	No	No	No	No	No	
SAV	3	30	3.75 (0.62)	No	No	No	Yes	No	
PD0	4	19	2.54 (0.57)	Yes	No	No	Yes	not ex	0 months
PD6	5	30	2.66 (0.76)	Yes	No	No	Yes	Yes	6.5 months
	6	20	3.41 (0.37)	Yes	No	No	Yes	Yes	7 months
PD12	7	17	4.13 (1.00)	Yes	No	Yes	Yes	Yes	11.5 months
	8	28	4.02 (1.20)	Yes	No	not ex	Yes	Yes	10.5 months
	9	20	3.75 (1.49)	Yes	not ex	not ex	Yes	Yes	14 months
PDchronic	10	30	3.30 (1.07)	Yes	Yes	Yes	Yes	Yes	14 & 5.5 months

[1] Summary of the production cyclus from sea water transfer until harvest

[2] Months after sea water transfer

not ex: not examined

Table 2

Condition factor, chemical parameters, muscle pH and melanin spots (LSmean±SE) in raw fillets of Atlantic salmon (*Salmo salar* L.) harvested after different time periods following PD outbreak.

	Control	SAV	PD0	PD6	PD12	PDchronic
Condition factor [1]	1.13±0.02^{a}	0.96±0.01^{c}	0.96±0.02^{bc}	0.96±0.03^{c}	1.02±0.02^{b}	0.92±0.02^{c}
Dry matter	30.4±0.1^{a}	30.4±0.4^{a}	27.9±0.9^{b}	29.3±0.2^{ab}	30.3±0.4^{a}	30.4±0.4^{a}
Fat content, %	9.2±0.4^{a}	8.8±0.4^{a}	7.7±1.0^{a}	8.3±0.6^{a}	9.3±0.4^{a}	9.5±0.5^{a}
Protein content, %	22.1±0.1^{a}	21.9±0.1^{a}	20.2±0.4^{c}	21.1±0.2^{b}	21.2±0.1^{b}	21.1±0.3^{b}
Muscle pH	6.10±0.01^{c}	6.18±0.01^{b}	6.21±0.01^{b}	6.22±0.01^{b}	6.29±0.02^{a}	6.21±0.02^{b}
Melanin, mm^{2}	8.3±4.7^{b}	19.8±12.7^{ab}	50.6±44.9^{ab}	25.4±17.9^{ab}	50.9±15.0^{a}	9.1±5.8^{b}

Different superscripts in the same row indicate significant variation ($P<0.05$).

[1] Condition factor: (Gutted body weight (g)* fish length (cm)$^{-3}$) *100

Table 3

Muscle properties and chemical parameters (LSmean±SE) of raw and smoked salmons sampled for analyses

		Control	SAV	PD0	PD6	PD12	PDchronic
NaCl, %	Plasma[1]	0.94±0.01^{b}	1.07±0.04^{a}	Not ex	0.89±0.02^{b}	0.96±0.04^{ab}	0.97±0.03^{ab}
	Smoked	3.7±0.22^{b}	4.4±0.14^{ab}	4.3±0.12^{ab}	4.7±0.23^{a}	4.3±0.24^{ab}	4.5±0.82^{a}
Gaping[2]	Raw	1.6±0.2^{b}	1.9±0.2^{ab}	1.7±0.4^{ab}	2.3±0.2^{a}	1.5±0.2^{b}	1.1±0.2^{b}
	Smoked	1.0±0.2^{a}	0.4±0.2^{a}	0.5±0.3^{a}	0.9±0.2^{a}	0.5±0.2^{a}	0.7±0.2^{a}
Drip loss, %	Raw	4.2±0.1^{b}	5.7±0.3^{a}	4.8±0.3^{b}	4.2±0.2^{b}	4.7±0.2^{b}	5.8±0.2^{a}
	Smoked	2.4±0.1^{b}	2.0±0.0^{c}	2.4±0.1^{b}	2.6±0.1^{b}	2.4±0.1^{b}	2.9±0.1^{a}

Different superscripts in the same row indicate significant variation ($P<0.05$).

not ex: not examined

[1] Plasma samples were sampled at slaughter

[2] Scale 0-5, where 0 is no gaping and 5 is extreme gaping (Andersen et al., 1994)

Table 4

Colorimetric parameter and coefficients of variance (CV) for raw fillets and colour differences (LSmean±SE) between raw and smoked fillets of salmon sampled for analyses

Colorimetric parameters [1]		Control	SAV	PD0	PD6	PD12	PD chronic
*L**	Raw	44.6±0.3^{e}	49.3±0.2^{c}	50.9±0.4^{ab}	50.9±0.4^{b}	47.5±0.2^{d}	51.9±0.3^{a}
*a**	Raw	30.2±0.2^{b}	27.1±0.2^{c}	25.3±0.5^{d}	30.7±0.4^{b}	27.0±0.3^{c}	32.1±0.3^{a}
*b**	Raw	32.2±0.2^{a}	26.4±0.2^{c}	24.2±0.3^{d}	32.2±0.4^{a}	27.8±0.4^{b}	32.0±0.3^{a}
H_{ab}	Raw	46.9±0.1^{a}	44.3±0.2^{cd}	43.8±0.2^{d}	46.3±0.1^{ab}	45.8±0.2^{b}	44.8±0.1^{c}
Variation within fillets[2]; %							
CV *L**	Raw	6.6±0.2^{ab}	6.9±0.3^{ab}	6.2±0.1^{ab}	6.5±0.3^{ab}	6.1±0.3^{b}	7.3±0.4^{a}
	Smoked	9.5±0.3^{a}	8.2±0.2^{b}	7.1±0.0^{c}	7.7±0.3^{bc}	8.3±0.3^{b}	9.1±0.3^{a}
CV *a**	Raw	4.3±0.2^{d}	5.0±0.2^{cd}	6.7±0.1^{a}	6.0±0.4^{a}	5.2±0.2^{bc}	5.9±0.2^{ab}
	Smoked	5.6±0.3^{b}	5.1±0.3^{b}	5.7±0.1^{b}	6.0±0.4^{b}	5.7±0.3^{b}	7.1±0.4^{a}
CV *b**	Raw	9.4±0.3^{ab}	9.3±0.4^{ab}	7.1±0.1^{c}	8.5±0.4^{bc}	8.8±0.4^{b}	10.4±0.4^{a}
	Smoked	18.9±0.7^{a}	14.8±0.6^{b}	15.6±1.3^{b}	17.0±0.8^{ab}	16.2±0.8^{b}	16.8±0.7^{ab}
CV H_{ab}	Raw	4.6±0.2^{ab}	5.3±0.2^{a}	4.6±0.4^{ab}	4.2±0.2^{b}	4.9±0.2^{ab}	4.9±0.2^{ab}
	Smoked	8.9±0.4^{a}	6.5±0.3^{b}	7.7±0.7^{ab}	7.9±0.4^{ab}	7.6±0.4^{ab}	7.4±0.5^{ab}
Reduction from raw to smoked fillets, %[3]							
Δ*L**		1.5±0.6^{d}	10.2±0.4^{c}	10.7±0.7^{c}	15.5±0.5^{b}	9.3±0.6^{c}	25.9±0.5^{a}
Δ*a**		23.8±0.8^{c}	14.0±0.6^{de}	17.4±1.0^{d}	27.2±1.3^{b}	13.4±1.1^{e}	44.9±0.5^{a}
Δ*b**		18.2±1.1^{b}	5.1±0.9^{cd}	1.8±1.3^{d}	17.0±1.7^{b}	7.4±1.1^{c}	34.7±0.9^{a}
ΔH_{ab}		-2.4±0.2^{a}	-2.8±0.3^{ab}	-4.9±0.4^{c}	-3.7±0.3^{bc}	-1-9±0.3^{a}	-4.8±0.3^{c}

Different superscripts in the same row indicate significant variation ($P<0.05$).

[1] Individual values represent an average of the seven areas (A-G) on each fillet (Fig. 1a).

[2] Data represent average coefficient of variation (CV, %) of colorimetric values between the 7 areas (A-G) on each fillet measured (Fig. 1a) within one group.

[3] Data represent average differences in colorimetric parameters between raw and smoked fillets.

Table 5

Classification matrix of Atlantic salmon (*Salmo salar* L.) harvested after different time periods following PD outbreak.

	Percent correct	Control	SAV	PD0	PD6	PD12	PDchronic
Control	94.7	54	0	0	0	3	0
SAV	75.9	0	22	0	0	7	0
PD0	62.5	0	5	10	0	1	0
PD6	67.9	2	1	1	19	0	5
PD12	66.7	8	9	3	1	42	0
PDchronic	96.4	0	0	0	1	0	27
Total	78.7	64	37	14	21	53	32

Rows: Observed classifications

Columns: Predicted classifications

A priori classification probabilities are proportional to group size

II

Paper II

Fillet quality and gene transcriptome profiling of heart tissue of Atlantic salmon with pancreas disease (PD)

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Submitted manuscript

Fillet quality and gene transcriptome profiling of heart tissue of Atlantic salmon with pancreas disease (PD)

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Abstract

This study determined the fillet quality and heart gene transcriptome in farmed Atlantic salmon (2.2 kg gutted weight) with varying pancreas disease (PD) pathology at slaughter: group A, SAV negative; group B, SAV positive; group C, SAV and PD positive. Gene expression and fillet quality characteristics of groups A and B were similar. Fish of group C had a thin body shape and pale and abnormally firm fillets with high collagen and low protein content, as well as an altered amino acid and fatty acid profile. They also had a marked reduction of energy metabolism and cell proliferation according to gene expression analyses. It is concluded that salmon infected with PD virus (SAV) may have acceptable fillet quality, but loss of pancreas may result in poor nutritional status of heart and muscle and unacceptable fillet quality. Hence such fish should not progress toward the market chain.

Keywords: Atlantic salmon, pancreas disease, salmonid alphavirus, fillet quality, heart pathology, microarray, gene expression

Introduction

Pancreas disease (PD) is a contagious disease caused by salmonid alphavirus (SAV) in Atlantic salmon and rainbow trout during the sea water phase. The disease was first recognized in Scotland in 1976 *(1)* and the first detected case in Norway was in 1989 *(2)*. Over the past years, PD has become a major problem for Norwegian salmon farmers, with 90 sites diagnosed for PD each year on average since 2006 (Norwegian Veterinary Institute). The losses associated with a PD outbreak for a standard farming location are estimated to be 1.8 million Euro due to e.g. mortality, growth retardation and reduced fillet quality *(3)*.

Pancreas is the primary organ damaged by PD, but severe cardiac and skeletal myopathies are also among the key features of this disease *(2, 4, 5)*. According to McLoughlin et al. *(5)* and Murphy et al. *(6)*, pancreatic and heart lesions often occur about one week after clinical signs of inappetence and lethargy, followed by an increase in the prevalence and severity of skeletal muscle pathology within 2-3 weeks *(5)*. The heart of diseased salmon in Norway seems to recover earlier than in Ireland, whereas more persistent damage of pancreatic tissue is reported in Norway in comparison with PD in Ireland and Scotland *(4)*. Due to the impaired function of the pancreas and hence the reduced ability to utilize nutrients from the feed, consequences of PD can persist long after the cessation of the outbreak.

Tissue damages caused by PD bear a strong similarity to the nutritional, toxic and exertional myopathies *(7)* that are common in other domestic animals under conditions of intensive culture *(8)*. The impact of PD related histopathological changes on important quality characteristics such as nutritional composition, sufficient and uniform color, and texture remains to be elucidated.

The objective of the current work was to study fillet quality properties and gene expression profile of heart tissue of Atlantic salmon within a population diagnosed with PD at the time of slaughtering. The salmon were collected from a farm that participated in a cohort study reported by *(9)*, and in a study that assessed quality of raw and smoked fillets of salmon from farms with different time from PD diagnosis to slaughter *(10)*.

Material and methods

Fish material

The fish in this study were Atlantic salmon (*Salmo salar* L.) obtained from a commercial fish farm, diagnosed with pancreas disease (PD) at slaughter without PD-related mortality, on the west coast of southern Norway. Results of average slaughter parameters, skeletal muscle composition, texture, color and drip loss (DL), as well as previous diagnostics, for this fish population are reported as farm number four by Lerfall et al. *(10)*.

As part of the cohort study *(9)*, samples for detection of SAV (heart and kidney) and SAV specific antibodies (serum), were collected at two and eight months after sea transfer. For these first two time points neither SAV nor SAV-specific antibodies were detected. At slaughter, samples for histopatology and heart and kidney samples for extraction of RNA were collected and subsequently used for real-time RT-PCR for detection of SAV *(9)* and for gene expression analyses of heart. For histopathology, samples from heart, pancreas and skeletal muscle were fixed in 10% phosphate-buffered formalin, embedded in paraffin wax and sections were stained with haematoxylin and eosin (HE) according to standard routines. The cellular changes in red and white muscle sections were characterized and graded as either 0 = none or sparse changes, or 1 = moderate to pronounced changes. No serum for antibodies was collected at slaughter. PD was diagnosed by the presence of characteristic tissue changes (*6)* combined with the detection of SAV *(9)*. The disease was classified as sub-clinical due to lack of abnormal mortality and behavior in the fish population.

Twelve fish with a similar body length and different PD pathology were selected among the 19 fish sampled from the farm in total *(10)*. The gutted body weight was 2.2 kg on average,

ranging from 0.9 kg to 3.3 kg (Table 1). Muscularity was expressed as the condition factor, CF: (gutted body weight (g) * fish length $(cm)^{-3}$) * 100. The selected fish were divided into three groups (n = 4 per group):

Group A, SAV negative.

Group B, SAV positive.

Group C, SAV and PD positive.

The salmon of group C had characteristic tissue changes (loss of exocrine pancreas and skeletal muscle lesions) for PD in combination with detection of SAV; hence these individuals were diagnosed with PD. The results of the diagnostic examinations for each of the 12 fish are presented in Table 1.

Fillet sampling

Locations on the fillet for analyses of texture, pH, and DL, as well as sectioning of the fillets for chemical analyses, seven days after slaughter are illustrated in Fig. 1. Section A (white muscle) was freeze dried (Christ Beta 1-16 LSC Freeze dryer, Martin Christ, Gefriertrocknungsanlagen, Osterode, Germany) and homogenized with dry ice before being used for analysis of amino acid (AA) profile and ash. Section B (white muscle) was stored at -80°C until required for analysis of chemical composition and tocopherols.

Texture, color, gaping, drip loss and pH

Texture analyses were performed instrumentally (TA-XT2, Stable Micro Systems Ltd., Surrey, England) by pressing a flat-ended cylinder (12.5 mm diameter, type P/0.5) into the fillet perpendicular to the muscle fibers at 1 mm/sec until reaching 90% of the initial fillet height (Fig. 1). The following parameters were registered from the resulting time-force curve:

the force (N) required to puncture the fillet surface (termed Fb), the force at 60% and 90% compression (termed F60 and F90, respectively), and the gradient (N/sec) of the linear part of the graph between the origin and Fb. Gaping was assessed using a scale from 0-5 according to the definition provided in *(11)*. Fillet color was expressed as *L*a*b**-values (*L** = lightness / translucence, 0-100, where 0 and 100 indicate black and diffuse white, respectively), *a** = redness (*a** > 0), *b** = yellowness (*b** > 0)), calculated from R (red), G (green) and B (blue) values, determined by image analysis according to *(12)* with some modifications *(10)*. Colorimetric parameters were analysed on seven areas placed manually on the fillet, avoiding dark spots (melanin) *(10)*, to enable calculation of variations in color between areas within fillets (CV%). DL was determined as the weight loss from a slice of white muscle during five days storage at 3°C *(13)* (modified as in *(14)*). Muscle pH was analyzed using a pH meter (330i, Wissenchaftlich-Techniche Werkstätten GmbH (WTW), Weilheim, Germany) connected to an electrode (BlueLine 21, Schott Instruments Electrode, SI Analytics GmbH, Mainz, Germany) and a temperature probe (TFK 325, WTW).

Composition

Protein was determined using the total Kjeldahl nitrogen method (Commission dir. 93/28/EEC) × 6.25. The astaxanthin and idoxanthin content were analyzed according to Vecchi et al. *(15)* and Aas et al. *(16)* respectively, and ash content was determined gravimetrically by drying samples at 105°C for 24 hours followed by combustion at 550°C *(17)*. Lipids were extracted according to *(18)* and used for the gravimetric determination of lipid content, analyses of fatty acids (FA) composition and tocopherols. The lipid class composition (triacyl glycerols, TAG and phospholipids, PL) was quantified using high-performance thin-layer chromatography (HP-TLC). Total lipids were applied using an automatic sample applicator (Linomat 5, CAMAG, Muttenz, Switzerland) onto a 10 x 20 cm

HP-TLC plate (Silicagel 60, Merck, Darmstadt, Germany) that had been pre-run in methanol and activated at 120°C for 20 minutes. The plates were developed to completion in hexane : diethyl ether : acetic acid (85 : 15 : 1) using an automatic developing chamber (AMD2, CAMAG, Muttenz, Switzerland). Lipid classes were visualized by dipping the plate for six seconds in a glass tank with 10% w/v copper sulfate and 4 % v/v phosphoric acid (85%) in methanol. The plate was dried and visualized (140°C for 5 minutes) on a TLC plate heater III (CAMAG). The lipid classes were identified by comparison with commercially available standards and quantified by scanning densitometry by absorption using a CAMAG TLC Scanner III (20 nm/sec, 400 nm), the amounts present being calculated using an integrator (WinCATS-Planar Chromatography, Version 1.4.3). Further, the quantities of lipid classes present were determined by establishing standard equations for each lipid class within a linear area. Furthermore, a standard mix of all the lipid classes was included on each HP-TLC plate to enable corrections of between-plate variations. FAs were determined from the fractions of phospholipids (PL) and triacylglycerides (TAG) by gas chromatography as described in *(19)*. α-tocopherols in white muscle was analyzed by HP-TLC using a modified method by *(20)*, as described by *(21)*.

The AA profile, including hydroxyproline (HYP) as a measure for collagen, and the AA metabolite taurine were analyzed according to Commission dir. 98/64/EC on a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, UK). Results for AA are presented as the percentage of total AA, based on water-corrected g AA per kg of freeze dried sample. Taurine is presented as g / kg freeze dried sample. The analytical methods for texture, gaping, color, DL, pH, protein and pigment are described in greater detail in *(10)*.

Glycosaminoglycans

In order to detect the presence of sulfated glycosaminoglycans (GAG) in white muscle, parallel sections from the formalin fixed and paraffin embedded muscle samples (collected for disease diagnostics, see above) were dyed with Alcian blue 8GX (Gurr Biological Stains, BDH, Poole, UK) *(22)*. The concentration of the staining solution ($MgCl_2$) was increased from 0.06 to 0.4 M to obtain specific staining of sulfated GAG.

Statistical methods

Statistical analyses were performed using SAS, version 9.1 for Windows (SAS Institute Inc., Cary, NC, USA). Groups A, B and C were compared by ANOVA, General Linear Model (GLM). The coefficient of variation (CV) was calculated as: 100 * standard deviation * mean^{-1}. The alpha level was set to 5% ($P < 0.05$).

Isolation of mRNA and gene expression analysis with microarray

RNA was isolated from heart tissue using the PureLink RNA Mini Kit (Invitrogen Corporation, Carlsbad, CA). Tissue was homogenized in TRIzol in a Precellys 24 (Bertin technologies, Montigny-le-Bretonneux, FRANCE). RNA integrity was assessed with Agilent 2100 Bioanalyzer. Analyses were performed in each of the 12 fish using Nofima's Atlantic salmon oligonucleotide microarray fabricated by Agilent Technologies (Santa Clara, CA, USA). The microarray contained 21 k unique probes to protein identified Atlantic salmon transcripts printed in duplicate *(23)*. Dual label hybridizations were carried out using a pool of all 12 samples as a common technical reference; all reagents and equipment were from Agilent Technologies. Microarrays were scanned with GenePix 4100A scanner (Axon Instruments, Union City, CA, USA) and GenePix Pro software version 6.0 was used for grid

alignment, quantification of signal intensities and evaluation of spot quality. Subsequent data analyses were carried out with the bioinformatic system STARS *(23)*. Lowess normalization was performed after filtration of spots flagged with GenePix; 11767 genes that passed quality control in at least 20 of 24 spots in 12 microarrays were selected. Given the minor differentiation between groups A and B, these groups were merged and compared with group C. Differentially expressed genes (DEG) were selected by criteria: $p < 0.05$ (t test) and > 1.6-fold difference. A search for enriched GO classes and KEGG pathways in the lists of DEG was performed. The numbers of genes corresponding to these classes were counted among DEG and all genes that passed quality control. Enrichment was assessed with Yates' corrected chi square test ($p < 0.05$); the terms with less than five genes were not taken into consideration.

Results

Biometrics

The average body length of the salmon was similar in all groups ranging from 62 to 65 cm. However, the muscularity (condition factor, CF) was significantly lower in group C (0.6) compared with groups A and B (1.0). The thin body and fillet shape of group C coincided with a one kg lower carcass weight on average (1.5 kg) compared with groups A and B (2.5 kg to 2.6 kg). Typical fillets of groups A, B and C are shown in Fig. 2.

Texture, gaping, color, pigment and drip loss

The breaking force (Fb) in group C (20.2 N) was 84% higher compared with groups A and B (11 N, Fig. 3). A similar pattern was observed among the other mechanical parameters (F60, F90 and gradient, data not shown). There were no significant differences in gaping or DL between the groups (Table 2). The photometric analyses showed that fillets in group C were more translucent (higher L^* value) and less red (lower a^* value) (Fig. 2.). On average the L^* values were 50.7, 49.9, and 54.3 in groups A, B, and C, respectively. The a^* values are depicted in Fig. 4. There was no significant difference in yellowness (b^* value) between the groups (23.6 on average). Fillets of fish from group C had a significantly higher CV of a^* (15%) than groups A and B (5-7%), but there were no differences in the CV of L^* and b^* (6-7%). Content of astaxanthin in fillets from group C was significantly lower (42%) than in groups A and B (Fig. 4), but idoxanthin content showed no difference between the groups (0.17 ± 0.05 mg/kg ww on average).

Gross composition and vitamin E (α-tocopherol)

The protein content differed significantly between the fish groups ($P < 0.01$; $R^2 = 0.74$), with the lowest significant content found in group C (4-5 percentage points) (Table 3). The fillet fat content of group C was 9.3% on average whereas that of groups A and B averaged 12.7% and 12.2%, respectively. The content of fillet fat, ash and α-tocopherols did not differ significantly between the groups but the pH was significantly higher in group C compared with groups A and B (Tables 2 and 4).

Lipid classes and fatty acid profile

The TAG and PL fractions were similar for all fish groups comprising 87.7% and 6.2% of the total lipids, respectively. The relative FA composition (% of total FA) of both the TAG and PL was significantly different in group C compared with groups A and B which had a similar composition. Numerically the most pronounced difference was seen in the PL fraction where the content of 22:6 *n*-3 was 14 percentage points higher in group C compared with groups A and B. The higher content of 22:6 *n*-3 was reflected in a significantly higher sum of *n*-3 PUFA whereas the content of saturated FA (SAFA) and monounsaturated FA (MUFA) was lower. The SAFA showed a similar variation pattern for the TAG and PL with a significantly lower content of 14:0 and 16:0, whereas for the MUFA, only the 16:1 *n*-7 was significantly lower in group C. The sum of MUFA of the TAG was not significantly different between the fish groups. Regarding *n*-6 FA, C20:4 *n*-6 of the PL in group C was significantly higher than groups A and B (Table 4).

Amino acid profile and taurine

The relative amounts of all amino acids (% of total AA) varied significantly between the groups, except for methionine, serine and lysine (Table 3). The relative contents of cysteine, aspartic acid, threonine, valine, isoleucine, leucine, phenylalanine and histidine were significantly lower in group C, whereas glutamic acid, proline, glycine, arginine and hydroxyproline were significantly higher. The content of tyrosine in group C was lower than in group A, but there was no difference between groups B and C. The content of alanine was lowest in group B. Group C had a significantly greater level of taurine (4.82 ± 0.98 g/kg) compared with groups A and B (2.68 ± 0.35 and 2.64 ± 0.59 g/kg, respectively).

Muscle histology

In fish from group C, Alcian blue stained white muscle showed more sulfated GAG around the muscle fibers and more connective tissue in certain areas. Overall, variation of muscle structure in group C was substantial whereas the fish from groups A and B had homogenous normal muscle cells. Typical images from Alcian blue staining of one fish from group A and one from group C are shown in Fig. 5. Muscle sections stained with HE showed degeneration and necrosis of muscle fibers mixed with seemingly normal fibers, as well as a chronic inflammatory reaction with fibrosis (images not shown). Grading of these changes in individual fish is shown in Table 1.

Gene expression of heart tissue

Hierarchical clustering combined all samples from group C in a sharply separated group while groups A and B were assigned to the same cluster (Fig. 6). To examine the effects of PD on gene expression, group C was compared with merged groups A and B and 800 genes were selected as DEG; 375 genes and 425 genes were respectively up- and down-regulated. The

search for enriched GO classes and KEGG pathways was achieved separately for up- and down-regulated genes. In the former, no consistent trend was revealed while thematic associations of down-regulated genes elucidated processes that were affected by the disease (Table 5). A coordinated decrease of expression was observed in genes involved in metabolism of amino acids and sugars, in mitochondrial processes and cell cycle related categories. Many genes from these groups and pathways showed sizeable expression changes; examples are given in Tables 6 and 7. Down-regulation of genes involved in the mobilization of glycogen was concordant with the decrease in expression of genes for glycolytic enzymes. There was also an increased expression of pyruvate dehydrogenase kinase, a negative regulator of metabolism. The TCA cycle was down-regulated together with genes involved in electron transfer and oxidative phosphorylation. The largest functional group depressed by PD in group C includes different aspects of the cell cycle (Table 7) starting from DNA repair and replication and biosynthesis of deoxyribonucleotides, the key material for the construction of DNA. Down-regulation was observed in a suite of genes coding for proteins required for formation and segregation of mitotic chromosomes, spindle assembly and microtubule based movement, which is the main driver of cytokinesis. A decrease in expression was observed in many genes that regulate the cell cycle at different stages. Genes encoding heat shock proteins were up-regulated, while several scavengers of free radicals were down-regulated. Regarding the extracellular matrix, type I collagen showed the greatest (11.4-fold) decrease in this study, with procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3, protein-lysine 6-oxidase and TGFbeta induced protein ig-h3 also being down-regulated. The angiopoietin-related protein 4 was up-regulated and there was an ectopic induction of pancreatic progenitor cell differentiation and proliferation factor.

Discussion

The fish examined in the present study were obtained from a commercial fish farm that was diagnosed with PD at slaughter, but with no previous PD related mortality. The fish examined were heterogeneous in regards to their health status. Four fish were diagnosed with PD and included in group C. Fish of group B were infected with SAV but were without tissue changes related to the disease, whereas fish of group A did neither have any detectable SAV nor PD related tissue changes. Different health status between individual fish in a farmed population has been reported earlier for PD *(9, 24)*. Accordingly, Kongtorp et al. *(25)* reported that an infection may be present sub-clinically in a population for a very long time, during which there may be a gradual increase in the number of fish showing lesions as well as the severity of the lesions.

The condition factor (CF), was relatively low in the salmon from groups A and B (1.0 on average), but within the normal range *(26)*. Conversely, group C had an abnormally thin body shape (CF 0.6 on average). It takes several months to develop changes of this magnitude due to disease *(4)* or starvation *(27)*.

Heart changes are among main characteristics of PD. However, in the present study, histopathological examination revealed no or only mild and unspecific changes. Likewise, microarray analysis of heart tissue revealed no indications of a viral infection. The Atlantic salmon microarray includes a large set of probes for virus responsive genes that are induced in the presence of RNA viruses, including SAV *(23)*. These genes did not show up-regulation in group C, suggesting that the virus was either absent from the heart or did not replicate. Microarray results showed no signs of inflammation or recruitment of immune cells that are

commonly recognized by distinct gene expression profiles. Therefore, profound changes in transcriptome profile in salmon hearts from group C were most likely due to PD-related dysfunction of the digestive system and malnutrition. Being functionally related, DEG have diverse roles. Enzymes of amino acid metabolism have different catalytic activities and substrate specificities (Table 6). Down-regulation of genes involved in glycogen metabolism, in addition to the up-regulation of pyruvate dehydrogenase kinase (a negative regulator of metabolism), suggest a reduced utilization of carbohydrates. Along with the down-regulation of aerobic cellular respiration, this suggests severe energy deprivation in the heart of salmon of group C. The regulation of heat shock proteins and free radical scavengers found in this study can be considered as evidence of cellular stress and as a sign of reduced protection from oxidative stress, respectively. The up-regulated angiopoietin-related protein 4 is a regulator of endothelial differentiation induced under hypoxic conditions. An intriguing finding was the ectopic induction of factors related to pancreatic cells, which may be indicative of a side effect of pancreas reparation. Suppression of amino acids and carbohydrate metabolism and mitochondrial processes could develop as a consequence of shortage in fuel supply and the need to reduce energy expenditure.

There was no consistent down-regulation of genes encoding myofiber proteins as would be expected with atrophy and loss of heart mass. However, a decrease of transcripts for type I collagen and enzymes involved in formation of extracellular matrix suggests structural changes in the heart in fish of group C. Most severe depressions were observed in a large group of genes involved in the cell cycle, an unexpected finding. Cardiac myocytes of mammals stop proliferation shortly after birth while fish hearts preserve high potential for regeneration *(28)*. However, the role of cell cycle arrest in the heart pathology of salmon was

unknown. Heart dysfunction impaired circulation and the supply of tissues with nutrients and oxygen and this most likely caused a further reduction in muscle quality.

The absence of antiviral and inflammatory transcripts in the hearts was not unexpected since only a mild, focal inflammation or absence of lesions in heart have been reported in a large proportion of fish with PD *(4)*. The heart is the first organ to recover *(29)*, and in the present work fish of group C were most likely in the late/regenerative stage of the disease *(4)*. To conclude, poor condition of PD diagnosed fish (group C) was most likely caused by impaired digestion and circulation, which resulted in a limited supply of nutrients to tissues.

In salmon fillets, the protein content is normally stable, ranging from 18 to 20% *(30, 31)*, whereas the fat content shows a pronounced variation ranging from 8-24% *(32)*. The muscle fat content of the salmon groups in the present study was similar and within the normal range of salmon unaffected by PD *(10)*. Accordingly, 86 days starvation of Atlantic salmon did not produce a significant effect on muscle fat content, but a significantly lower CF *(27)*. The low CF of group C corresponded with low protein content in the fillet. A dramatic decrease of protein content (up to 60%) takes place in feed deprived Pacific salmon during spawning migration *(33)*. The salmon of group C had 20 to 24% lower protein content compared with groups A and B, respectively. The degraded muscle proteins are most likely used as fuel *(33)*. The significantly firmer texture of fish of group C was probably related to an accumulation of collageneous scar tissue, supported by higher levels of fibrosis and HYP. Furthermore, a greater content of sulfated GAG *(34, 35)* may have contributed to firmer texture in these fish.

Fish of group C had lower levels of astaxanthin (Ax) and paler fillets (lower a^* and higher L^* values). Inappetence and a reduced ability to digest feed probably caused reduction of

pigmentation in the fish in group C. The retention of Ax is low (<20%) *(36, 37)* and hence, further digestive problems may be detrimental to pigment deposition. In addition to being paler, fillets of fish from group C also showed irregular coloration. Structural damage of white muscle and accumulation of scar tissue probably reduced the number of binding sites for Ax on its major binding protein alpha-actinin *(38)* and altered the light scattering of the muscle. Additionally, oxidative stress caused by the disease might have lowered Ax contents in fish from group C, as this pigment functions as an antioxidant in interactions with vitamin E *(39)*. The average vitamin E showed no significant difference between the fish groups, although the level was numerically highest of group A. These results correspond to those of McCoy et al. *(40)* who did not find an association between vitamin E deficiency and PD-caused myopathy. The lack of significant difference in muscle idoxanthin, a metabolite of Ax deposited in the muscle *(41)*, may indicate a slower pigment metabolism in the fish from group C.

The results on FA composition indicate a preservation of important membrane lipids (DHA), while SAFA and MUFA may be utilized to a higher degree for energy metabolism in fish of group C. Accordingly, in starved horse mackerel, high levels of *n*-3 PUFA were consistently preserved in dorsal muscle, whereas SAFA and MUFA were metabolized for energy production *(42)*.

All essential AAs except arginine were similar or significantly lower in group C. These results are in line with those observed from microarray analysis of heart tissue, also indicating a shortage in fuel supply in white skeletal muscle of this group. Elevated Cl^- plasma levels in SAV infected salmon indicated osmotic disturbances in the study of Lerfall et al. *(10)*. It is

interesting to notice that levels of taurine, which is an important regulator of osmosis, were significantly higher in white muscle of salmon belonging to group C.

The results demonstrated that a group of farmed salmon at the time of PD diagnosis may show a great variation among individual fish in pathological profile, gene expression profile in heart tissue, and fillet quality characteristics. Salmon infected with SAV, but with no signs of disease otherwise, were similar to those uninfected, whereas salmon infected with SAV in combination with loss of pancreas had thin body shape, poor nutritional status of heart and muscle, and unacceptable fillet quality. Hence such fish should not progress toward the market chain.

Abbreviations used

*a** = redness (*a** > 0)

AA = amino acid

*b** = yellowness (*b** > 0)

CF = condition factor

DEG = differentially expressed genes

DHA = docosahexaenoic acids

DL = drip loss

FA = fatty acid

GAG = glycosaminoglycans

GO = gene ontology

HE = haematoxylin and eosin

HPC = histopathological changes

HP-TLC = high-performance thin-layer chromatography

HYP = hydroxyproline

KEGG = Kyoto Encyclopedia of Genes and Genomes

*L** = lightness / translucence (0-100, 0 = black, 100 = diffuse white)

MUFA = monounsaturated fatty acids

NQC = Norwegian quality cut

PD = pancreas disease

PL = phospholipids

PUFA = polyunsaturated fatty acids

SAFA = saturated fatty acids

SAV = salmonid alphavirus

TAG = triacylglycerides

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Figure captions

Figure 1: Schematic illustration of sampling locations and analyses performed. A: analysis of hydroxyproline and ash; B: analysis of lipids, fatty acids, tocopherols, protein and pigment; C and E: texture analyses; D: pH and drip loss.

Figure 2: Fillets of farmed Atlantic salmon diagnosed with pancreas disease (PD) at slaughter. From top to bottom: example fillet from group A = SAV negative, B = SAV positive, and C = SAV and PD positive.

Figure 3: Fillet firmness (breaking strength) (1) and content of hydroxyproline (2) of farmed Atlantic salmon diagnosed with pancreas disease (PD) at slaughter. Results are presented as average per group: group A = SAV negative, B = SAV positive and C = SAV and PD positive. n = 4 fish / group.

Figure 4: The colorimetric value of a^* (1) and the level of astaxanthin (2) of farmed Atlantic salmon diagnosed with pancreas disease (PD) at slaughter. Results are presented as average per group: group A = SAV negative, B = SAV positive and C = SAV and PD positive. n = 4 fish / group.

Figure 5: Example images of histology sections of Alcian blue stained white muscle of farmed Atlantic salmon diagnosed with pancreas disease (PD) at slaughter. (1) Salmon from group A = SAV negative; (2) salmon from group C = SAV and PD positive.

Figure 6: Hierarchical clustering of microarray results, by 11.8 k genes, of four fish per group A, B and C. Euclidean distances were used as a metric and the tree was constructed with Ward's method.

Tables

Table 1.
Biometrics, histopathological changes in muscle and heart tissue, detection of salmonid alphavirus (SAV), and pancreas disease (PD)-diagnoses in 12 farmed Atlantic salmon from a population diagnosed with PD at slaughter. The fish were assigned to three groups: Group A = SAV negative, B = SAV positive and C = SAV and PD positive.

	Group A				Group B				Group C			
	1	2	3	4	1	2	3	4	1	2	3	4
Gutted weight (g)	2689	2070	2520	3262	2320	2756	2036	2801	1496	1488	949	2012
Body length (cm)	63	63	65	69	61	65	59	65	68	60	54	67
Condition factor[a]	1.1	0.8	0.9	1.0	1.0	1.0	1.0	1.0	0.5	0.7	0.6	0.7
Histopathological changes in[b]												
white muscle	0	1	0	1	0	0	0	0	1	1	1	1
red muscle	0	0	1	1	1	1	0	0	1	1	0	1
heart tissue	0	0	0	0	0	0	0	0	0	0	0	0
SAV	no	no	no	no	yes	yes	yes	yes	Yes	yes	yes	yes
Loss of pancreas	no	no	no	no	no	no	no	no	Yes	yes	yes	yes
PD	no	no	no	no	no	no	no	no	Yes	yes	yes	yes

[a] Condition factor: (Gutted body weight (g)* fish length $(cm)^{-3}$) *100
[b] Histopathological changes: None to sparse = score 0. Moderate to pronounced = score 1.

Table 2. Muscle pH, content of ash, α-tocopherols, fillet gaping and drip loss (DL) (mean ± SE) of farmed Atlantic salmon diagnosed with pancreas disease (PD) at slaughter. Group A = SAV negative, B = SAV positive and C = SAV and PD positive.

	Group A	Group B	Group C	P-value
pH	6.19 ± 0.02 b	6.20 ± 0.00 b	6.36 ± 0.04 a	0.002
Ash (% freeze dried muscle)	4.1 ± 0.4	3.7 ± 0.1	3.9 ± 0.6	0.841
α-tocopherol (µg / g fat)	137.9 ± 24.4	109.4 ± 1.7	111.0 ± 25.5	0.672
Gaping (score)	2.0 ± 1.2	1.5 ± 0.9	1.0 ± 1.0	0.805
DL (%)	4.7 ± 0.5	5.7 ± 0.6	5.7 ± 0.7	0.392

Different lower case superscripts in the same row indicate significant variation ($P < 0.05$) by one-way ANOVA.

Table 3. Protein content and amino acids (AA) of farmed Atlantic salmon diagnosed with pancreas disease (PD) at slaughter. Results are shown as average per group ± SE. Group A = SAV negative, B = SAV positive and C = SAV and PD positive.

	Group A	Group B	Group C	P-value
Total protein (% wet weight)	21.0 ± 1.4 a	19.8 ± 1.4 a	15.9 ± 1.5 b	0.004
Amino acid (% of total AA)				
Cysteine	1.14 ± 0.01 a	1.14[a] ± 0.03	1.07[b] ± 0.03	0.023
Methionine	3.29 ± 0.02	3.27 ± 0.02	3.29 ± 0.03	0.735
Aspartic acid	10.64 ± 0.05 a	10.67 ± 0.03 a	10.46 ± 0.05 b	0.011
Threonine	5.25 ± 0.03 a	5.20 ± 0.04 a	5.08 ± 0.02 b	0.002
Serine	4.40 ± 0.06	4.42 ± 0.04	4.41 ± 0.04	0.184
Glutamic acid	15.24 ± 0.20 c	15.41 ± 0.19 b	15.61 ± 0.22 a	0.002
Proline	3.63 ± 0.07 b	3.62 ± 0.04 b	3.79 ± 0.11 a	0.015
Glycine	4.69 ± 0.12 b	4.61 ± 0.14 b	5.04 ± 0.18 a	0.023
Alanine	5.90 ± 0.02 ab	5.80 ± 0.08 b	5.90 ± 0.05 a	0.082
Valine	5.53 ± 0.12 a	5.52 ± 0.12 a	5.38 ± 0.13 b	<0.001
Isoleucine	4.93 ± 0.06 a	4.94 ± 0.07 a	4.82 ± 0.08 b	0.006
Leucine	8.18 ± 0.03 a	8.21 ± 0.02 a	8.08 ± 0.03 b	0.018
Tyrosine	3.59 ± 0.15 a	3.56 ± 0.10 ab	3.55 ± 0.14 b	0.066
Phenylalanine	4.25 ± 0.04 b	4.30 ± 0.04 a	4.18 ± 0.05 c	<0.001
Histidine	3.01 ± 0.02 a	2.99 ± 0.04 a	2.68 ± 0.06 b	<0.001
Lysine	9.42 ± 0.12	9.41 ± 0.11	9.38 ± 0.17	0.515
Arginine	6.51 ± 0.07 b	6.55 ± 0.07 b	6.61 ± 0.08 a	0.009
Hydroxyproline	0.32 ± 0.07 b	0.30 ± 0.05 b	0.53 ± 0.04 a	0.071

Different lower case letters in the same row indicate significant variation ($P < 0.05$) by one-way ANOVA.

Table 4. Total lipids and fatty acid (FA) composition from the triacylglycerol (TAG) and phospolipid (PL) fractions in white muscle of farmed Atlantic salmon diagnosed with pancreas disease (PD) at slaughter. Results are shown as average per group ± SE. Group A = SAV negative, B = SAV positive and C = SAV and PD positive.[a]

	Fraction	Group A	Group B	Group C	P-value
Total lipid (% of ww)		12.7 ± 2.0	12.2 ± 4.3	9.3 ± 2.7	0.627
Fatty acid (% of total FA)					
C14:0	TAG	5.2 ± 0.1 a	5.5 ± 0.2 a	4.8 ± 0.1 b	0.019
	PL	2.1 ± 0.2 a	2.2 ± 0.2 a	1.2 ± 0.2 b	0.017
C16:0	TAG	14.0 ± 0.1 a	14.5 ± 0.1 a	12.7 ± 0.5 b	0.041
	PL	28.3 ± 2.2 a	30.3 ± 4.2 a	20.5 ± 0.6 b	0.028
C18:0	TAG	2.7 ± 0.0	2.7 ± 0.0	2.6 ± 0.1	0.817
	PL	5.3 ± 0.5	6.0 ± 0.6	4.6 ± 0.3	0.254
Σ SAFA	**TAG**	**22.5 ± 0.2 a**	**23.8 ± 0.4 a**	**21.0 ± 0.6 b**	**0.014**
	PL	**36.6 ± 2.7 a**	**39.1 ± 4.5 a**	**27.5 ± 0.5 b**	**0.025**
C16:1 *n*-7	TAG	5.3 ± 0.1 ab	5.8 ± 0.3 a	5.1 ± 0.1 b	0.037
	PL	2.2 ± 0.25 a	2.0 ± 0.2 a	1.2 ± 0.1 b	0.017
C18:1 *n*-7	TAG	2.9 ± 0.1	3.2 ± 0.1	2.6 ± 0.3	0.361
	PL	2.9 ± 0.2 a	3.0 ± 0.4 a	2.2 ± 0.1 b	0.046
C18:1 *n*-9	TAG	19.1 ± 0.5	18.3 ± 1.1	18.6 ± 1.2	0.843
	PL	9.0 ± 0.9	9.0 ± 1.8	6.7 ± 0.6	0.187
C20:1 *n*-9	TAG	9.6 ± 0.4	8.4 ± 0.3	9.5 ± 0.6	0.361
	PL	1.9 ± 0.2 a	1.6 ± 0.0 a	1.0 ± 0.1 b	0.006
C22:1 *n*-11	TAG	10.4 ± 0.6	9 ± 0.3	9.7 ± 0.6	0.442
	PL	1.4 ± 0.2 a	1.3 ± 0.4 ab	0.7 ± 0.1 b	0.073
Σ MUFA	**TAG**	**52.2 ± 0.9**	**49.9 ± 0.8**	**51.3 ± 2.0**	**0.656**
	PL	**20.5 ± 2.0 a**	**19.9 ± 2.8 a**	**12.3 ± 1.7 b**	**0.038**
C18:3 *n*-3	TAG	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	0.968
	PL	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.142
C20:5 *n*-3	TAG	5.3 ± 0.2	6.1 ± 0.9	5.4 ± 0.5	0.523
	PL	8.4 ± 0.8	8.3 ± 1.8	9.2 ± 0.5	0.693
C22:5 *n*-3	TAG	2.5 ± 0.1 b	2.7 ± 0.3 ab	3.1 ± 0.1 a	0.033
	PL	2.3 ± 0.3	2.0 ± 0.2	2.6 ± 0.1	0.270
C22:6 *n*-3	TAG	8.1 ± 0.4	8.6 ± 0.6	9.0 ± 1.0	0.661
	PL	26.8 ± 3.3 b	25.6 ± 5.0 b	40.3 ± 1.3 a	0.015
Σ PUFA *n*-3	**TAG**	**17.9 ± 0.7**	**19.5 ± 1.4**	**19.9 ± 1.4**	**0.468**
	PL	**38.5 ± 4.3 b**	**36.5 ± 6.8 b**	**52.9 ± 1.2 a**	**0.033**
C18:2 *n*-6	TAG	3.8 ± 0.1	3.8 ± 0.7	3.8 ± 0.2	0.978
	PL	1.3 ± 0.1	1.2 ± 0.4	1.0 ± 0.1	0.298
C20:4 *n*-6	TAG	0.5 ± 0.0 a	0.3 ± 0.0 b	0.4 ± 0.0 ab	0.119
	PL	0.9 ± 0.1 b	1.0 ± 0.2 b	1.6 ± 0.2 a	0.009
Σ PUFA *n*-6	**TAG**	**5.0 ± 0.2**	**4.7 ± 1.1**	**5.2 ± 0.1**	**0.581**
	PL	**2.7 ± 0.2**	**2.3 ± 0.3**	**3.0 ± 0.2**	**0.176**

[a] Not all fatty acids are reported. Values are mean ± SE. Different lower case letters in the same row indicate significant variation ($P < 0.05$) by one-way ANOVA.

Table 5. Enrichment of terms in the list of genes that were down-regulated in heart of salmon with PD (group C).

Term	Genes	Enrichment	P-value	Vocabulary
Alanine and aspartate metabolism	5	2.9	0.044	KEGG
Arginine and proline metabolism	8	4.4	0.000	KEGG
Glycine, serine and threonine metabolism	5	2.9	0.044	KEGG
Phenylalanine metabolism	6	5.7	0.000	KEGG
Tyrosine metabolism	6	3.2	0.013	KEGG
Valine, leucine and isoleucine degradation	6	2.8	0.030	KEGG
Citrate cycle (TCA cycle)	5	3.4	0.021	KEGG
Oxidative phosphorylation	12	3.0	0.001	KEGG
Pyruvate metabolism	5	2.9	0.048	KEGG
PPAR signaling pathway	7	2.9	0.015	KEGG
Fructose and mannose metabolism	12	6.2	0.000	KEGG
Glycolysis / Gluconeogenesis	22	7.9	0.000	KEGG
Pentose phosphate pathway	7	5.1	0.000	KEGG
Insulin signaling pathway	11	2.7	0.003	KEGG
Cell cycle	19	4.8	0.000	GO
DNA repair	6	3.5	0.008	GO
DNA replication	9	7.3	0.000	KEGG
Microtubule-based movement	9	5.4	0.000	KEGG
p53 signaling pathway	11	5.3	0.000	KEGG

Table 6. Differentially expressed genes involved in metabolism. Data are fold difference between groups C and A, B (mean ± SE).

Gene	Fold difference	Role
Amino acids metabolism		
Aspartate aminotransferase	-1.98 ± 0.22	aminotransferase
Serine--pyruvate aminotransferase	-2.19 ± 0.20	aminotransferase
Branched-chain-amino-acid aminotransferase	-1.99 ± 0.08	aminotransferase
Methionine-R-sulfoxide reductase B2	-3.24 ± 0.41	reductase
Sodium-coupled neutral amino acid transporter	-2.42 ± 0.53	transporter
Dihydropteridine reductase	-2.34 ± 0.24	degradation
Methylcrotonoyl-CoA carboxylase beta chain	-2.83 ± 0.20	degradation
Phosphoglycerate dehydrogenase	-2.05 ± 0.48	biosynthesis
Adenosylhomocysteinase B	-2.35 ± 0.49	cysteine, methionine metabolism
Mitochondria		
ATP synthase subunit alpha	-4.16 ± 0.94	oxidative phosphorylation
Cytochrome c oxidase subunit VIIa	-2.17 ± 0.37	oxidative phosphorylation
Cytochrome c oxidase subunit 4	-2.08 ± 0.12	oxidative phosphorylation
Cytochrome b-c1 complex subunit 6	-1.81 ± 0.11	oxidative phosphorylation
Isocitrate dehydrogenase 3 (NAD+) alpha	-2.03 ± 0.26	TCA cycle
Succinate dehydrogenase complex, subunit D	-1.93 ± 0.34	TCA cycle
Aconitase 2	-2.26 ± 0.51	TCA cycle
Cytochrome b-c1 complex subunit 2	-1.95 ± 0.36	oxidative phosphorylation
Cytochrome c	-2.24 ± 0.34	oxidative phosphorylation
ADP/ATP translocase 2	-2.38 ± 0.55	transport
Sugar metabolism		
Pyruvate dehydrogenase kinase isozyme 2	2.08 ± 0.28	negative regulator
Pyruvate kinase	-3.26 ± 0.61	glycolysis
Phosphoglycerate mutase	-2.22 ± 0.20	glycolysis
Glyceraldehyde phosphate dehydrogenase	-8.50 ± 0.87	glycolysis
6-phosphofructokinase type C	-4.09 ± 0.59	glycolysis
Triosephosphate isomerase	-4.63 ± 0.44	glycolysis
Phosphoglycerate kinase	-4.38 ± 0.38	glycolysis
Alpha-enolase	-2.46 ± 0.40	glycolysis
Glyceraldehyde-3-phosphate dehydrogenase	-7.89 ± 1.34	glycolysis
Fructose-bisphosphate aldolase A	-3.62 ± 0.71	glycolysis
Glucose-6-phosphate isomerase	-2.86 ± 0.18	glycolysis
Aldolase a, fructose-bisphosphate 1	-2.37 ± 0.04	glycolysis
Glycogen synthase	-1.86 ± 0.16	glycogen metabolism
Glycogen phosphorylase	-4.23 ± 0.64	glycogen metabolism
Phosphoenolpyruvate carboxykinase	-1.97 ± 0.07	gluconeogenesis

Table 7. Differentially expressed genes with roles in cell cycle, stress responses and tissue homeostasis. Data are fold difference between groups C and A, B (mean ± SE).

Gene	Fold difference	Role
Cell cycle		
DNA repair protein RAD51 homolog A	-4.03 ± 0.48	DNA repair
DNA replication complex GINS protein SLD5	-3.18 ± 0.67	DNA repair
Minichromosome maintenance complex 10	-1.96 ± 0.10	DNA replication
DNA primase large subunit	-2.22 ± 0.14	DNA replication
DNA replication licensing factor MCM3	-3.28 ± 0.60	DNA replication
DNA primase small subunit	-2.88 ± 0.27	DNA replication
DNA replication licensing factor mcm6-B	-1.76 ± 0.20	DNA replication
DNA replication helicase 2 homolog	-2.11 ± 0.12	DNA replication
DNA polymerase epsilon catalytic subunit	-3.08 ± 0.33	DNA replication
DNA replication licensing factor mcm2	-5.50 ± 0.75	DNA replication
Origin recognition complex subunit 5	-2.28 ± 0.16	DNA replication
Retinoblastoma-associated protein	-5.88 ± 1.44	regulator
G-2 and S-phase expressed 1	-2.51 ± 0.36	regulator
Cell division protein kinase 2	-3.26 ± 0.60	regulator
Cell division cycle protein 20 homolog	-3.65 ± 0.45	regulator
CDC45-related protein	-2.29 ± 0.28	regulator
Cyclin-A2	-4.38 ± 0.87	regulator
G2/mitotic-specific cyclin-B3	-1.95 ± 0.10	regulator
G2/mitotic-specific cyclin-B1	-5.87 ± 1.77	regulator
Cell division control protein 2	-5.04 ± 1.28	regulator
Cyclin-dependent kinase 2-interacting protein	-2.09 ± 0.54	regulator
Cyclin-dependent kinases regulatory subunit	-3.62 ± 0.36	regulator
Regulator of cytokinesis 1	-4.46 ± 0.71	regulator
Cyclin-dependent kinase inhibitor 1C	-2.64 ± 0.09	regulator
Dedicator of cytokinesis 7	-1.92 ± 0.35	regulator
cell division cycle associated 7	-2.08 ± 0.28	regulator
Cell division protein kinase 6	-5.58 ± 1.19	regulator
Histone H3-like centromeric protein A	-1.80 ± 0.08	chromosome maintenance
Centromere protein J	-4.21 ± 0.47	chromosome segregation
Chromosome transmission fidelity factor 8	-1.83 ± 0.14	chromosome segregation
Centromere protein N	-2.53 ± 0.34	chromosome segregation
Homologous-pairing protein 2	-2.69 ± 0.34	chromosome segregation
Sister chromatid cohesion protein DCC1	-4.26 ± 1.02	chromosome segregation
Centrosomal protein of 27 kDa	-2.16 ± 0.12	chromosome segregation
Kinetochore protein Spc24	-2.27 ± 0.22	chromosome segregation
Centromere protein H	-2.56 ± 0.39	chromosome segregation
Spindle and kinetochore-associated protein 1	-3.29 ± 0.66	chromosome segregation
Centromere protein M	-2.97 ± 0.25	chromosome segregation
Centromere protein S	-2.71 ± 0.50	chromosome segregation
Inner centromere protein B	-2.35 ± 0.32	chromosome segregation
Spindle pole body component 25	-5.29 ± 1.31	chromosome segregation
Targeting protein for Xklp2	-2.60 ± 0.34	spindle assembly
Mitotic spindle assembly checkpoint protein MAD2A	-2.76 ± 0.30	spindle assembly
Kinesin family member 20A	-3.44 ± 0.62	cytokinesis
Kinesin family member 2C	-2.61 ± 0.46	cytokinesis

Kinesin family member 4	-2.76 ± 0.22	cytokinesis
Microtubule-associated protein RP/EB	-1.89 ± 0.26	cytokinesis
Ribonucleoside-diphosphate reductase subunit M2	-2.31 ± 0.14	deoxyribonucleotides biosynthesis
Ribonucleoside-diphosphate reductase large subunit	-2.65 ± 0.18	deoxyribonucleotides biosynthesis
Thymidylate synthase	-7.79 ± 4.59	deoxyribonucleotides biosynthesis
Thymidine kinase	-5.53 ± 1.33	deoxyribonucleotides biosynthesis
Dihydrofolate reductase	-3.95 ± 0.77	folate metabolism
Putative c-Myc-responsive isoform 1	-3.51 ± 0.57	deoxyribonucleotides biosynthesis
Cellular and oxidative stress		
Heat shock protein 70b	1.80 ± 0.22	protein folding
Heat shock protein HSP 90-alpha	2.51 ± 0.66	protein folding
Heat shock protein hsp90	2.35 ± 0.45	protein folding
Small heat shock protein-like	2.06 ± 0.25	protein folding
Glutathione peroxidase type 2	-2.09 ± 0.07	oxidative stress response
Glutathione S-transferase theta-1	-2.05 ± 0.13	oxidative stress response
Peroxiredoxin-1	-1.84 ± 0.27	oxidative stress response
Peroxiredoxin-5	-2.26 ± 0.14	oxidative stress response
Peroxiredoxin-6	1.81 ± 0.08	oxidative stress response
selenoprotein H	-1.84 ± 0.13	oxidative stress response
Superoxide dismutase	-1.74 ± 0.10	oxidative stress response
Tissue structure and reparation		
Alpha 1 type I collagen	-11.39 ± 3.07	extracellular matrix
Procollagen C-endopeptidase enhancer 1	-4.06 ± 2.40	extracellular matrix
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	-4.79 ± 1.19	extracellular matrix
Protein-lysine 6-oxidase	-6.19 ± 2.15	extracellular matrix
Transforming growth factor-beta-induced protein ig-h3	-4.32 ± 1.71	extracellular matrix
Angiopoietin-related protein 4	4.57 ± 0.38	differentiation
Pancreatic progenitor cell differentiation	6.52 ± 1.21	differentiation

Figure 1

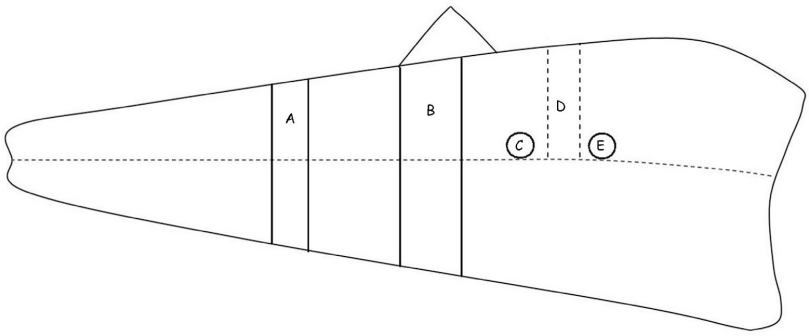


Figure 2

Figure 3

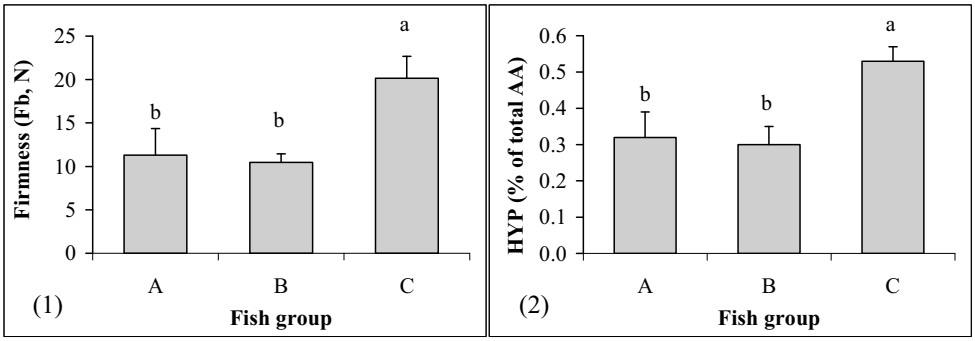

Firmness (Fb, N)
25
20
15
10
5
0
b
b
a
A
B
C
(1)
Fish group
HYP (% of total AA)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
b
b
a
A
B
C
(2)
Fish group

Figure 4

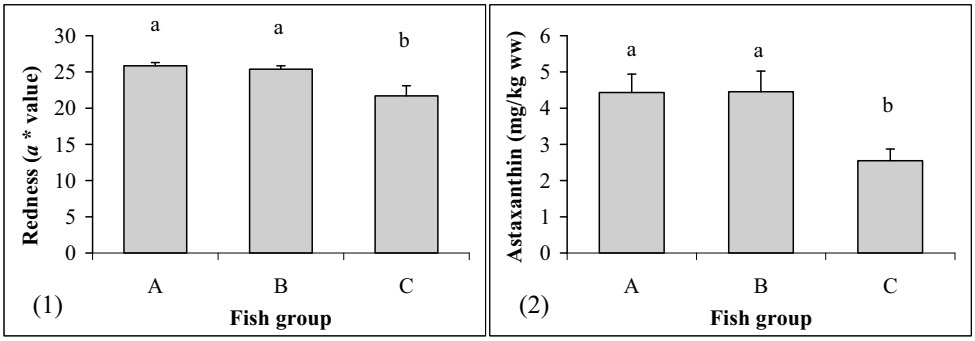

Redness (a* value)
30
25
20
15
10
5
0
a
a
b
A
B
C
(1)
Fish group
Astaxanthin (mg/kg ww)
6
5
4
3
2
1
0
a
a
b
A
B
C
(2)
Fish group

Figure 5

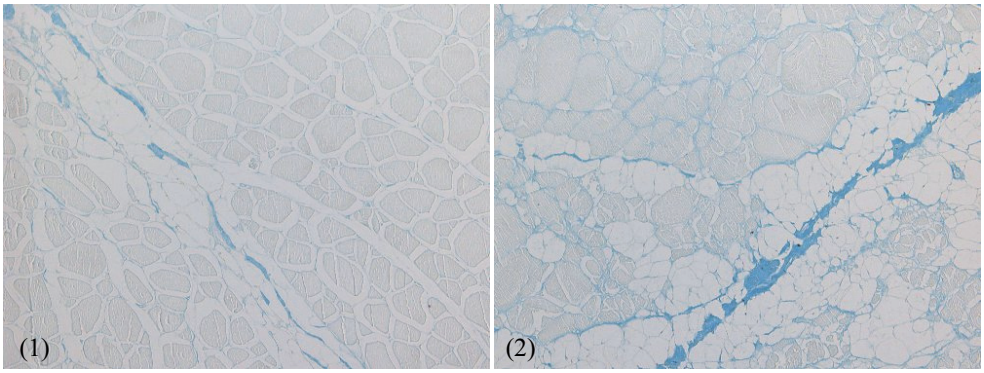
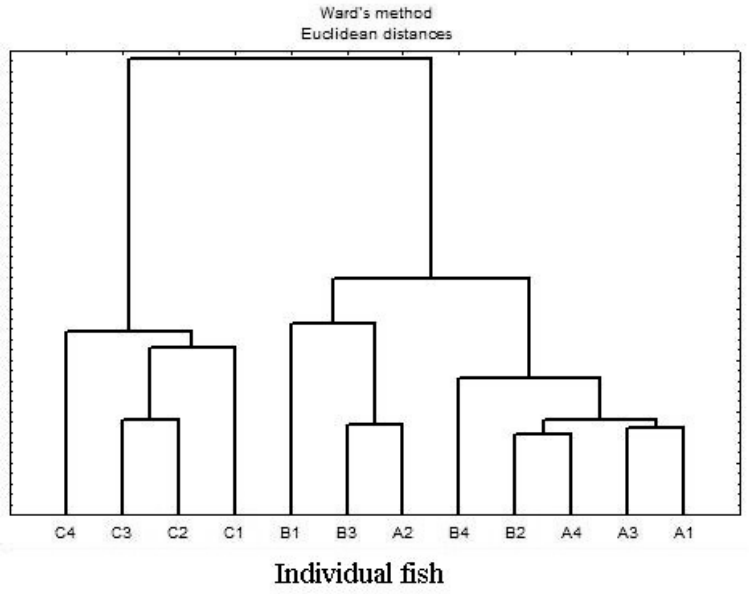


Figure 6


Ward's method
Euclidean distances
C4
C3
C2
C1
B1
B3
A2
B4
B2
A4
A3
A1
Individual fish

Paper III

Use of sodium nitrite in salt-curing of Atlantic salmon (*Salmo salar* L.) – Impact on product quality

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III

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Use of sodium nitrite in salt-curing of Atlantic salmon (*Salmo salar* L.) – Impact on product quality

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ABSTRACT

Quality changes during processing and quality differences in smoked fillets of Atlantic salmon (4–5 kg) salted with nitrite salt compared to table salted fillets were measured. Quality parameters from right-side fillets dry salted with nitrite salt were compared with the respective left-side fillets treated the same way with table salt. Ten raw right-side fillets were analysed and used as raw material reference. Use of nitrite salt in salt-curing of smoked salmon affected colour to a more reddish hue, tended to increase carotenoid stability and displayed positive effects on NaCl diffusivity. Only slight weight changes and change in texture properties were revealed. The use of nitrite salt displayed no adverse effects like increased content of *N*-nitrosoamines in smoked products. In fact, significant lower contents of *N*-nitrosoamines were found in nitrite salted smoked fillets compared to smoked fillets salted with table salt. Relatively high amounts of residual nitrite in nitrite-treated fillets seem to be the most prominent adverse effect caused by the use of nitrite salt in salt-curing of smoked Atlantic salmon.

1. Introduction

Among various seafood in Europe, fresh and cold-smoked Atlantic salmon (*Salmo salar* L.) are important products. Traditionally, wild salmon was used in the smoking industry, but during the last decades farmed salmon has been a more easily accessible raw material. Norway is one of the main suppliers to the European salmon marked, producing 755,000 metric tons (2008) of which 1850 metric tons (EFF, 2009) is sold as smoked salmon at the domestic market. In the European farmed salmon market, nearly 40–50% is consumed as smoked products (Borch & Aaker, 1997).

Salting, drying and smoking in combination is one of the oldest methods of food preservation (Horner, 1997). The process extends storage stability, enhances flavour and texture and modifies colour in the product. Nowadays cold-smoked salmon appears as a lightly preserved product with sodium chloride (NaCl) content in the range of 2.0–3.9% (Birkeland, 2004).

Salmonid fishes accumulate astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′dione) in muscle tissue (Torrisen, Hardy, & Shearer, 1989) and is the main carotenoid used for pigmentation of farmed Atlantic salmon. Fillet colour is held to be an important quality parameter for salmonid fishes (Choubert, Cravedi, & Laurentie, 2009). Redness contributes significantly to overall enjoyment of cooked salmonid flesh, and have a signalling value as an indicator of product quality (Sylvia, Morrissay, Graham, & Garcia, 1996), hence astaxanthin concentration and colour have the most marginal effects on eating quality and flavour (Østerlie, Bjerkeng, Karlsen, & Storrø, 2001).

Drying is used in the smoking industry to remove water from the fillets and hence to decrease the water and water activity of the products (Horner, 1997). Choubert, Blanc, and Courvalin (1992) noted that smoking of farmed rainbow trout induces loss of water, with an increase of carotenoid concentration and a decrease of hue and lightness. Other studies have reported a reduction in the three stimuli measurement (CIE $L^*a^*b^*$, 1994) parameter *alpha* (a^*) (Rørå et al., 1998), as well as an increase of *beta* (b^*), and a reduction of *lightness* (L^*). In curing of meat, nitrite salt (0.6% sodium nitrite and 99.4% sodium chloride) is commonly used. When added to meat, nitrite salt has several functions. Nitrite give the products a stable red colour, acts as an antioxidant, prevent or retards microbiological growth and finally, the curing agent gives the products a pleasant flavour.

Nitrite is unstable at acidic pH values and can disproportionate into nitrous acid, which again decomposes to NO and reacts with food components (Reinik, Tamme, & Roasto, 2008). Both nitrite salting and smoking of cured meat and fish will produce NO in

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the product. Yurchenko and Mölder (2006) found that NO produced in the wood smoke could react with secondary amines to form volatile *N*-nitrosoamines. This reaction will only occur when heating the products (>130 °C) at the same time as the pH is low enough to produce NO^+ or when a reduction–oxidation reaction with metal ions takes place to form NO^+. The NO formed from N_2O_3 can also bind to myoglobin (Fe^{2+}) and form a heat stable nitroso-myoglobin complex (NOMg) (Pacher, Beckman, & Liaudet, 2007). On heating NOMg the protein moiety is denatured but the red NO-porphyrin ring system still exists (Honikel, 2008).

When nitrite salt is used in salmon curing, formation of nitrosoastaxanthin can occur from NO reacting with peroxides under acidic conditions. Nitrosoastaxanthin has lower λ_{max} and less reddish hue compared to astaxanthin (Yoshioka et al., 2006).

Due to the complex chemistry of nitrite, nitric oxide and related compounds it is difficult to establish the level associated with health risk (Pacher et al., 2007). Natural levels of nitrite in food are usually lower than the detection limit. The European Commission's Scientific Committee for Food (SCF) set an acceptable daily intake (ADI) value for nitrite in 1995, which resulted in a present value of 0–0.06 mg nitrite per kg body weight (EU Scientific Committee for Food, 1995). However, nitrite in cured food is considered a health problem because it may lead to formation of carcinogenic *N*-nitrosoamines in the body. Acute nitrite toxicity will appear when the ferrous II ion (Fe^{2+}) in oxyhaemoglobin (Hb) is oxidised to ferric III ion (Fe^{3+}), which in turn can produce methaemoglobin (Met-Hb) when nitrate from food intake is reduced to nitrite (Pacher et al., 2007). Methaemoglobin is unable to bind and transport oxygen, and 60% Met-Hb of total Hb is considered lethal level (Hill, 1996).

The main goal of this research was to examine how nitrite salt affected colour and other quality parameters of smoked Atlantic salmon.

2. Materials and methods

2.1. Fish and processing materials

Farmed Atlantic salmon (4–5 kg, $n = 40$) were anaesthetized using CO_2, slaughtered, gutted and washed at a local salmon farmer in Trøndelag, Norway. The fish was immediately transported to Sør-Trøndelag University College, Trondheim, Norway where it was filleted, trimmed by hand and processed within 36–72 h. Raw material characteristics were carried out on the right-side (A) fillets of ten salmon. The average fat and dry matter content in raw fish were 21.4 ± 2.9% and 38.7 ± 2.0%, respectively (ranges 17.2–26.3% and 35.5–42.4%). The average α-tochopherol and nitrite content in raw fish were 17.6 ± 1.4 mg kg^{-1} and 0.41 ± 0.01 mg kg^{-1}, respectively (ranges 16.1–19.6 mg kg^{-1} and 0.40–0.44 mg kg^{-1}). Average total content of carotenoids in raw fish was 5.1 ± 0.4 mg kg^{-1} (range 4.6–5.9 mg kg^{-1}). Astaxanthin and idoxanthin comprised 5.0 ± 0.4 mg kg^{-1} and 0.08 ± 0.02 mg kg^{-1} of total carotenoids, respectively (range 4.5–5.8 mg kg^{-1} and 0.05–0.12 mg kg^{-1}). Colourimetric assessments; lightness (L^*), redness (a^*), yellowness (b^*) and hue (H^0_{ab}) average values were 43.0 ± 1.5, 8.9 ± 0.8, 16.3 ± 1.9 and 61.1 ± 1.1, respectively (ranges 40.7–45.2, 7.8–10.4, 13.9–19.5 and 59.4–63.3).

Different types of salt were used in the experiment: Nitrite salt (nutrient quality with 0.6% sodium nitrite, Akzo Nobel, GC Rieber, Norsal, Trondheim, Norway) and table salt (fine refined salt, minimum 99.8% NaCl, GC Rieber, Norsal, Trondheim, Norway).

Cod (*Gadus murhua*) (3 kg, $n = 3$) and herring (*Clupea harengus*) (400–500 g, $n = 3$) were bought at the local fish marked. They were filleted and trimmed by hand before processing.

2.2. Experimental design

2.2.1. Quality differences between smoked salmon fillets salted with nitrite and table salt (experiment 1)

Quality differences in smoked fillets salted with nitrite and table salt were measured on 30 Atlantic salmon. Right-side (A) fillets ($n = 30$) were dry salted with nitrite salt and quality parameters were compared with respective left-side (B) fillets ($n = 30$) treated with table salt. A and B fillets were divided into 3 groups ($n = 10$) and respectively subjected to 3 different salting times (15, 35 and 60 h) to obtain different salt concentrations. To measure quality differences between fillets salted with nitrite salt and table salt, fillets were analysed for texture, colour ($L^*a^*b^*$) and content of fat, dry matter, sodium chloride (NaCl), nitrite, astaxanthin, idoxanthin, nitrosoastaxanthin, vitamin E and *N*-nitrosoamines.

2.2.2. Changes in quality parameters from raw to smoked salmon fillets (experiment 2)

Quality changes during processing were measured on 10 Atlantic salmon. Raw material analyses were carried out on right-side (A) fillets ($n = 10$), used as raw material reference. To follow changes in fillets salted (35 h) with nitrite or table salt from raw material to final products, left-side (B) fillets were split into two groups and dry salted with nitrite salt ($n = 5$) and table salt ($n = 5$), respectively. Changes were measured by analysing weight, texture, colour ($L^*a^*b^*$) and content of dry matter, NaCl, astaxanthin and idoxanthin. To consolidate experiment 2, weight and colour changes between raw and smoked fillets of 30 Atlantic salmon from experiment 1 (salted for 15, 35 and 60 h) were added.

2.2.3. Colour changes in white fish (experiment 3)

To test the hypothesis that nitrite reacts with myoglobin to nitroso-myoglobin, cod ($n = 3$) and herring ($n = 3$) were split into right-side (A) and left-side (B) fillets and salted with nitrite salt and table salt, respectively. Colour changes ($\Delta L^*\Delta a^*\Delta b^*$ and ΔH^0_{ab}) between fillets salted with nitrite salt and table salt were compared.

2.3. Salting procedure

All fillets except the raw material reference in experiment 2 were dry salted in polyethylene boxes. To obtain different NaCl concentrations to compare quality changes between fillets salted with nitrite salt and table salt (experiment 1), different salting times were used. Fillets ($n = 10$) of each group were salted for 15, 35 and 60 h, respectively. To measure quality changes during processing (experiment 2), fillets were salted for 35 h ($n = 5$). In experiment 3, cod and herring were dry salted for 15 and 5 h, respectively. Before drying and smoking all fillets were steeped for one hour in cold water (8 °C).

2.4. Drying and smoking

Salt-cured salmon fillets from experiments 1 and 2 were sent to a commercial processing company, Trondheim, Norway, dried and cold-smoked before being returned to Sør-Trøndelag University College, Trondheim, Norway. Fillets were randomized on grid trolleys and dried at 20 °C for 180 min, then smoked for 300 min in a smoking cabinet. The fillets were stored at 4 °C for 12 h before analysing colour, texture and weight, and sample preparation. In experiment 3, cod and herring were dried and smoked at Sør-Trøndelag University College, Trondheim, Norway. Fillets were randomized on a grid trolley and dried at 20 °C for 180 min, then smoked for 300 min in a smoking cabinet.

2.5. Sample preparation

Salmon samples (experiments 1 and 2) for nitrite, NaCl, carotenoids, vitamin E and *N*-nitrosoamine analyses were prepared by homogenising a defined piece of raw and smoked fillets (350 g, Fig. 1.) on a Robot Coupe®Blixer 6 v.v. Homogenised samples were packed in 70 ml OLA-LT boxes, Landteknikk AS, Oslo, Norway (~50 g) and frozen at −80 °C until further analyses.

2.6. Weight change during processing

Weight changes (ΔM_t°) were calculated by a method after Gallart-Jornet et al. (2007).

$$\Delta M_t^\circ = \left(\frac{M_t^\circ - M_0^\circ}{M_0^\circ}\right),$$

where, M_0°, weight raw salmon fillet; M_t°, weight smoked salmon fillet.

2.7. Total fat

Fat was extracted and total amount calculated by a method after Bligh and Dyer (1959) with slight modifications.

2.8. Dry matter and sodium chloride content

Dry matter content of homogenised, raw and smoked salmon fillets was calculated gravimetrically after drying at 105 °C for 24 h (ISO 6496, 1983). Sodium chloride content was measured by a Chloride Analyser (Model 926 Sherwood Scientific Ltd.). Samples (1–1.5 g) were added hot deionised water (30 ml), homogenised (9500 rpm, 45 s.) by an Ultra-Turrax T25, Janke & Kunkel IKA®-Labortechnik, Staufen, Germany and heated in a water bath (100 °C, 10 min), cooled to room temperature and diluted to 100 ml in a volumetric flask before analyses.

2.9. Nitrite content

Nitrite content of homogenised, raw and smoked salmon fillets was extracted and analysed by a modified NCFA method (NCFA, 1982). Nitrite was quantified by using a standard curve prepared from nitrite solutions in the range 0–500 $\mu g\, l^{-1}$ $NaNO_2$.

Theoretical nitrite values in nitrite-treated fillets were calculated from amount sodium chloride detected. Amount nitrite in commercial nitrite salt is 0.6% w/w.

2.10. Colourimetric measurements

Colourimetric assessments** (CIE $L^*a^*b^*$, 1994; Birkeland, 2004) were performed on all raw and smoked fillets (Atlantic salmon, cod and herring). The measurements were taken at tree defined points (Fig. 1.) with a Minolta Chroma meter, CR200 Minolta, Japan. L^* describes the lightness of the sample, a^* intensity in red ($a^* > 0$), b^* intensity in yellow ($b^* > 0$) and the hue angel H_{ab}^0, where $H_{ab}^0 = 0$ for red hue and $H_{ab}^0 = 90^\circ$ for yellowish hue. Chroma (C^*) is the colour saturation where low chroma indicate greyscale and high chroma brightness. Average values per fillet were used for statistical analyses.

Changes (Δ) of colour parameters and colour difference (ΔE) between the raw fillet ($L_1a_1b_1$) and the smoked fillet ($L_2a_2b_2$) were calculated for Atlantic salmon as described by Birkeland (2004) and CIE $L^*a^*b^*$ (1994).

2.11. Carotenoid content

Astaxanthin and idoxanthin in muscle tissue were extracted by a method after Bligh and Dyer (1959) with slight modifications. The HPLC used was an Agilent1100 liquid chromatograph (Agilent Technologies, Paolo Alto, CA, USA) connected to an Agilent photodiode array UV–VIS detector. Astaxanthin was analysed on a $H_3PO_4^-$ modified silica gel HPLC column (Lichrosorb SI60–5, 125 × 4.0 mm, 5 μm, Hichrom, Reading, UK) and detected at 470 nm with hexane:acetone (86:14) as mobile phase (isocratic, flow 1.0 ml min^{-1}). Idoxanthin analyses was carried out on a Luna CN 100A, 250 × 4.6 mm, 5 μm, Phenomenex®, USA, HPLC column and detected at 470 nm with hexane:acetone (80:20) as mobile phase (isocratic, flow 1.5 ml min^{-1}). Astaxanthin and idoxanthin were quantified by response factors (RF) prepared from standards of known concentrations. These standards were prepared from crystalline all-*E*-astaxanthin (AcrosOrganics, 328612500) and 3′,4′-*cis* and 3′,4′-*trans* isomers of idoxanthin reduced from astaxanthin by a method after Aas, Bjerkeng, Hatlen, and Storebakken (1997) with slight modifications. The concentration of the standard solutions were measured using a spectrophotometer (UV-1700, Shimadzu) using molar absorbtivity $E_{1\%,\ 1\ cm} = 2100$ (acetone, $\lambda_{max} = 472$ nm) for all-*E*-astaxanthin and $E_{1\%,\ 1\ cm} = 2245$ (acetone, $\lambda_{max} = 458$ nm) for 3′,4′-*cis* and 3′,4′*trans* isomers of idoxanthin (Aas et al., 1997). To identify peaks, retention times (t_R) and VIS-spectra were compared with those of the standard solutions. As a final control, samples were spiked with standards to ensure that they were coeluated.

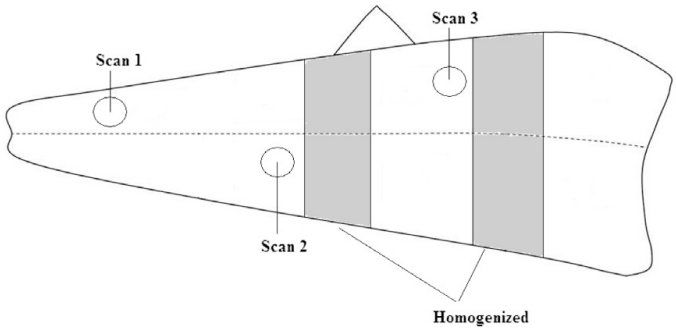

Fig. 1. Gray areas were homogenised and used for chemical analyses (stored at −80 °C until analysis). Scan 1, 2 and 3 indicated areas selected for colour measurements of Atlantic salmon, cod and herring fillets.

2.12. Nitrosoastaxanthin content

Nitrosoastaxanthin was synthesised and analysed by a method after Etoh, Yoshioka, Hayakawa, Kulkarni, and Maoka (2006). Salmon samples were extracted by a method after Bligh and Dyer (1959) with slight modifications.

2.13. Texture measurements

Textural properties were measured with a TA-XT2 Texture Analyser (Stable Micro Systems, UK). The probe used was a flat-ended cylindrical plunger with diameter 12 mm. Measurements was made in front of the dorsal fin, about 2 cm above the lateral line. The probe was pressed 5 mm into the fillets and the resistance force (sample firmness) at the endpoint was measured.

2.14. Vitamin E (α-tocopherol) content

Homogenised samples (5 g) were weighed out in plastic tubes. Ethanol (96%, 6 ml) was added and homogenised (9500 rpm, 2 min) using an Ultra-Turrax T25, Janke & Kunkel IKA®-Labortechnik, Staufen, Germany. Samples were saponified with KOH (0.5 M in CH_3OH, BHT 0.2%) and extracted with hexane:diethyl ether (4:1; v/v). Vitamin E (α-tocopherol) content was analysed on HPLC, Agilent1100 liquid chromatograph (Agilent Technologies, Paolo Alto, CA, USA) connected to an Agilent photodiode array UV–VIS detector using a not endcapped silica gel HPLC column (Suplex PKB-100, 250 × 4.6 mm, 5 μm, Supelco, USA). Vitamin E (α-tocopherol) was detected at 295 nm (21 °C) with methanol:methyl-*tert*-buthyl ether + water (80:20 + 5; v/v) as mobile phase (isocratic, flow 0.8 ml min^{-1}) and quantified by response factors (RF) prepared from standard α-tocopherol (Calbiochem, Germany).

2.15. N-Nitrosoamine content

N-Nitrosoamines were analysed on a GC–MS, Varian CP 3800 Gas Chromatograph (Varian Inc., Paolo Alto, CA, USA) connected to a Varian Saturn 2200 GC/MS CI detector using a Varian VF-1301MS (30 m × 0.25 mm i.d. df = 0.25 μm) column. Acetonitrile were used as CI agent. MS parameters were raised after an EPA method (EPA, 2004) with modifications on retention time window and ion scan range for *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodipropylamine-d14 (NDPA-d14, internal standard), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP) and *N*-nitrosodibuthylamine (NDBA). Salmon samples were extracted and prepared as described by Yurchenko and Mölder (2006) before analysis. NDPA-d14 was used as an internal standard for quantification of *N*-nitrosoamines.

2.16. Statistical analyses

MINITAB Statistical Software for Windows, Release 15, Minitab Inc., USA was used for statistical analysis. The significance ($P < 0.05$) of any difference between different salting times or the impact of treatment was determined using one and two-factor analysis of variance with replication (ANOVA) combined with Tuckey's pairwise comparison test. Where the design was unbalanced, a general linear model (GLM) was used. Correlations were carried out using a Pearson correlation test. To obtain *P*-values between different groups, two-sampled *t*-tests assuming equal variances ($n < 30$) and unequal variances ($n > 30$) were carried out using Microsoft Office Excel 2003 for Windows (Microsoft Corporation, USA). Where changes between parameters were close to being significant, the terminology "tends to" was used in combination with exact *P*-values.

3. Results and discussion

3.1. Quality differences between smoked salmon fillets salted with nitrite and table salt (experiment 1)

Experiment 1 was set up to show differences in quality parameters between salmon fillets salted with nitrite and table salt. Parameters measured were content of NaCl, nitrite, total fat, astaxanthin, idoxanthin, nitrosoastaxanthin, α-tochopherol and *N*-nitrosoamines together with resistant force and colourimetric assessments. To examine how different concentrations of NaCl and nitrite affected examined parameters, fillets were salted for 15, 35 and 60 h to obtain different NaCl and nitrite concentrations (Table 1).

A significant interaction between salting time and type of salt was found. Nitrite salted fillets displayed no significant differences in NaCl absorption between 15 and 35 h salting, but significantly higher NaCl absorption after 60 h, compared to corresponding fillets salted with table salt. This could probably be explained by faster diffusion of $NaNO_2$ into the matrix, followed by more rapid protein denaturation and higher degradation of the cell structure. Correlation analysis between weight change and NaCl concentration of all samples (experiment 1 and 2 together) displayed a significant negative correlation ($R^2 = -0.48$). Nitrite salted fillets ($R^2 = -0.63$) displayed a better correlation compared to fillets salted with table salt ($R^2 = -0.10$, not significant).

Table 1
Content of NaCl, $NaNO_2$, astaxanthin, idoxanthin and vitamin E (α-tochopherol) in smoked Atlantic salmon fillets salted with nitrite salt and corresponding fillets salted with table salt.

Salting time and type of salt used	NaCl (g 100 g^{-1})	Nitrite (mg kg^{-1})	Nitrite (theoretical) (mg kg^{-1})	Astaxanthin (mg kg^{-1})	Idoxanthin (mg kg^{-1})	α-tochopherol (mg kg^{-1})
15 h						
Nitrite salt	2.6 ± 0.2^{c}	72.1 ± 17.2^{a}	157.3	5.4 ± 1.0^{a}	0.1 ± 0.0^{a}	17.3 ± 2.8^{a}
Table salt	3.0 ± 0.2^{bc}	1.3 ± 0.5^{b}	0	5.4 ± 0.7^{a}	0.2 ± 0.0^{a}	17.5 ± 3.1^{a}
35 h						
Nitrite salt	3.6 ± 0.2^{b}	73.8 ± 16.8^{a}	213.2	5.1 ± 1.1^{a}	0.1 ± 0.1^{a}	19.2 ± 3.2^{a}
Table salt	3.3 ± 0.3^{bc}	1.0 ± 0.2^{b}	0	5.0 ± 0.7^{a}	0.2 ± 0.1^{a}	19.6 ± 3.4^{a}
60 h						
Nitrite salt	4.4 ± 0.1^{a}	88.3 ± 25.5^{a}	263.5	6.0 ± 0.8^{a}	0.1 ± 0.1^{a}	16.9 ± 3.0^{a}
Table salt	3.5 ± 0.7^{b}	1.3 ± 0.3^{b}	0	5.1 ± 0.7^{a}	0.2 ± 0.0^{a}	17.7 ± 2.5^{a}

The data represent average value ± standard deviation of 10 salmon fillets, except for [NO_3^-] of fillets salted with table salt where $n = 6$. Significant differences ($P < 0.05$) between means in the same column are indicated with different superscript a, b and c. Theoretical nitrite content (mg kg^{-1}) was calculated according to the formula: ([NaCl] (g 100 g^{-1})) × 0.06) × 100 × 10).

Nitrite content in fillets in connection to salting time is shown in Table 1. Nitrite content in nitrite-treated fillets tended to increase with increased NaCl concentration and displayed a good and significant correlation ($R^2 = 0.72$). The content of nitrite in muscle tissue is decreased in proportion to theoretical values for high NaCl concentrations (Table 1). A possible explanation is that NO_2^- in acidic environment can react to nitrous acid. Thorarinsdottir, Arason, Bogason, and Kristbergson (2004) found that pH in cod muscle decreased with increased NaCl concentrations and decreased water content. After nitrous acid is formed, the corresponding anhydride could be in equilibrium with the oxides NO and NO_2 (Honikel, 2008). NO reacts with myoglobin, which led to decreased nitrite content in muscle tissue. In smoked fillets salted with table salt, small amounts of nitrite was found, which can be explained by the fact that nitrite oxide is a biological molecule (Pacher et al., 2007) parallel to the formation of nitrite under the smoking process.

Residual nitrite found in nitrite-treated salmon averaged 78.1 mg kg^{-1}. Acceptable daily intake (ADI) given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and European Commission's Scientific Committee for Food (SCF) for a 70-kg person is 4.9 mg day^{-1}. Environmental Protection Agency (EPA) is more liberal and has set ADI for a 70-kg person to 7 mg kg^{-1} body weight (Erkekoglu, Sipahi, & Baydar, 2009). However, nitrite intake from one meal (100 g) nitrite-treated smoked salmon will exceed the limit of JECFA and SCF. With long-term daily intake of high nitrite levels exceeding ADI, the risk of mild to moderate methemoglobinemia would be increased, especially for susceptible populations such as young children and elderly (Erkekoglu et al., 2009). In the case of nitrite-treated smoked salmon, this should be taken under consideration.

Average content of total fat in nitrite-treated fillets salted for 15, 35 and 60 h were 20.75 ± 2.15%, 23.73 ± 3.64% and 18.23 ± 1.91%, respectively, while total fat content of fillets salted with table salt were 20.93 ± 3.08%, 23.88 ± 2.77% and 21.57 ± 1.38%, respectively. The fat content increased significantly with increased salting time independent of type of salt used, except for fillets salted for 60 h. This could probably be explained by biological variations according to fat content in salmon, and a significant negative correlation between contents of fat and water ($R^2 = -0.51$).

Within groups of nitrite-treated fillets, a slight tendency of higher astaxanthin content were found in the group salted for 60 h ($P = 0.077$) compared to the group salted for 15 h. For fillets salted with table salt, no significant differences were found (Table 1). Between fillets salted with nitrite and table salt (all groups together), significant differences were found in astaxanthin and idoxanthin (a metabolite of astaxanthin) content. Nitrite-treated fillets showed lower content of idoxanthin and higher content of astaxanthin compared to fillets salted with table salt; an explanation could be a tendency of increased stability of astaxanthin caused by the preserving effect of nitrite (Cuppett, 1988). However, no differences in α-tochopherol content between different groups were observed, revealing that no peroxidation has occurred. This supports that nitrosoastaxanthin was not found in any of the smoked fillets. To understand the increased stability of astaxanthin in nitrite-treated fillets, other factors have to be taken under consideration. Birkeland (2004) showed differences in solubility of astaxanthin in different brine concentrations and reported that astaxanthin was washed out during the salting process. In this study, the incorporation of nitrite in the salt probably affects the solubility of astaxanthin and might result in lower loss of astaxanthin during the salting step. Another explanation could be that loss of water due to increased NaCl concentrations in nitrite-treated fillets resulted in higher amounts of astaxanthin in these fillets. Further research has to be carried out to fully understand these mechanisms.

Table 2
Colour measurements (CIE $L^*a^*b^*$, 1994) of smoked salmon fillets salted with nitrite salt and corresponding fillets salted with table salt.

Salting time and type of salt used	L^*	a^*	b^*	H^0_{ab}
15 h				
Nitrite salt	37.7 ± 1.4^{b}	7.3 ± 0.7^{a}	15.3 ± 1.1^{a}	64.5 ± 1.4^{b}
Table salt	39.4 ± 1.1^{a}	4.7 ± 0.7^{b}	15.1 ± 1.4^{a}	72.5 ± 1.8^{a}
35 h				
Nitrite salt	37.7 ± 1.1^{b}	7.2 ± 1.1^{a}	16.5 ± 1.0^{a}	66.6 ± 2.4^{b}
Table salt	38.7 ± 1.3^{ab}	5.2 ± 1.3^{b}	16.2 ± 1.7^{a}	72.2 ± 3.9^{a}
60 h				
Nitrite salt	38.4 ± 0.6^{ab}	6.7 ± 0.6^{ac}	15.4 ± 0.8^{a}	66.4 ± 1.3^{b}
Table salt	40.0 ± 1.4^{a}	5.6 ± 1.7^{bc}	16.2 ± 1.7^{a}	71.0 ± 5.5^{a}

The data represent average value ± standard deviation of three measurements (Fig. 1) from each sample of 10 fillets. Significant differences ($P < 0.05$) between means in the same column are indicated with different superscript a, b and c.

Nitrite-treated fillets displayed significantly higher a^*-values and lower H^0_{ab} and L^* values compared to the corresponding fillets salted with table salt, i.e. more reddish hue in nitrite-treated fillets (Table 2). For intensity in yellow (b^*), no significant differences were found (Table 2). No significant differences in colour were found between the three groups processed with nitrite salt. The colour of the product will be influenced by all operating steps in the smoking procedure. Brining, dry salting or injection-salting are believed to have different affect on quality and yield of smoked salmon fillets (Birkeland, 2004; Gallart-Jornet et al., 2007). A sensory evaluation study of cold-smoked salmon available on the European market revealed the main discriminating factors to be colour, intensity and characteristic of smoke note, amine note and salty perception (Cardinal et al., 2004). No sensory evaluation of nitrite-treated cold-smoked salmon has yet been carried out, but a study by Cuppett (1988) did not find any differences in taste of smoked Great Lakes Whitefish treated with and without nitrite after 14 days of storage. Other studies by Froehlich, Gullett, and Usborne (1983) on ham, and Pluta, Zmarlicki, Gawel, and Ostrowski (1988) on cheese supported that sensory properties are probably not affected by low levels of nitrite. Sensory evaluation of nitrite-treated cold-smoked salmon could be an aim of further research.

Average resistant force of nitrite-treated fillets salted for 15, 35 and 60 h were 437.4 ± 106.1 g, 517.9 ± 94.2 g and 478.2 ± 80.3 g, respectively, while resistance force of fillets salted with table salt were 419.3 ± 98.0 g, 448.1 ± 105.6 g and 426.9 ± 111.2 g, respectively. In these study nitrite-treated fillets showed higher content of NaCl compared to fillets salted with table salt. According to Gallart-Jornet et al. (2007) one could expect a positive correlation between resistant force and content of NaCl, but texture analysis in this study showed only a slight tendency ($P = 0.074$) of increased resistant force in fillets salted with nitrite salt compared to table salt.

Average amounts *N*-nitrosoamines found in smoked salmon salted with nitrite salt and table salt were 13.5 ± 2.1 μg kg^{-1} and 18.1 ± 5.2 μg kg^{-1}, respectively (ranges 9.8–16.5 μg kg^{-1} and 11.1–26.6 μg kg^{-1}). *N*-Nitrosoamines found were NPIP and NPYR (Table 3). No traces of NDMA, NDEA and NDBA were found. This could be explained by the freshness of the sample, low temperature in the cold smoking process, low content of trimethylamine *N*-oxide (TMAO) in farmed Atlantic salmon and the fact that salmonid fish have relatively low contents of secondary amines (Belatti & Parolari, 1982). All groups together displayed a tendency of lower content of NPYR ($P = 0.056$) and significantly lower content of NPIP and total amounts of *N*-nitrosoamines in nitrite-treated fillets compared to fillets salted with table salt. This is probably due to

Table 3
Content of *N*-nitrosoamines (μg kg^{-1}) in smoked salmon fillets salted with nitrite salt and corresponding fillets salted with table salt.

Salting time and type of salt used	NDMA NDEA NDBA	NPYP	NPIP	Total amount *N*-nitrosoamines
15 h				
Nitrite salt	n.d	7.2 ± 0.6^{a}	6.7 ± 0.4^{ab}	13.9 ± 0.8^{a}
Table salt	n.d	7.0 ± 1.5^{a}	11.8 ± 5.1^{ab}	18.9 ± 6.5^{a}
35 h				
Nitrite salt	n.d	7.0 ± 1.3^{a}	6.5 ± 1.8^{ab}	13.5 ± 3.2^{a}
Table salt	n.d	9.2 ± 2.2^{a}	10.4 ± 3.9^{a}	19.6 ± 6.0^{a}
60 h				
Nitrite salt	n.d	7.9 ± 1.7^{a}	5.1 ± 0.6^{b}	12.9 ± 2.2^{a}
Table salt	n.d	9.9 ± 2.1^{a}	6.2 ± 2.6^{ab}	16.1 ± 3.4^{a}

The data represent average value ± standard deviation of 5 salmon fillets. Significant differences ($P < 0.05$) between means in the same column are indicated with different superscript a and b. Not detected is indicated with n.d.

the preservative effect of nitrite, which may lead to increased stability of secondary amines and lower contents of *N*-nitrosoamines. In another study by Yurchenko and Mölder (2006), a lower content of *N*-nitrosoamines compared to our study were found, i.e. total amounts of *N*-nitrosoamines in hot and cold-smoked salmon of 4.6 and 1.7 μg kg^{-1}, respectively. Differences between experimental designs, analytical artefacts and the fact that age, environment, bacterial flora, and storage conditions affect amine concentrations in the fish can state this (Fiddler, Doerr, Ertel, & Wassermann, 1971). Yurchenko and Mölder (2006) detect NDMA and NDEA in smoked Atlantic salmon. These *N*-nitrosoamines were not detected in our study.

Amounts of *N*-nitrosoamines found in smoked salmon fillets in our study and by Yurchenko and Mölder (2006), compared to amounts in bacon and beer found by Lijinsky (1999), displayed that one typical meal of smoked salmon (0.2–2 μg 100 g^{-1}) has a lower content of *N*-nitrosoamines than a typical meal of bacon (10 μg 100 g^{-1}) or beer (35 μg 500 ml^{-1}). Limits for adverse effects caused by *N*-nitrosoamines are diffuse in the literature. Belitz, Grosch, and Schieberle (2004) claims that estimates of average daily intake of nitrosoamines are approximately 1 μg. In addition, an endogenic dose should be included. This may result from ingestion of amines, and of nitrate- and nitrite ions, which are abundant in food. Lijinsky (1999) emphasised the importance of not ignoring the tiny concentrations of *N*-nitrosoamines for two reasons. First because of the great carcinogenic potency of this group of carcinogens (animal studies), and second because *N*-nitrosoamines might well be more effective (dose-by-dose) in humans than they are in experimental rodents. According to the US EPA, the maximum admissible concentration of NDMA and NDEA in drinking water is 7 ng l^{-1} and 2 ng l^{-1}, respectively with the risk estimation of 10^{-5} (Andrzejewski, Kasprzyk-Hordern, & Nawrocki, 2005). In Estonia, maximum total dose of NDMA and NDEA in fish and fish products is 3 μg kg^{-1} (Yurchenko & Mölder, 2006). Both amounts of food of and how often food with *N*-nitrosoamines is eaten, are factors that affect these values. Cautiousness has to be taken because *N*-nitrosoamines can be formed during process and storage conditions different from those in this study.

3.2. Changes in quality parameters from raw to smoked salmon fillets (experiment 2)

Experiment 2 was set up to show the effect of nitrite salt compared to table salt on weight changes, loss of carotenoids, changes in colour and resistant force during processing of smoked salmon fillets.

Average weight changes ($\Delta M^0 t$) of smoked nitrite salted fillets salted for 15, 35 and 60 h were −0.144 ± 0.011, −0.165 ± 0.011 and −0.168 ± 0.009, respectively, while weight changes ($\Delta M^0 t$) of smoked fillets salted with table salt were −0.147 ± 0.012, −0.163 ± 0.011 and −0.154 ± 0.008, respectively. The highest weight loss was obtained for fillets salted for 35 and 60 h. Nitrite salted fillets displayed significant weight changes between fillets salted for 35 and 60 h compared to fillets salted for 15 h. No significant difference was observed between fillets salted for 35 and 60 h. Fillets salted with table salt displayed significant weight changes between 15 and 35 h salting time. No significant differences were observed between the 35 and 60 and the 15 and 60 h groups. These results displayed that salting time influenced on salt absorption, while no significant effect of type of salt used was found. Dry salting leads to protein denaturation, (the myofibril proteins rapidly loose water due to the salting-out process) and reduced water holding capacity (Offer & Trinick, 1983), which will consequently lead to weight loss.

Decreased carotenoid concentration was observed during processing, but no significant difference between types of salt used was observed. Total loss of carotenoids for nitrite-treated fillets and fillets salted with table salt were, −3.4 ± 5.1% and −11.1 ± 11.6%, respectively. Comparable results for smoked salmon fillets salted with table salt have been reported elsewhere (Birkeland, 2004).

Both nitrite and table salt showed significant effects on changes in colourimetric characteristics from raw to smoked fillets (Table 4). Change in redness (Δa^*) and hue (ΔH^0_{ab}) were higher than changes in lightness (ΔL^*), yellowness (Δb^*), chroma (ΔC^*) and overall colour difference (ΔE). Comparable results have been reported elsewhere (Rørå et al., 1998). Nitrite-treated fillets showed significantly lower changes in a^*-values, H^0_{ab} and C^* compared to fillets salted with table salt. Lower ΔE-values were found for nitrite-treated fillets, i.e. better colour stability in nitrite-treated fillets during processing (Table 4). Increase of redness and lower

Table 4
Colour changes (CIE $L^*a^*b^*$, 1994) between raw and smoked salmon fillets salted with nitrite salt and corresponding fillets salted with table salt.

Salting time and type of salt used	ΔL^*	Δa^*	Δb^*	ΔH^0_{ab}	ΔC	ΔE
15 h						
Nitrite salt	6.3 ± 1.4^{a}	0.9 ± 0.5^{b}	−0.8 ± 1.2^{a}	1.1 ± 0.2^{b}	−0.3 ± 1.3^{a}	3.4 ± 0.7^{a}
Table salt	5.0 ± 1.3^{a}	3.9 ± 0.6^{a}	0.3 ± 1.7^{a}	4.1 ± 0.8^{a}	1.8 ± 1.9^{a}	4.0 ± 0.4^{a}
35 h						
Nitrite salt	5.8 ± 1.5^{a}	1.6 ± 0.8^{b}	−0.0 ± 1.9^{a}	2.3 ± 1.4^{bc}	0.7 ± 2.0^{a}	3.7 ± 0.7^{a}
Table salt	4.9 ± 1.8^{a}	3.8 ± 1.3^{a}	0.8 ± 2.5^{a}	4.4 ± 1.6^{a}	2.2 ± 2.6^{a}	4.2 ± 1.0^{ac}
60 h						
Nitrite salt	4.7 ± 2.1^{a}	1.7 ± 0.9^{b}	−0.3 ± 2.0^{a}	1.7 ± 0.4^{b}	0.5 ± 2.1^{a}	3.0 ± 0.6^{ab}
Table salt	4.5 ± 1.7^{a}	3.2 ± 1.7^{a}	−0.6 ± 2.3^{a}	3.7 ± 1.7^{ac}	0.7 ± 2.4^{a}	4.2 ± 1.3^{ac}

The data represent average value ± standard deviation of three measurements (Fig. 1) from each sample of 10 salmon fillets, except for the 35 h group where $n = 15$. Significant differences ($P < 0.05$) between means in the same column are indicated with different superscript a, b and c.

ΔE in nitrite-treated fillets versus those salted with table salt, could probably be explained by formation of NOMg. NOMg display an increased absorbance at $\lambda = 455$ nm compared to myoglobin (Mg) (Fig. 2), and formation of NOMg will thus be visualised more reddish compared to Mg. Increased content of the more yellow carotenoid idoxanthin (Table 1) in fillets salted with table salt could affect the colour to a less reddish hue (Table 4). Depending on pH and brine concentration, astaxanthin could be washed out as a protein-pigment complex and cause colour loss during processing (Birkeland, 2004). Birkeland (2004) showed that astaxanthin retention was influenced by smoking temperature but not by salting method in an experiment comparing salting of fillets by dry-curing and injection salting. An overall loss of 13% astaxanthin was observed. Fish muscle has low levels of myoglobin and other heme-compounds (Olsen, Sorensen, Stormo, & Elvevoll, 2006), but clearly sufficient levels to produce NOMg (pink colour) and cover carotenoid loss during processing of smoked salmon.

No significant differences in resistant force were observed during processing between nitrite-treated fillets and fillets salted with table salt. Changes in resistant force of fillets salted with nitrite salt compared to table salt were 168.6 ± 22.0% and 244.2 ± 110.9%, respectively. In proportion to no significant differences in NaCl concentrations (3.6 ± 0.2% and 3.3 ± 0.3%) in respective groups (salted 35 h), this was expected.

3.3. Colour changes in white fish (experiment 3)

Experiments 1 and 2 displayed a more reddish hue in nitrite-treated salmon fillets compared to the corresponding fillets salted with table salt. Increased redness of nitrite-treated salmon fillets were confirmed in experiment 3 with cold-smoked cod and herring fillets. The aim of this experiment was to show that sufficient amounts of myoglobin (Mg) are present in fish muscle to produce nitrosomyoglobin (NOMg) and consequently increase red colour on the surface of fish fillets.

Average values of L^*, a^*, b^* and H^0_{ab} of nitrite-treated cod fillets were 43.67 ± 0.48, −0.70 ± 0.20, 16.81 ± 0.51 and 93.80 ± 0.71, respectively, while L^*, a^*, b^* and H^0_{ab} of fillets salted with table salt were 45.49 ± 0.96, −2.59 ± 1.13, 17.32 ± 0.21 and 102.13 ± 3.38, respectively. Cod fillets salted with nitrite salt displayed significantly higher a^* values and lower H^0_{ab} and L^* values compared to corresponding cod fillets salted with table salt, i.e. more reddish hue in nitrite salted smoked cod fillets. No significant difference in yellowness (b^*) was observed between cod fillets salted with nitrite salt and table salt.

Average values of L^*, a^*, b^* and H^0_{ab} of nitrite-treated herring fillets were 40.31 ± 0.39, 3.86 ± 0.25, 14.86 ± 0.26 and 75.63 ± 0.89, respectively, while L^*, a^*, b^* and H^0_{ab} of fillets salted with table salt were 39.88 ± 0.63, 2.59 ± 0.83, 15.71 ± 0.41 and 80.59 ± 2.44, respectively. Herring fillets salted with nitrite salt tended to show higher a^* values ($P = 0.065$) and displayed significantly lower H^0_{ab} and b^* values compared to corresponding herring fillets salted with table salt, i.e. more reddish hue in nitrite salted smoked herring fillets. No significant difference in lightness (L^*) was observed between herring fillets salted with nitrite salt and table salt.

Results from smoked fillets of cod and herring confirmed that nitrite salt made fish fillets more reddish and that use of nitrite can increase colour quality in smoked salmon fillets. This indicates that fish muscle contains sufficient amounts of Mg to produce NOMg and consequently increase red colour on the surface of fish fillets.

3.4. Summary

Nitrite salt affected colour of cold-smoked Atlantic salmon to a more reddish hue, which is associated with good quality. Increased redness in nitrite-treated fillets could be explained by formation of NOmyoglobin and a tendency of increased stability of astaxanthin with increased nitrite concentration ($R^2 = 0.48$). Nitrite salt also showed positive effects on salt diffusion but only a tendency of increased resistant force. No effects were observed on α-tochopherol content. During processing, slightly increased weight loss was observed of nitrite-treated fillets, due to the positive effect of nitrite on NaCl diffusivities, followed by increased loss of water in these samples. The use of nitrite salt in salt-curing led to significant amounts of residual nitrite in the smoked product. These amounts are relatively high according to ADI set by JECFA and SCF. However, the risk of methemoglobinemia is probably low, since an average European does not eat smoked salmon on a daily basis. Another consideration about high intakes of nitrite is endogenic formation of *N*-nitrosoamines. In this study, nitrite-treated fillets showed lower content of *N*-nitrosoamines than fillets salted with table salt, but further work has to be carried out to fully understand what happened under storage and endogenous in humans. To optimise the processing conditions according to product quality, further work has to be done to find the best salting time and amounts of sodium nitrite incorporated in the sodium chloride.

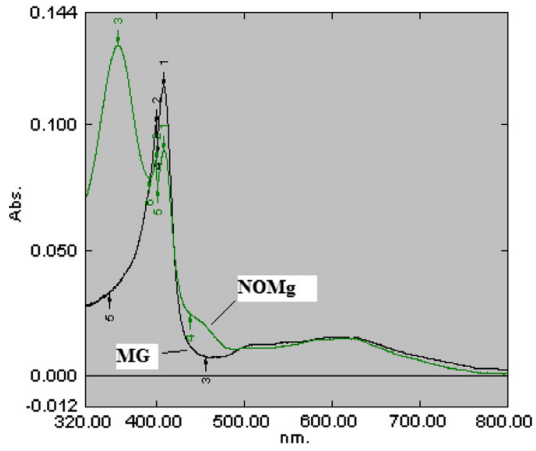

Fig. 2. UV/VIS spectra of myoglobin (Mg) and nitrosomyoglobin (NOMg).

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Paper IV

Salting method affects the retention of carotenoids in the fillet surface of cold-smoked Atlantic salmon (*Salmo salar* L.)

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IV

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Short communication

Salting method affects the retention of carotenoids in the fillet surface of cold-smoked Atlantic salmon (*Salmo salar* L.)

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Introduction

The colour of salmon fillets is affected by the carotenoid concentration in the muscle tissue (Skrede & Storebakken, 1986), and the surface colour is an important decision-maker for consumers when purchasing smoked salmon (Gormley, 1992; Rørå *et al*., 2004a). The consumer have been shown to prefer red-coloured salmon products (cf. Bjerkeng, 2000); thus, it is important to maintain muscle pigmentation and coloration as far as possible in the surface of smoked fillets. Astaxanthin is the major carotenoid in salmon flesh, with α-actinin as the major binding protein (Matthews *et al*., 2006).

Processing protocols affect the quality characteristics of smoked salmon products (Cardinal *et al*., 2001; Espe *et al*., 2001, 2002; Rørå *et al*., 2004b; Birkeland & Bjerkeng, 2005). However, relatively little specific information is available regarding the stability (retention) of carotenoids throughout processing and storage of cold-smoked salmon, especially when prerigour raw material is used. Birkeland *et al*. (2004) reported the retention of astaxanthin in postrigour salmon fillets to be 85% after smoking and 24-day chilled storage. The authors did not report the retention of astaxanthin in the surface of smoked fillets with regard to salting method applied.

Fillet rigour status at the time of salting affects the uptake of salt in muscle tissue during dry salting, and reduced uptake and distribution of salt have been reported in prerigour salmon fillets compared with those of postrigour salmon fillets (Wang *et al*., 2000; Rørå *et al*., 2004b). Birkeland *et al*. (2007) concluded that injection-salting was a suitable salting technology to obtain acceptable salt content and distribution in early (prerigour) processed salmon fillets. Dry salting is driven by diffusion (Dyer, 1942), which results in relatively high salt concentrations in the fillet surface during the smoking step. Furthermore, it is generally accepted that salt affects the protein properties (Offer & Trinick, 1983) and the water activity. Differences in surface water activity of salmon fillets during smoking affect the absorption of smoke components (Sikorski *et al*., 1998), which may further affect the surface colour because of alterations in the fillet surface and/or decreased stability of the carotenoids. Hence, because of the importance of surface colour as a decision-maker for consumers when purchasing smoked salmon products, more detailed information regarding the effects of raw material and processing conditions on the fate of carotenoids in the fillet surface throughout processing and storage are needed. Therefore, the aim of the experiment was to study the retention of carotenoids in the fillet surface throughout processing and 14-day vacuum storage of cold-smoked Atlantic salmon using dry or injection salting and raw material of different rigour status at time of salting.

Materials and methods

Farmed Atlantic salmon (*Salmo salar* L., weight: 3623 ± 280 g, condition factor: 0.9 ± 0.05, $n = 10$) were slaughtered at a local slaughterhouse in Rogaland, Norway, and immediately transported as gutted fish, on ice, to Nofima Norconserv AS, Stavanger, Norway. The right fillet was cut-off and trimmed by hand 5 h after slaughter (prerigour), while the left fillet was stored on the spine, on ice, for 4 days before filleting (postrigour). To proof the rigour status at time of filleting, fillet contraction was measured after salting, smoking and 14-day storage. Prerigour and postrigour

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fillets were subjected to either injection or dry salting (20 h) according to Birkeland *et al*. (2004) with slight modifications. Five fillets were used for each protocol that were named *prerigour injection salted* (PRE-I), *prerigour dry salted* (PRE-D), *postrigour injection salted* (POST-I) and *postrigour dry salted* (POST-D). After different salting procedures, the fish groups were dried separately for 60 min followed by four cycles of 50-min smoking and 10-min drying (23.0–23.3 °C, relative humidity: 75–83%, air velocity: 0.4–0.8 m s^{-1}) (Birkeland *et al*., 2003). Vacuum-packaged fillets from all protocols were stored in a refrigerator (4.3 ± 0.5 °C) for 14 days. Cylinders for chemical analyses in the surface layer (5 mm) were punched out from raw fillets, after salting, after smoking and after 14-day vacuum storage (Fig. 1). The muscle samples were homogenised individually and analysed for dry matter, DM (ISO, 6496), NaCl (Lerfall & Østerlie, 2011) and carotenoids (astaxanthin, idoxanthin and lutein). The carotenoids were extracted after a method by Bligh & Dyer (1959) with slight modifications and analysed according to Aas *et al*. (1997). The content of DM was adjusted in proportion to salt content for each sample to show the relative water flux in and out from the salmon fillet. The retention of carotenoids after each processing step was adjusted in proportion to weight changes during processing and expressed as percentage of initial weight. Main effects of rigour status and salting method applied on retention of astaxanthin, idoxanthin and lutein following all processing steps and vacuum storage were calculated as an average of protocols involving equal rigour status and salting method, respectively. Surface colour (CIE, 1994) was measured on a DigiEye full system, VeriVide Ltd., Leicester, UK. The software Digipix was used to calculate $L^*a^*b^*$ values from red, green and blue (RGB) values obtained from the fillet image (Fig. 1).

Data were analysed by one-way ANOVA combined with Tukey's pairwise comparison test and correlation (Pearson's correlation coefficient) analyses using the MINITAB statistical software (Version 15; Minitab Inc., State College, PA, USA). The alpha level was set to 5% ($P < 0.05$).

Results and discussion

Analyses of DM and carotenoid content of three randomly chosen salmon showed no significant differences between the four sampling locations along the fillet (Fig. 1, $P > 0.19$) or between prerigour and postrigour fillets (filleted 5 h and 4 days after slaughter, respectively), i.e. respective left and right fillet from the same individual ($P > 0.56$). However, colorimetric analyses of the raw material showed that prerigour and postrigour fillets had significantly different colour characteristics (Table 1).

Dry or injection salting of prerigour salmon fillets resulted in a contraction of 6.8 ± 0.7% (PRE-D) and 10.4 ± 4.3% (PRE-I) after salting, respectively, whereas maximum contraction was observed after smoking (9.5 ± 0.3% and 13.2 ± 2.8%, respectively). No changes in fillet length were observed of postrigour fillets, except for a small contraction during 14-day storage (2.5%). The supply of NaCl to the muscle tissue accelerates the development of rigour mortis (Larsen *et al*., 2008). Injection salting gives a more uniform salt distribution in the muscle tissue (Birkeland *et al*., 2007), explaining a stronger contraction of fillets from the protocol PRE-I as compared to PRE-D (3.6% points). Diffusion of salt throughout the muscle structure is a time-consuming process (Lautenschlager, 1985), explaining decreasing salt content in the fillet surface throughout smoking and storage of dry salted fillets (Table 2). Lower salt content in the surface layer after injection salting as compared to dry-salting is a result of mechanical and physical differences between the salting methods. Dry-salting is driven by diffusion, i.e. salt in, solute out, and injection salting is driven by mechanical supply of salt and water.

The variation pattern in surface DM content after salting was preserved throughout the smoking step and until after storage (Table 2). It is presumed that dry-salting caused loss of solutes during the salting and

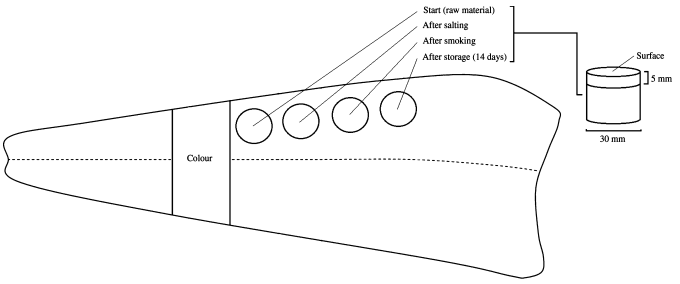

Figure 1 Schematic illustration of the fillet areas used for colorimetric and chemical analyses.

Table 1 Colorimetric parameters for raw fillets and colour differences between raw and smoked salmon fillets stored 14 days (average ± SE, $n = 5$)

Colorimetric parameters	Protocol[†]				Effect of protocol[‡]
	PRE-I	PRE-D	POST-I	POST-D	
Raw					
L^*	61.2 ± 0.7^b	59.2 ± 0.6^b	68.9 ± 1.1^a	68.8 ± 0.9^a	***
a^*	39.6 ± 0.7^b	40.9 ± 0.7^b	44.6 ± 0.7^a	45.0 ± 0.5^a	***
b^*	38.4 ± 0.5	37.9 ± 0.4	39.5 ± 0.4	39.1 ± 0.8	ns
Reduction (%)[§]					
ΔL^*	−7.2 ± 1.4^b	17.4 ± 1.3^a	7.9 ± 3.0^a	11.6 ± 1.0^a	***
Δa^*	1.3 ± 1.4^d	31.6 ± 0.5^a	12.0 ± 1.1^c	21.9 ± 2.0^b	***
Δb^*	−9.5 ± 2.5^c	20.3 ± 0.7^a	−2.2 ± 2.5^{bc}	5.7 ± 1.8^b	***

[†]PRE-I, prerigour injection salted; PRE-D, prerigour dry salted; POST-I, postrigour injection salted; POST-D, postrigour dry salted.
[‡]Different lowercase superscripts within each row indicate significant differences between processing protocols by one-way ANOVA and Tukey's pairwise comparison test. Level of significance is indicated by $P < 0.001$ (***) and not significant (ns)
[§]Reduction in colorimetric parameters from raw to smoked fillets stored 14 days: $\Delta Lab = \frac{(Lab_{raw} - Lab_{stored})}{Lab_{raw}} \times 100\%$

smoking step because of salting-out effects (Gallart-Jornet *et al*., 2007), whereas increased DM content of injected fillets after smoking and 14-day storage was caused by physical ablution of solutes from the fillets.

Retention of carotenoids in the surface layer showed no significant difference between the protocols (PRE-I, PRE-D, POST-I and POST-D), except for the retention of idoxanthin that was significantly lowest in PRE-I after 14-day storage (Table 2).

Rigour status at the time of salting did not show any significant main effects on the retention of astaxanthin or lutein (Table 3). Significantly lower retention of idoxanthin was found in the surface layer of fillets salted prerigour as compared to fillets salted postrigour. Not correcting the retention for fillet weight changes during processing would give significant effects of rigour mortis on the retention of astaxanthin because of different fillet weight changes caused by lower salt uptake in prerigour as compared to postrigour fillets (Wang *et al*., 2000).

Retention of astaxanthin and idoxanthin was significantly influenced by the salting method applied (Table 3). After the salting step, no significant effects on retention were found. However, an indication ($P = 0.117$–0.125) of lower retention of astaxanthin and idoxanthin was observed in dry salted as compared to injection-salted fillets. After smoking, significantly lower retention of idoxanthin and an indication ($P = 0.074$) of lower retention of astaxanthin were found in dry salted as compared to injected fillets. Moreover, after 14-day storage, significantly lower retention of astaxanthin and idoxanthin was found in dry-salted fillets as compared to injected fillets. Thus, the effect of salting method applied on retention of carotenoids in the fillet surface seems somewhat time-dependent. Lower retention of astaxanthin and idoxanthin in dry-salted fillets may be a result of differences in surface water activity during the smoking step because of differences in salt content, which may affect the absorption of smoke components on the fillet surface (Sikorski *et al*., 1998).

The smoking step was found to be the main contributor to denaturation of astaxanthin in the fillet surface. Moreover, the retention of astaxanthin after smoking and storage was significantly different from the initial level and the level found after the salting step. Birkeland *et al*. (2004) reported the average retention of astaxanthin in the whole fillet to be mostly influenced by the salting step, whereas the smoking step was found to have minor effects on the average retention. The contradiction between these studies indicates that the surface layer is more affected by smoke components than the whole fillet. The main contributor to decomposition of idoxanthin in the fillet surface was found to be the smoking step except for prerigour fillets, which show greatest loss of idoxanthin during 14-day storage (Table 3). No significant loss of lutein was observed throughout processing and storage. Hence, lutein seems more stable throughout processing and storage as compared to astaxanthin and idoxanthin.

Retention of astaxanthin in the surface of smoked fillets correlated significantly with the surface salt content after both salting ($r = -0.488$) and smoking ($r = -0.524$), but after 14-day storage, the correlation was insignificant ($r = -0.417$ and -0.431, $P = 0.058$ and 0.067, respectively). Correlations between the salt content and retention of astaxanthin were probably due to alterations in fillet surface properties during smoking and changes in the protein structure, which may influence the physical ablution and the stability of carotenoids throughout processing and 14-day storage. Because of this, release of astaxanthin caused by higher

Table 2 Salt, dry matter, astaxanthin, idoxanthin and lutein content and retention (% of initial) of astaxanthin, idoxanthin and lutein (average ± SE, $n = 5$) in the fillet surface layer at different processing steps following different processing protocols

Parameter	Processing step	Protocol[†]				Effect of protocol[‡]
		PRE-I	PRE-D	POST-I	POST-D	
Salt (NaCl, %)	Raw	0.2 ± 0.0^{b}	0.2 ± 0.0^{d}	0.2 ± 0.0^{c}	0.2 ± 0.0^{d}	ns
	Salted	2.2 ± 0.2^{aD}	5.3 ± 0.2^{aB}	3.0 ± 0.1^{aC}	8.3 ± 0.1^{aA}	***
	Smoked	2.6 ± 0.1^{aC}	4.2 ± 0.1^{bB}	2.9 ± 0.1^{aC}	6.4 ± 0.1^{bA}	***
	Stored	2.2 ± 0.1^{aBC}	1.9 ± 0.1^{cC}	2.4 ± 0.1^{bB}	3.2 ± 0.1^{cA}	***
	Effect of processing step[2]	***	***	***	***	
Dry matter[§] *(%)*	Raw	30.3 ± 0.2^{b}	30.0 ± 0.3^{c}	30.3 ± 0.2^{b}	30.0 ± 0.2^{c}	ns
	Salted	30.4 ± 0.3^{bB}	36.5 ± 0.4^{bA}	29.3 ± 0.2^{bB}	36.3 ± 0.5^{bA}	***
	Smoked	31.5 ± 0.4^{bB}	39.6 ± 0.6^{aA}	32.2 ± 0.3^{aB}	39.0 ± 0.2^{aA}	***
	Stored	33.6 ± 0.5^{aB}	39.5 ± 0.5^{aA}	33.6 ± 0.6^{aB}	39.1 ± 0.3^{aA}	***
	Effect of processing step[‡]	***	***	***	***	
Astaxanthin						
Initial (mg kg^{-1} *ww)*	Raw	5.7 ± 0.1	6.0 ± 0.2	5.7 ± 0.1	5.9 ± 0.2	ns
Retention (%)[¶]	Salted	98.8 ± 1.2^{a}	95.5 ± 3.1^{a}	97.0 ± 1.9^{a}	93.1 ± 2.4^{a}	ns
	Smoked	87.6 ± 2.2^{b}	84.0 ± 1.4^{b}	84.9 ± 3.4^{b}	78.2 ± 3.3^{b}	ns
	Stored	88.0 ± 2.6^{b}	83.4 ± 2.0^{b}	87.4 ± 3.5^{ab}	81.5 ± 0.9^{b}	ns
	Effect of processing step[‡]	**	**	*	**	
Idoxanthin						
Initial (mg kg^{-1} *ww)*	Raw	0.17 ± 0.02	0.11 ± 0.01	0.15 ± 0.02	0.14 ± 0.03	ns
Retention (%)[¶]	Salted	97.6 ± 2.5^{a}	92.4 ± 4.7^{a}	97.4 ± 1.6^{a}	93.0 ± 3.0^{a}	ns
	Smoked	82.5 ± 3.0^{b}	73.9 ± 3.7^{b}	80.8 ± 4.6^{b}	72.8 ± 3.9^{b}	ns
	Stored	51.9 ± 4.5^{cAB}	33.6 ± 1.9^{cB}	76.2 ± 1.0^{bA}	60.1 ± 8.2^{bA}	**
	Effect of processing step[‡]	***	***	**	**	
Lutein						
Initial (mg kg^{-1} *ww)*	Raw	0.40 ± 0.02	0.36 ± 0.04	0.40 ± 0.02	0.39 ± 0.03	ns
Retention (%)[¶]	Salted	98.4 ± 1.0	92.3 ± 4.7	97.0 ± 2.1	93.1 ± 3.2	ns
	Smoked	94.4 ± 3.2	89.3 ± 3.8	91.0 ± 3.2	90.6 ± 3.4	ns
	Stored	95.4 ± 3.4	94.4 ± 2.3	91.0 ± 4.4	96.1 ± 2.5	ns
	Effect of processing step[‡]	ns	ns	ns	ns	

[†]PRE-I, prerigour injection salted; PRE-D, prerigour dry salted; POST-I, postrigour injection salted; POST-D, postrigour dry salted.

[‡]Different lowercase superscripts within each column indicate significant differences between processing steps, while different capital letter superscripts within each row indicate significant differences between processing protocols by one-way ANOVA and Tukey's pairwise comparison test. Level of significance is indicated by $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and not significant (ns).

[§]Dry matter values are adjusted in proportion to salt content.

[¶]Retention (% of initial) was adjusted in proportion to weight changes during processing. Retention values above 100% occur after salting probably due to analytical artefacts. These values are set to be 100% before statistical analyses.

salt concentrations in the surface of dry-salted fillets makes astaxanthin more exposed to decomposition caused by light, oxygen and probably chemical components absorbed from the smoke (Schiedt & Liaaen-Jensen, 1995). Carotenoids are quite labile compounds where the stability of astaxanthin in salmon muscle is related to the strength of the protein–astaxanthin binding (Latscha, 1990). Moreover, supply of smoke components (influenced by surface water activity) may as well affect the proteins and lower the binding strength between astaxanthin and the proteins.

During processing and storage, dry-salted protocols (PRE-D and POST-D) showed highest reduction in translucence (positive ΔL^*), redness (positive Δa^*) and yellowness (positive Δb^*) (Table 1). Moreover, ΔL^* correlated significantly with retention of astaxanthin in the final product ($r = -0.540$), indicating lower translucence of fillets with the most prominent loss of astaxanthin. In addition, the low and insignificant correlation ($r = -0.354$, $P = 0.163$) observed between retention of astaxanthin and Δa^* support that colour stability might be affected by other factors. For example, differences between the protocols in absorption of smoke components and Maillard browning on the fillet surface (Sikorski *et al.*, 1998).

It is concluded that the smoking step is the major contributor to loss of astaxanthin in the fillet surface of cold-smoked Atlantic salmon. Furthermore, the retention of astaxanthin and idoxanthin seems somewhat dependent on the salting method applied, but only minor effects of rigour mortis on retention of carotenoids were observed in this study. In addition, colour of the final

Table 3 Main effects of rigour status and salting method on retention (% of initial) of astaxanthin, idoxanthin and lutein (average ± SE, $n = 10$) throughout processing and 14 days storage of cold-smoked Atlantic salmon

Parameter	Processing step	Rigour status		Effect of rigour status†	Salting method		Effect of salting method†
		Pre	Post		Dry	Injection	
Astaxanthin							
Retention (%)‡	Salted	97.2 ± 1.6	95.1 ± 1.6	ns	94.3 ± 1.9	97.9 ± 1.1	ns
	Smoked	86.0 ± 1.4	81.6 ± 2.5	ns	80.8 ± 2.1	86.3 ± 2.0	ns
	Stored	85.7 ± 1.7	84.5 ± 2.0	ns	82.5 ± 1.1	87.7 ± 2.1	*
Idoxanthin							
Retention (%)‡	Salted	95.0 ± 2.7	95.2 ± 1.8	ns	92.7 ± 2.6	97.5 ± 1.4	ns
	Smoked	78.2 ± 2.7	77.3 ± 3.2	ns	73.4 ± 2.5	81.7 ± 2.6	*
	Stored	41.8 ± 3.8	68.2 ± 4.9	**	45.4 ± 5.8	64.1 ± 5.0	*
Lutein							
Retention (%)‡	Salted	95.3 ± 2.5	95.1 ± 1.9	ns	92.7 ± 2.7	97.7 ± 1.1	ns
	Smoked	91.8 ± 2.5	90.8 ± 2.1	ns	89.8 ± 2.6	93.1 ± 2.3	ns
	Stored	94.9 ± 2.0	90.8 ± 3.1	ns	93.2 ± 1.9	93.2 ± 2.7	ns

†Effects of rigour status and salting method were calculated by one-way ANOVA and Tukey's pairwise comparison test. Level of significance is indicated by $P < 0.05$ (*), $P < 0.01$ (**) and not significant (ns).

‡Retention (% of initial) was adjusted in proportion to weight changes during processing. Retention values above 100% occur after salting probably due to analytical artefacts. These values are set to be 100% before statistical analyses.

product seems, in this study, more affected by other process-related factors than retention of astaxanthin.

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