Quality of salted cod (*Gadus morhua* L.) as influenced by raw material and salt composition

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Kristin Lauritzsen,
Tromsø 6th of February 2004

FYLG TANKEN

Fylg tanken – men ikkje
til ytste egg,
og plukk ikkje kvar ein blom
på nysprotten hegg.

Lat tankane kome
og blåne or augneleite,
og blomar på draumtreet hanga,
kva dei so heiter.

Olav H. Hauge

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Paper V Lauritzsen, K. and Olsen, R.L. (2004). Effects of antioxidants on copper induced lipid oxidation during salting of cod (*Gadus morhua* L.). J. Food Lipids (In press).

1 INTRODUCTION

The ancient Egyptians may have been the first to cure meat and fish with salt. Salted fish and birds were found in Egyptian tombs prior to the first Chinese record of preserving fish in salt approximately 2000 $_{B.C.}$. Whether the Egyptians discovered the process first or not, they were certainly the first civilization to preserve food on large scale. Salt production techniques were spread through southern Europe by the Roman Empire (Kurlansky, 2002). In 1987, the world production of cured fish products was 13.2 billion tons. Although world production of cured fish has been growing, the percentage of the total annual catch of fish has declined during the 1980s. Asia is by far the largest producer of dried, salted or smoked fish products and among the highest producers are China, Japan, Indonesia, the Philippines, India and Korea (Ismail and Wootton, 1992). During the production of salted fish products water, proteins and minerals are lost from the raw material. The losses from catch to cured product have been estimated to 25% by FAO (1981), based on observations and experience in the global fisheries.

In the North Atlantic countries, methods of salting and drying cod (*Gadus morhua* L.) have continued practically unchanged for centuries up to the present time. Though this type of cod is found only in northern waters, salted cod entered the repertoire of most European cuisines, especially in southern Europe where fresh cod was not available. Fish provided the predominant source of protein for both rich and poor. Salted or salted and dried cod was the stable food for explorers and colonisers. When the Catalans took control of Naples in 1443 they brought salted cod to southern Italy and the Italian population became great salted cod enthusiasts. The traditional products are mostly consumed by the Mediterranean and Caribbean populations but there is also a growing group of consumers in the Scandinavian countries asking for salted cod dishes in restaurants. Usually, the fish has been eaten during the religious fasting periods or at weekends. All the fishing nations of northern Europe have been participants in the extremely profitable salted cod market. Northern countries had the cod but needed the salt. The salt was produced by solar evaporation from seawater locked into ponds in the sunny Mediterranean countries and was exchanged with the salted fish from the North Atlantic countries.

Drying and salt curing of fish have been used as preservation methods for times of scarcity but they have however, lost importance as preservation methods due to the widespread use of refrigeration technology in the developed countries. Nevertheless, during the years 2000-02 the frozen cod filet block market has been decreasing in percentage of the total export of fish products from Norway (Source: Norwegian Fisheries Export Council). The fish is being marketed differently, with more and more emphasis on fillets, portions and value-added fresh or marinated products. During the period from 1970 to 2000, catching quotas for wild cod were at a maximum in 1997 and then went down dramatically. Due to decreasing quotas, the farming of cod began to be an interesting possibility and the processes

of producing heavily salted niche products from farmed fish became the subject of scientific discussions.

Salted cod products are highly appreciated not only because of their characteristic taste, texture and aroma, but also because of their high storage stability and nutritional value. Methods of cod salting have been modified and improved during the last fifty years in order to obtain specified value-added products. In 1997, salted and smoked fish and clip-fish products accounted for approximately 5.3 billion Norwegian Kroner, approximately 20% of the total fish export from Norway (Source: Norwegian Fisheries Export Council). The price of the cured cod is set mainly according to the size of the fish (length and weight), the smell, colour and stiffness of the surface, the eventual presence of production errors and the water content of the flesh. Specialised graders of salted cod categorise the fish into 3 to 4 quality classes: imperial/superior, universal, popular, and mixed, the latter being the bottom class of quality. During the nineteen seventies and eighties, increasing quantities of salted cod of high and stabile quality were exported with great success from Island and the Faroe Islands to the traditional salt fish markets. In Spain and Portugal these products were highly appreciated in front of the Norwegian salted and clip-fish of cod. The sales pitch included a heavy emphasis on reliability of supply, stringent quality control and inspection procedures. Due to the increasing demand for high quality and stability of these salted products, scientific studies on the quality aspects of heavily cured cod started in Norway during the 1970s and 1980s.

This thesis was initiated in 1997 at the Norwegian Institute of Fisheries and Aquaculture Research (Fiskeriforskning) in Tromsø. It contains information about biochemical mechanisms which take place at high ionic strength in salt cured cod muscle.

2 BACKGROUND

Salt curing preserves fish by lowering of the water activity (aW) and muscle-pH. In salted fish, denaturation of proteins always occurs, but normally it proceeds more slowly than the penetration of the salt (Tülsner, 1978). The Na^+ and Cl^- ions act as counterions toward negatively and positively charged groups, respectively, disturbing the native conformation of the proteins (Sikorski and Ruiter, 1994). The salt penetration throughout the flesh causes swelling of the myofibrillar matrix, though this is restricted in intact muscle by the sarcolemma. Denaturation by salt and by change in pH results in a decreased extractability of fish muscle proteins. Heavily salted fish loses much water, its texture is tough, and the flavour is much less developed than, for example, in fatty herring salted with a low amount of salt. Lipids are always broken down to some extent during heavy salting of fish. It has been suggested that the presence of transition elements in salt used for curing of cod enhances oxidation of lipid components (Shewan, 1955). Every year salted cod with yellow and brown discolouration of the surface has been found. This has been explained to be due to iron or copper contaminations of the salt (Beatty and Fougere, 1957). The lipid oxidation end products themselves and their interactions with the fish muscle proteins influence the sensory properties of the product. Traces of iron, copper, calcium and magnesium ions originating in the salt, the processing equipment and/or in the tap water used in the process may accelerate both lipid oxidation and protein denaturation processes of the salted product. The use of antioxidants may forestall the lipid oxidation of the salted cod (Martinsen, 1995; Joensen *et al.,* 1996).

In dry salting of cod, it is generally experienced that the salt should not be too finely grained because the fine-grained salt dissolves quickly in the fish muscle fluids causing a too rapid withdrawal of moisture from the surface tissue. As a consequence, a rapid protein denaturation and coagulation occurs, preventing further penetration of the salt into the fish and giving rise to a condition known in the trade as “salt burn”. Due to these considerations and low prices, solar salts with a broad grain size distribution are normally used for heavy salting of cod.

It has been found that traces of calcium and magnesium in the salts have marked effects on both the colour and texture of salted fish. As little as 1% calcium or magnesium in salt causes a remarkable whitening and stiffening of the flesh. Since impure salts have been used for salting of cod for centuries, buyers demand a firm white-fleshed fish. It is generally experienced that the amount of calcium and magnesium in the salt should be in the range of 0.3 to 0.6% (w/w) (Lauritzsen and Akse, 1995). Salts of both calcium and magnesium, have been reported to give the strong acrid flavours characteristic of commercially salted fish (Borgstrom, 1968). The most commonly used methods for salting of cod today are kench curing (also called dry salting), picklesalting, injection salting, brine salting and vacuum salting. Short descriptions of these methods are given below.

Kench curing (dry salting)

The solid salt, which is distributed over the fish surface, extracts moisture from the fish muscle and forms brine. During the curing process, the initial outward flow of water and soluble proteins from the muscle to the brine, by virtue of the higher osmotic pressure of the latter, is eventually reversed. This is because the salt, which diffuses inwards, forms a complex with the proteins of the meat which has a higher osmotic pressure than the brine. Kench curing of cod involves the following stages (Kvande-Pettersen and Losnegaard, 1991; Akse *et al*., 1993):

1. The fish is manually or by machine filleted or split, removing most of the backbone. It is placed in stacks with salt interspersed between the layers. The stack of fish and salt are left up to 7-14 days to let the salt penetrate the muscle.
2. When the salt has penetrated the fish, it extracts the fish fluid through plasmolysis. The extracted fluid is called pickle and it is allowed to drain away continuously. Used salt is removed from the fish surfaces and the fish is restacked with new dry salt between the layers once or twice during the ripening process. The water content of the cod muscle is usually reduced from approximately 82 to about 54 per cent during the salt curing process.

Picklesalting

This is the other dry salting method in which the moisture extracted while the salt is penetrating, is not drained away, thus the fish is gradually immersed in a salt pickle of extracted fluids. Usually, when the cod has been picklesalted for 5-7 days, the fish is kench cured once or twice to saturated NaCl level of the muscle tissue. During the kench curing, the used salt is removed from the fish surface and the fish is restacked with new dry salt between the layers (Kvande-Pettersen and Losnegaard, 1991). The product is thought to ripen during this part producing the characteristic sensory properties of salted cod.

Injection salting

Salting by automatic needle injections has become common during the last decades to increase the speed of salt penetration and to ensure a uniform salt concentration throughout the flesh. The speed of the salt penetration increases due to the fact that salt is forced mechanically at high pressure into the muscle tissue prior to passive salt diffusion. Usually, the injection needles are penetrated into the flesh from the open fillet surface down to the inner side of the skin. Injection of saturated brine solution starts as the needles reach the skin and the brine flows continuously out of the needles into the flesh as they return through the fillet back to the start position. The injection pressure and number of injections into the fish can be adjusted in the automatic injection machine. Usually, the salt concentration increases from 0.15% in fresh cod muscle to 2-5 % in ready salt injected fish muscle. Afterwards, the cod is brined, picklesalted and/or kench cured once or twice for 10 days in cold storerooms as

described above, until the fish muscle has reached saturated salt level. The weight yield is usually experienced to be higher by this salting method than by traditional dry salting methods, due to a lower moisture loss from the fish during the kench curing stages (Davidson, 1989; Akse and Pedersen, 1993).

Brine salting

This is a wet salting method in which the fish is soaked in ready made brine (usually 18-25% NaCl, w/w). The salt content of the final product can be regulated by altering the duration and temperature of the brine. When meat is initially placed in curing brine, the exterior muscles will be exposed to a much higher concentration of salt than when equilibrium between meat and brine is subsequently attained. On the other hand, interior muscle locations will be subjected to a slow increase in salt concentration from physiological to equilibrium level. Usually, the cod is brined for 2-4 days in 20% NaCl (w/w), then picklesalted and/or kench cured once or twice for 14-21 days to saturated salt level of the muscle tissue. This method is also experienced to give a higher weight yield than by traditional dry salting, due to a lower moisture loss at the kench curing stages (Pedersen, 1981; Bøgh-Sørensen *et al*., 1986; Kvande-Pettersen and Losnegaard, 1991; Joensen, 1994; Joensen *et al*., 1996).

Vacuum salting

The vacuum osmotic dehydration (VOD) method has been tried for salting of cod and has given a faster uptake of NaCl and lower water loss from the muscle compared to ordinary dry salting methods. The weight yield of salted cod was higher by the VOD method but the muscle surface of the fish had a more yellow colour than by ordinary dry salting methods (Halsebakke, 1996; Joensen *et al*., 1997). VOD is probably a more efficient method used on particularly porous foodstuffs such as fruit and vegetables, than on the more compact fish muscle structure. When porous foodstuffs are soaked into an osmotic liquid at vacuum conditions, a hydrodynamic mechanism (HDM) occurs (Fito and Pastor, 1994). This mechanism causes transportation of the external liquid into the foodstuff. HDM is a result of pressure differences between the gas in the pores of the foodstuff and that in the environment. When the pressure on the outside of the pores increases, the gas inside the pores will be compressed. The external liquid, which is hardly compressed, will flow into the pores. The penetration may be due to capillary forces, but the temperature and changes in pressure will also have an influence. As a consequence of the hydrodynamic mechanism, VOD causes a faster mass transport compared to osmotic dehydration at atmospheric pressure. The reason for this is that the pores are filled up with osmotic solution to a larger extent than by ordinary osmotic dehydration, making a larger interface area between the foodstuff and the osmotic solution (Fito, 1994).

The rate of salt penetration into the fish muscle may increase up to 100-fold by splitting, filleting and skinning the fish. On the one hand, a slow penetration of salt is wanted to promote the development of the typical ripened sensory properties of the product. On the other hand, a fast penetration of salt is needed to retard spoilage reactions by enzymes and micro-organisms. The optimal rate usually lies in between these rates depending on the specifications of the product. Spoilage processes are mainly controlled by the salt concentration of the muscle tissue, temperature, water activity, pH and availability of oxygen (Sikorski and Ruiter, 1994). The sensory properties of the cured muscle may be so altered that the product turns suitable for eating without heating during culinary preparation. Heavily salted cod can be eaten for instance as "Tapas" dishes, usually in Spain, by dipping thin slices of fish into spiced olive oils.

2.1 Requirements to the cod, salt and water properties

The Norwegian quality regulations relating to fish and fishery products state several requirements to be fullfilled regarding the fish, salt and water properties for production of salted products (Directorate of Fisheries, Department of Quality Control, 1998). The most important factors that may influence the quality of salt cured cod are presented below.

*§*5-3. *Stockfish and heavy salting of fish.*

1. The raw material shall satisfy the following organoleptic and chemical requirements:

A. The fish shall not be soft and deformed.
B. There may be a moderate odour of spoilage products.
C. The mucous membranes shall not show yellow slime (be discoloured yellow or brown).
D. The fish shall not have ragged bellies.
E. The meat along the backbone shall not show pink or red discolouration.
F. Samples of meat shall not on average contain more than 10 mg, and no single sample more than 15 mg of trimethylamine nitrogen per 100 g.
G. When tested in accordance with the method stipulated by the Directory General of Fisheries, samples of meat shall not on average contain more volatile nitrogen per 100 g of fish than: 35 mg TVN for cod.

2. Raw materials for salted fillet shall meet the requirements laid down in *§*5-2(1).
3. Processing of fish that is to be heavy salted shall begin as soon as possible after loading/landing. Fish shall not be kept on ice for more than 12 days before salting/drying.

The Norwegian regulations state the following criteria for the quality of the salt.

§ 16-2. Salt properties

1. The salt should be used just once for production of seafood.
2. Additional requirements:

A. The salt must have a pure clean appearance and it should not contain noticeable coloured particles or foreign crystals. Abnormal smells are not permitted and the salt must have a clear and pure taste.

B. The total chloride content, estimated as dry weight of NaCl, should be at least 97,0 % (w/w).

C. The total water content of the salt should not be above 6% (w/w).

D. The total iron content must be less than 10 mg/kg (ppm) and the total copper content less than 0.1 mg/kg (ppm).

E. Noticeable dirt, oil or other foreign particles, including proteins, are not permitted in the salt.

F. No other chemicals should be added to the salt without specified permission.

Salt occurs widely in nature, both in the sea and in deposits in the earth. Salt is obtained by the mining of rock salt, the evaporation of sea water in solar ponds, or by the refining, evaporation and recrystallization of vacuum salt in industry plants. The main impurities found in rock salt are anhydrite ($CaSO_4$) and water-insoluble mineral constituents (Kvande-Pettersen, 1964; 1969). Rock salt contains only minor proportions of soluble impurities and therefore the quality of this salt cannot be improved to any great extent by washing procedures. The anhydrite ($CaSO_4$) fraction is homogenously distributed in the salt crystals. Solar salt from evaporation ponds contains both soluble and insoluble impurities (Kvande-Pettersen, 1964; 1969). Solar salts originating from 30 countries from South America and to the Far East were analysed by Schmitz and Wöhlk (1993) with particular emphasis on the analysis of NaCl, KCl, $MgCl_2$, $MgSO_4$, $CaSO_4$, water insolubles, drying losses and particle-size distribution. The results are as shown in Table 1. On condition that the solar ponds are properly operated and the washing plants optimally designed, it is possible to obtain a NaCl content of > 98.5%, in most cases even 99%. The soluble impurities can be reduced to 0.3%, and the $CaSO_4$ content to 0.3%. If the purity obtainable in washing plants is not sufficient for the intended purpose, removal of the impurities in the brine by chemical precipitation, separation of the precipitated products and subsequent crystallization of the salt solution is possible. Such procedures are usual for vacuum salt production, table salt having purities of 99.95% NaCl (Schmitz and Wöhlk, 1993; Grisolia and Clementi, 1993). Typical grain-size ranges of salt packaged for direct sale to consumers are 1.25-8 mm (coarse salt) and 0.25-0.7 mm (fine salt).

TABLE 1

Typical characteristics of sea salt from solar ponds

		Average	Most frequent value class	Zone of dispersion
NaCl	%	96.10	97–98	92.4–99.7
KCl	%	0.24	0–0.1	0.03–0.44
$MgCl_2$	%	0.66	0–0.2	0–1.28
$MgSO_4$	%	0.59	0–0.2	0.16–1.05
$CaSO_4$	%	0.62	0.4–0.6	0.28–0.92
Insolubles	%	0.29	0–0.2	0.08–0.44
Dry loss	%	4.36	1–2	1.8–7.9
RRSB plot:				
Mean size	mm	3.75	2–3	1.9–5.9
Uniformity		1.80	1.75–2	1.4–2.1

Typical solar salts which have recently been used by Norwegian and Islandic salt fish processing plants are summarized in Table 2. It is shown in Table 2 that the total content of iron lies above the limits given in § 16-2 in all of the solar salts. Additionally it is shown that the chloride content is below the limit of 97% in the Ibiza salt.

Table 2. Chemical composition and grain size distribution of solar salts used for the production of heavily salted cod in Island and Norway during the 1990s.

Solar salt name:		Earth	Torrevieja	Tunis	Ibiza
Chemical Components:					
Water	(%)	0.40-0.50	2.50-1.80	2.30-1.30	2.60-3.10
NaCl	(%)	98.0-99.0	97.3-97.7	97.1-97.8	96.2-94.5
$CaSO_4$	(%)	0.53-0.72	0.15-0.32	0.17-0.30	0.82-1.58
$MgSO_4$	(%)	0.04-0.17	0.00-0.10	0.10-0.30	0.16-0.38
$MgCl_2$	(%)	0.00-0.02	0.00-0.12	0.10-0.30	0.16-0.48
Na_2SO_4	(%)	0.04-0.49	0	0	0
Unsoluble	(%)	0.10-0.40	0.10	0.10	0.10
Fe	(ppm)	4-30	12-25	10-15	13-17
Cu	(ppm)	-	<0.01	<0.01	<0.01
Grains size distribution:					
>2.83	(mm)	28	32	35	42
2.06-2.83	(mm)	14	33	26	22
1.68-2.06	(mm)	9	12	13	12
1.41-1.68	(mm)	7	7	11	9
0.84-1.41	(mm)	15	11	13	11
0.50-0.84	(mm)	10	4	2	3
<0.05	(mm)	17	1	0.2	0.6
Total	(%)	100	100	100	100

Lauritzsen *et al*., unpublished results.

The Norwegian Regulations also state criteria for the quality of tap water for food processing. Some of these chemical criteria are presented below in Table 3 (Norwegian tap water requirements).

Table 3. General criteria for the quality of tap water for food processing in Norway.

Parameter	Unit	Limit
Iron (Fe)	mg/ L	0.2
Chloride (Cl)	mg/L	200
Copper (Cu)	mg/L	0.1
Sodium (Na)	mg/L	200
Sulphate (SO_4)	mg/L	100
pH		6.5-9.5

In a study on tap water quality from seven Norwegian food processing plants, the total copper content was found to vary in the range of 0.009 - 0.23 ppm Cu (Gulstad, 1994). The highest copper levels were twice as high as the copper limit given in Table 3.

2.2 Muscle chemical composition

The average composition of gutted fish is about % 73 flesh, 21% bone and 6% skin. From the composition of the flesh, the fish is classified as fat or lean fish. Lean fish muscle is approximately 78-83% water, 15-20% protein, 0.2-4% fat, 0.5% carbohydrate and 1-1.3% ash (Love, 1980; Spinelli and Dassow, 1982). Cod is classified as a lean fish with only about 0.5% lipid (Love, 1992; Ingolfsdottir *et al.,* 1998), where protein and water content are usually inversely related, i.e., amount to approximately 99% of the cod muscle. Cod composition varies according to sex, age, season, water temperature, type and abundance of available food (Dambergs, 1964; Ross and Love, 1979; Love, 1980; Eliassen and Vahl, 1982; Takama *et al*., 1985; Black and Love, 1986; Ingolfsdottir *et al*., 1998). Prior to spawning, the activity of the cod is directed towards building up reproductive organs (roe or milt) and the flesh may be depleted of protein and replaced with water (Eliassen and Vahl, 1982; Love, 1980; Rustad, 1992; Lauritzsen, 1993). The protein content of the muscle tissue is very large and the quality of this protein is very high, containing types and ratios of amino acids that are similar to those required for the maintenance and growth of human tissue (Nettleton, 1985). Of the total nitrogen content of the muscle, approximately 95% is protein and 5% is smaller peptides, amino acids and other compounds.

In cod, storage of fat is carried out in the liver. The muscle contains on average 0.5% lipid, mostly phospholipids located in the membranes (Ackman 1967; Sidwell *et al*., 1978). Although the fat content is low, the fat is rich in polyunsaturated fatty acids (PUFA), particularly omega-3-fatty acids such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) which help to reduce blood lipids in humans, thereby helping to protect against heart disease (Nettleton, 1985). The beneficial effects of PUFAs have been associated with their ability to increase membrane fluidity and by conversion to

eicosanoids (which are compounds associated with the physiology of the vascular system) to reduce thrombosis.

Light muscle fibers of saithe (*Pollachius virens*) contain 0.5-1.0 ppm Cu and 2-4 ppm iron while the level of dark muscle fibers are three times higher for both metals respectively (Dulavik *et al.,* 1998). Heme pigments are abundant in dark muscle of fish, which is also richer in unsaturated lipids and thus more susceptible to oxidation than white muscle. The mean content of calcium and magnesium of the fresh cod muscle is reported to be 20 and 230 ppm respectively (Akse *et al*., 1993). Pro-oxidant components that are present in cod muscle include: transition metals and metalcontaing proteins, mitochondria, nicotinamide adenine dinucleotide phosphate (NADP), lipoxygenase, ascorbate, ferritin and transferrin. Antioxidant components that are naturally present in cod muscle are: tocopherols, ubiquinone, ascorbate, citrate, histidine-depeptides, superoxide dismutase, catalase, and gluthathione peroxidase. Heme pigments are abundant in the dark muscle of cod, which is also richer in unsaturated lipids and thus more susceptible to oxidation than the white muscle (Pokorný, 1987; Hultin, 1994; Dulavik *et al*., 1998).

2.3 Muscle structure

Cod muscle is not a uniform material, but contains varying proportions of three principal tissues, white (fast) muscle fibres, dark (red or slow) muscle fibres and connective tissue (Greer-Walker, 1970). The main bulk of any fillet is white muscle fibres. Changes in properties affect the texture of the cooked or raw fish as it is chewed, and if the fillet has toughened too much, it is these fibres or bundles of fibres that tend to lodge between the teeth of the eater.

Although the muscles vary in size and shape, all are characterized by an external covering of connective tissue, the fascia or epimysium. This layer of connective tissue binds the individual bundles of muscle fibres into place and binds groups of muscles together (Hultin, 1976). In fish muscle, fibre types are more distinctive than in mammalian or avian muscles. The arrangement of these muscles in codfish is shown in Figure 1 (Greer-Walker, 1970). The dark muscle is concentrated on the surface, particularly near the lateral line, which is a group of skin sensory organs extending in a single row, from head to tail, along the surface of each side of the body. Some deviation from the pattern in Figure 1 is not uncommon. Generally, the dark muscle fraction in fish increases toward the tail region. At a point one-third of the way forward from the tail, red fibres constitute 15-30% of the total. For bottom-feeding cod, the percentage of red fibres is low, ranging from 2 to 12% at this same location (Greer-Walker, 1970). White muscle from fish is very uniform in composition no matter where it is located. Dark muscle, however, varies in composition as a function of its location, containing more lipid in the anterior part of the fish and more water and protein in the posterior part.

Muscle colour and susceptibility to cold shortening and lipid oxidation are also influenced greatly by fibre type; that is, muscles with a preponderance of white fibres are generally much less susceptible to cold shortening and lipid oxidation than muscles with a majority of red fibres (Marsh *et al*, 1974; Locker *et al*, 1975). It is believed that cold shortening is directly associated with the relative amount of mitochondria and inversely accociated with the amount of sarcoplasmic reticulum (Cornforth *et al.,* 1980). Mitochondria have a high content of metal catalysts, serve as prime energy transducers of the muscle cell and are situated throughout the cell.

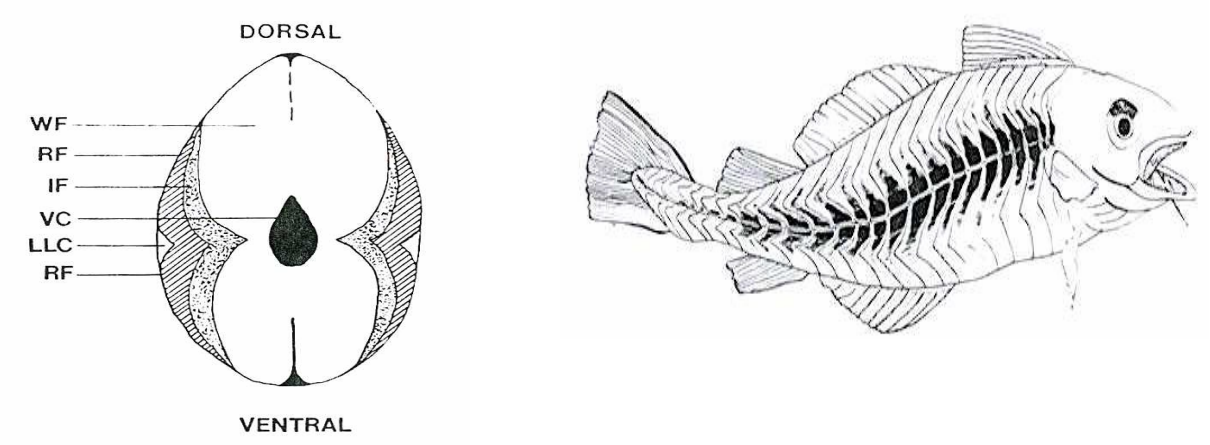

Figure 1. Left: Transverse section of cod at point of maximal flexure: WF, white fibres; RF, red fibres; IF, intermediate fibres, VC, vertebral coloumn, LLC, lateral line canal (From Greer-Walker, 1970). Right: Cod with skin removed to show dark muscle (From Love, 1988).

Extracellular matrix

Most muscle cells in fish are in contact with an intricate meshwork of interacting, extracellular macromolecules that constitute the extracellular matrix. The term connective tissue is often used to describe the extracellular matrix plus the cells (fibroblasts, macrophages and mast cells) found in it. The main bulk of connective tissue in a fillet is the skin. Much of the skin of fish is converted to soluble gelatine during cooking and causes little difficulty for chewing or swallowing. More important to the concept of fish as food is the particularly connective tissue which is widely distributed throughout the musculature and amounts to about 3% of the flesh (Dunajski, 1979). Connective tissue is the material which holds the fillet together and if it weakens for any reason, the musculature may be difficult to fillet and after filleting cannot be skinned mechanically or hung for smoking. Further, a fillet which gapes or disintegrates is unlikely to be bought by a customer. Although the fillet consists of large numbers of parallel muscle cells, the mass can be seen to be greatly subdivided by thin membranes (myocommata) of collagen. The dividing lines, which are easily visible, are the exposed edges of thin membranes of collagen, extending from the flat surface of the fillet through to the skin, often forming “cones” on the way. Collagen is the major protein of the extracellular matrix.

Intracellular proteins

Delicate extensions of fine connective tissue from the perimysium, the endomysium, surround the individual muscle fibers, which constitute 75 to 92 % of the muscle volume (Forrest *et al*., 1975). Inside the endomysium is a thin membrane, the sarcolemma, which encloses the soft sarcoplasmic content of the muscle fiber. The sarcolemma appears as a homogenous, apparently structureless, membrane, which can be clearly distinguished from the finest divisions of the connective tissues. Invaginations of the sarcolemma form the transverse system called T-tubules. The ends of the T-tubules meet in the interior of the cell close to two sacs of the sarcoplasmic reticulum. The sarcoplasmic reticulum is a membranous system located in the cell (fibre) and generally arranged parallel to the main axis of the cell.

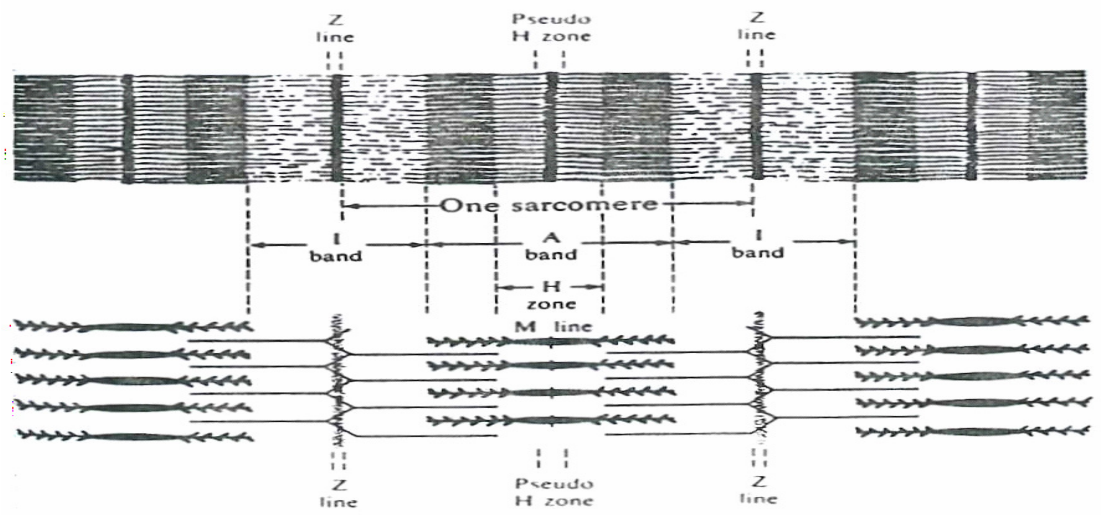

Figure 2. Thick and thin filaments of striated myofibrils, located transversely to the fibre direction.

The major inner components of the muscle fibre are the myofibrils, which constitute the contractile apparatus (Figure 2). The myofibril fraction contains at least 20 distinct and unique proteins. Myofibrils are threadlike structures lying in the sarcoplasm within the mitocondria, T-tubules and sarcoplasmic reticulum of the muscle (Bendall, 1973). A sarcomere is comprised of thick and thin longitudinal filaments. The A band is comprised of thick (mostly myosin) and thin filaments (mostly actin), whereas the I band is composed of thin filaments. The thin filaments extend outward from the Z disks in both directions, and the thin filaments overlap the thick filaments in parts of the A band. The lighter zone of the A band, the H zone, is the area where thin filaments do not overlap thick filaments. The contractile state of the muscle has an important bearing on the size of these various bands and zones, since during contraction the thin and thick filaments slide past each other. During contraction, the length of the A band remains constant, but the I band and the H zone both shorten. Thin filaments are embedded in or connected to the Z disk, and because of this, the Z disk presumably serves as an anchor during the contractile process.

2.4 Water holding capacity (WHC)

Water holding capacity, solubility, swelling, surface activity, adhesion and gelation are the main functional properties conferred on meat by its functional proteins. These properties are mostly determined by the myofibrillar fraction of the muscle (Morrissey *et al.*, 1987; Honikel, 1989). Factors that influence water binding by proteins include amino acid composition, conformation, surface characteristics, pH, ionic species and concentrations and temperature. The extent of hydration depends on the content of polar groups (ionised and unionised) (Kuntz, 1971; Kuntz and Kauzmann, 1974).

Water exists in at least two environments in the muscle, and in each of these a proportion is "bound" or "free" (Pearson *et al*., 1974). In detailed studies of myofibrils, Offer and Trinick (1983) presented evidence that most of the water in muscle is held by capillary forces between the thick and thin filaments. Most of the water in muscle is present in the myofibrils, in the spaces between the thick filaments of myosin and the thin filaments of actin/tropomysosin. The interfilament space has been observed to vary between 320 Å and 570 Å in relation to pH, sarcomere length, ionic strength, osmotic pressure and whether the muscle is *pre-* or *post-rigor* (Offer and Trinick, 1983). This corresponds to a threefold change in volume, and it signifies a far greater change in the interfilament water content than can be accounted for by the binding of water to muscle proteins. Hamm (1960) pointed out that more than 5 per cent of the total water in muscle could be directly bound by hydrophilic groups on the proteins. The stepwise release of water from muscle food by the application of different temperatures has indicated that water is bound by the proteins in several layers (Hamm, 1960; Wiberbicki *et al*., 1963; Ofstad *et al*., 1993, 1996a,b). The relative unimportance of the binding of water to the surface of proteins was demonstrated by the fact that maximum water holding by myofibrils occurred under conditions when a considerable amount of the A-band proteins had been extracted. Interfilament spacing determines the major water-holding capacity of myofibrils, and that spacing is mainly determined by long-range electrostatic forces. Constraints opposing swelling when the negative charges on the protein filaments are increased at elevated pH, include the attachments of the actin filaments to the z-line, those of the myosin filaments to the M-line proteins and the cross-links between the actin and myosin filaments themselves (Millmann and Nickel, 1980; Offer and Trinick, 1983). Offer and Trinick believe that the increased interfilament spacing is due not only to increasing negative charges on the filaments but also to an effect of the salt on the restraining links. As long as the cross-links between the thin (actin) and thick (myosin) filaments remain attached, the lattice cannot swell appreciable. Conversely, if the lattice cannot swell, the cross-links cannot remain attached. When the cross-links dissociate, they must all do so at the same time to allow swelling. Swelling must therefore be a highly co-operative phenomenon (Offer and Trinick, 1983). Both sodium chloride and pyrophosphate exert these two effects (Bendall, 1954; Greene, 1981).

2.5 Biochemical aspects of salt curing

The biochemical mechanisms of curing meat muscle were first extensively investigated by Callow (1932, 1933, 1936). Studies on salt curing of cod started approximately 20 years later and were reported at first during the 1950s and 1960s (Dyer, 1949; Duerr and Dyer, 1952; Dyer and Gunnarsson, 1954; Arnesen, 1954; Shewan, 1955; Beatty and Fougere, 1957; Castell *et al*, 1965; Del Valle and Nickerson, 1967; Borgstrom, 1968; Zaitsev *et al*, 1969).

Intrinsic factors of the muscle tissue *post mortem*

Rigor mortis is one of the most prominent changes in muscle occurring soon after death. If the fish is killed while relaxed, creatine phosphate is degraded prior to breakdown of adenosine triphosphate (ATP) (Iwamotho *et al.*, 1988). When the creatine phosphate reach about the same concentration as ATP, ATP begins to decrease (Watabe *et al.*, 1991) and *rigor mortis* starts (Iwamoto *et al.*, 1987). The muscle enters full *rigor mortis* as ATP concentration decreases to 1.25 μmol/g (Fraser *et al*., 1967). *Rigor mortis* occurs when cross-bridge cycling between myosin and actin in the myofibrils ceases, and permanent actin and myosin linkages are formed (Pate and Browkow, 1980). *Rigor mortis* is resolved after some time. Possible causes of *post-mortem* tenderization include a wakening of Z-discs of myofibrils (Hultin, 1976; Seki and Tsuchiya, 1991) a degradation of connective tissue (Seki and Wantanbe, 1984; Ando *et al.*, 1993), or a wakening of myosin-actin junctions (Yamanoue *et al*., 1998).

Salt added to *pre-rigor* meat has an inhibitory effect on the enzymes involved in *post-mortem* glycolysis, the pH attained is about 0.3-0.4 units higher than that in corresponding comminuted meat which has been salted *post-rigor*, and with the result that the added salt is less detectable to the palate (Honikel *et al*., 1981a,b). The rate of *post-mortem* glycolysis has an effect on tenderness in addition to that on shortening during the onset of *rigor mortis* in meat muscle (Lawrie, 1998). Where the rate of pH fall is inordinately fast, as with the PSE (pale soft exudative) condition in pigs (Bendall and Wismer-Pedersen, 1962), sarcoplasmic proteins are denaturated and precipitate on those of the myofibrils; the latter may also be denaturated to some extent, since they become less soluble in these circumstances. It has been shown that the ratio of insoluble myofibrillar protein to total protein in muscle is directly correlated with toughness (Hegarty *et al.*, 1963).

As glycolytic activity slows down in the muscle with increasing time *post mortem*, the ultimate pH is principally brought about by the hydrolysis of ATP and secondly by the anaerobic breakdown of glycogen to lactate (Lee *et al*., 1976). Both the extent and rate of change in pH are important. The raw cod muscle develops a softer texture and increased fillet gaping when the ultimate pH decreases below 6.6 (Dunajski, 1979; Love, 1988; Rustad, 1992; Lauritzsen, 1993; Ofstad *et al*., 1993; 1996a,b). Gaping occurs when the myotomes of fish muscle separate and it is probably caused by one or more of several independent factors: low pH, *rigor*, high temperature (Love, 1980; Love, 1982) and disintegration of collagen fibres in the pericellular connective tissue (Ando *et al*. 1991a,b; 1992; 1993). The texture of cooked

fish is also closely related to the *post-mortem* pH of the flesh, that is the lower the ultimate pH, the tougher the texture. The pH apparently exerts its effect on the texture of fish muscle by influencing the contractile elements, since fish collagen is disrupted by normal cooking. In meats of all species, decreasing meat pH results in shrinkage of the filament lattice due to the equalization of charge. Attachment of the myosin heads to actin at *rigor* causes further shrinkage (Offer *et al*., 1988a,b). The ultimate muscle pH also affects the enzymatic activity of the fish muscle *post mortem*. This is described in detail below in the chapter on salt ripening.

Salt diffusion

Salt diffusion means the net transport of NaCl molecules from one area to another due to the gradient of NaCl-concentration across the areas. Solute transfer can occur because of convection, molecular diffusion or eddy diffusion. Convection and eddy diffusion influence transfer in the extract, but diffusion due to random molecular motion dominates in the solid. Convection and eddy diffusion are fast compared to molecular diffusion; in the leaching of foods, diffusion in the solid is usually rate-controlling. The extent of control is indicated by the Biot number, Bi = kma/Ds, where k is the mass-transfer coefficient in the extract, a is the characteristic dimension of the solid, Ds is the diffusivity in the solid, and $m = (Y/X)\infty$ is the equilibrium distribution ratio between Y, the solute concentration in the extract, and X, the solute concentration in the solid. Y and X are concentrations on a mass-per-unit-volume basis or a mole-per-unit-volume basis (Schwartsberg and Chao, 1982). Fick's first law provides the basic definition for the Ds:

$J = - Ds (\partial X/\partial r)$, where J is the diffusion-induced solute flux in the r direction and $\partial X/\partial r$ is the solute concentration gradient in that direction (Peters, 1971).

The factors which influence the uptake of salt into the muscle tissue may be divided into internal factors of the muscle and external factors of the environment.

Internal factors:

- The size of the muscle, the fibre type (light or dark), fibre direction (Wood, 1966) and the *rigor* state (Hamm, 1960; Honikel *et al*., 1981a,b; 1983; Akse and Joensen, 1996)
- Chilled or frozen storage prior to salting (Kvande-Pettersen, 1971, 1973; Deng, 1977; Bello *et al*., 1981; Akse, 1995)
- The ratio of connective tissue to muscle fibres (age of the fish) (Borgstrom, 1968; Wilding *et al*., 1986)
- The pH (Hamm, 1960; Farouk and Swan, 1997; Körmendy and Gantner, 1958)
- The fat content (Wood, 1966) and temperature of the fish (Körmendy and Gantner, 1958; Del Valle and Nickerson, 1967).

External factors:

- Brine temperature (Callow, 1934; Wistreich *et al*., 1959; Holmes, 1960; Henrickson *et al*., 1969; Peters, 1971)
- NaCl concentration of the brine (Duerr and Dyer, 1952; Crean, 1961; Peters, 1971; Bøgh-Sørensen *et al*., 1986; Slabyj *et al*., 1987; Berhimpon *et al*., 1991; Poernomo *et al*., 1992)
- The volume of the brine in relation to the volume of the muscle (Borgstrom, 1968; Peters, 1971)
- Salting method (Crean, 1961; Voyle *et al*., 1986; Akse *et al*., 1993; Lauritzsen *et al*., 1994; Halsebakke, 1996; Joensen *et al*., 1997)
- Salt or brine composition (Wilding *et al*., 1986; Bernthal *et al*., 1989; Joensen, 1994; Lauritzsen and Akse, 1995; Martinsen, 1995; Joensen *et al*., 1996)
- The microbial activities (Borgstrom, 1968)

Normally, diffusion of sodium chloride into muscles is rapid, equilibrium being established in about 48 hours in 25 % NaCl, w/w (Callow, 1930; Peters, 1971). The slower the diffusion inwards however, the longer is the period of outflow of water from the muscle. Slow inward diffusion is favoured by immersion of the meat in relatively weak salt solutions and by a closed micro-structure in the tissue.

Ionic strength and ionic composition

Minimum WHC of the meat muscle occurs at the isoelectric pH (~5) where the actomyosin has zero net charge (Morrisey *et al*., 1987). The WHC of wild and farmed cod muscle tissue increase with increasing pH above the isoelectric point post mortem (Love, 1988; Rustad 1992; Lauritzsen, 1993; Ofstad *et al*. 1993; 1996a,b). Rao *et al.* (1989) found that WHC also increased from pH 5.1 to 4.0 in beef muscles, which swelled both across and along the fibre axis. Interactions between the swelling of the muscle fibres and that of the connective tissue determined the total swelling of the muscle between pH 4.5 and 4.0. Gault (1991) demonstrated that mildly acid conditions during marinating were associated with an increased toughness in the cooked meat when the pH was ca. 5.0. Such low pH values would not arise naturally in fish meat since the enzymes affecting *post-mortem* glycolysis tend to be inactivated as the pH falls to 5.4-5.5 close to the isoelectric point of the muscle proteins. Only very rarely does the pH fall below 5.0. The preservation of foods by organic acids in traditionally marinated meat with vinegar and spices, however, involves conditions which enhance the WHC of muscle proteins on the acidic side of the iso-electric point.

The amount of water bound to the muscle proteins depends on the steric availability of polar groups, the surface area and general surface topography (Kuntz, 1971; Bull and Breese, 1976; Foegeding *et al*., 1996). Uptake of NaCl causes a displacement of the isoelectric point

of meat proteins in the direction of decreasing pH-values. The amount of water bound to proteins increases with increasing neutral electrolyte concentration up to 0.1-0.15 M (salting-in). The amount of water uptake may be as high as 40% and depends almost entirely on the ionic strength of the medium. Maximum WHC occurs at an ionic strength of 0.8-1.0 M or at ~6% NaCl (w/w). The pH-values of the isoelectric points decrease gradually until there is a salt concentration of 6%. A concentration of 1 M NaCl (6%, w/w) corresponds thus to an isoelectric point at pH 4.35. The increase in water absorption of meat caused by sodium chloride is correlated to and is probably due to a displacement of the isoelectric points of meat proteins. Salting-in depends on the surface charge distribution and polar interactions with the solvent. At higher concentrations (~ 2M) there is a decrease in bound water as ions compete with protein groups for water. An increase of the salt concentration beyond 20% NaCl, causes a displacement of the isoelectric points in the alkaline direction. A 20% (w/w) NaCl concentration corresponds with an isoelecric point at pH 4.73 (Niinivaara and Pohja, 1954). The electrical double layer surrounding the protein molecules is suppressed. Protein conformation is altered, resulting in reduced protein hydration and probable precipitation.

When the NaCl concentration of the muscle increases from 1 to 5 M NaCl, the muscle tissue starts to shrink and the salting-out effects take place. Salting-out is often described as loss of stable hydrophilic surface, causing exposed hydrophobic areas of proteins to interact, aggregate and precipitate (von Hippel and Schleich, 1969). Knight and Parsons (1988) have observed the swelling of myofibrils in concentrated salt solutions. They described the effect as entropic swelling pressure caused by a steric resistance to the rotational movement of the tails of myosin molecules imposed by the actin filaments to which they were attached. They attributed the apparent dehydrating effect of higher salt concentrations to the precipitation of myosin, a feature which would reverse its depolymerization at high ionic strength (>1 M NaCl). Knight and Parsons (1988) also found that when myofibrils were exposed to solutions of increasing molarity in 1 M steps, the extractability of protein was more complete than when the muscles were exposed to 5M sodium chloride directly, as Callow's empirical observations indicated (Callow, 1930; 1931). On the other hand, less protein was extracted from the A-band by 1 M sodium chloride if the myofibrils had first been subjected to 5 M brine, possibly because the latter had caused some denaturation of the mysosin.

Salts markedly affect water binding by proteins because of their effects on electrostatic interactions (Damodaran and Kinsella, 1982). The effects of salts vary with the cationic and anionic species involved and are related to size of the hydrated radii of the particular salt species (Hofmeister series) (Kinsella, 1982; Fennema, 1985). In general, according to the Hofmeister series, the cations $Ca^{2+}>Mg^{2+}>Li^{+}>Cs^{+}>Na^{+}>K^{+}>NH4^{+}$ tend to enhance the solubility of proteins when added with the chloride ion, while, with the sodium cation, the anions $SCN^{-}>CLO_4^{-}>I^{-}>NO_3^{-}>Br^{-}>Cl^{-}>CH3COO^{-}>F^{-}>HPO_4^{2-}>SO_4^{2-}$ have a similar effect (von Hippel and Wong, 1964; Hatefi and Hanstein, 1969). The salt effects on

the WHC of the muscle are mainly explained by the binding of the cations at low salt concentration (<0.1 M) and by binding of the anions to the proteins at high salt concentration (0.1-1 M) (Sarkar, 1950). The addition of the salts to the meat muscle affects the WHC of the muscle tissue differently according to whether the pH is on the acidic or alkaline side of the isoelectric point. Generally, when on the alkaline side, the salts increase the WHC while on the acidic side the salts hardly increase the WHC of the muscle (Hamm, 1960; Hamm, 1986). In addition, interaction effects between different salts may affect the solubility of the muscle proteins. When pyrophosphate is added together with NaCl, the amount of NaCl needed to reach maximal swelling of the proteins is reduced. With only NaCl added, the swelling of the proteins is caused by the extraction of the mid section of the A-band. In the presence of both NaCl and pyrophosphate, the A-band gets completely extracted (Offer and Trinick, 1983). The critical factor seems to be the cross-bridges (M and Z lines) between the thick and thin filaments that are overlapped, and to what extent they are resolved by the salts to able further expansion of the muscle network. The sarcomere length seems to have less influence on the swelling. Divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}), in addition to increase the WHC of the muscle proteins at low concentrations (von Hippel and Wong, 1964; Hatefi and Hanstein, 1969), are able to crossbind the polypeptide chains within the muscle proteins dependent on the pH. In general, such crossbinding reduces the WHC of the muscle proteins (Hamm, 1986).

Salt ripening of the fish muscle

The sensory characteristics in salt ripened cod are mainly determined by increased salt concentration, enzymatic activity, non-enzymatic changes in the water, protein and lipid fractions of the fish muscle and by interactions between these processes. Microbial growth is minimal in cod muscle at high NaCl concentrations (>17%) and has therefore probably little influence on the sensory properties of heavily salted cod (Lauritzsen and Akse, 1994). Ripening processes due to microbial growth are therefore not focused in this thesis.

Changes in the protein and water fractions

Immediately after slaughtering, the deterioration of the muscle proteins starts due to enzyme activity of endogenous muscle proteases, fibroblasts, bacteria on the fish surface or of residual blood cells. If the fish muscle is additionally contaminated with digestive enzymes from the gutting operation, these can also be activated. The activity of all enzymes will depend on the amount of active enzyme, the presence of inhibitors, activators and required co-factors and the muscle pH (Foegeding *et al*., 1996). The gutting operation, washing and storage time and temperature prior to salting are therefore of great importance to the enzyme activity in the flesh during salt ripening. The enzymes from various sources have different pH optima and areprobably affected differently by sodium chloride.

Cathepsins are lysomal proteinases that show optimal activity at acid pH. Those known to occur in muscle and to act on muscle proteins are (type, pH optimum) cathepsin B1, pH 3.5-6.0; cathepsin H, pH 6.0; cathepsin L, pH 5.0; cathepsin D, pH 3.0-5.0. Calpains are muscle proteinases that are activated by calcium at neutral pH (Gildberg, 1988; Foegeding *et al*., 1996). Trypsin-like serine proteinases have been found in fish (Toyohara *et al*., 1991). These proteinases cause the undesirable breakdown of surimi-based fish products. The activity of the calpains in fish muscle is relatively low (Foegeding *et al*., 1996). It may therefore be relatively low during salt ripening of cod compared to the activity of the cathepsins, trypsin-like serine proteinases, being active at low pH.

The major changes in the protein fraction of the cured fish are caused by the increased NaCl concentration which lowers the muscle pH and increases protein denaturation. Protein denaturation caused by salts and changes in pH differ from that of heat denaturation (Privalow and Makhatadze, 1993; Khechinashivili *et al*., 1995). Specific ionic effects on the proteins may also occur during heavy salting of cod. Magnesium chloride has a salty bitter taste and by binding with the muscle proteins it may influence the taste of the cured product (Borgstrom, 1968). Particularly calcium salts with >0.3 to 0.5% (in terms of Ca) have been reported to affect the ripening of cured fish. This impurity gives rise to pronounced protein coagulation and hardens the flesh (Borgstrom, 1968; Zaitsev *et al*., 1969).

Cod contains a significant concentration of trimethylamine oxide (TMAO) which is dissolved in the water fraction of the muscle tissue. In some individuals the content of TMAO may exceed 0.1 M in *post-mortem* fish muscle (Sikorski and Kostuch, 1982). Micro-organisms can reduce TMAO to trimethylamine, a compound responsible in part for the "fishy" odour that develops in fish on storage. Trimethylamine oxide also decomposes in fish to dimethylamine and formaldehyde. This reaction is catalysed by an enzyme, and the rate is particularly great in muscle tissue of cod. The rate of this reaction is also increased by freezing or disrupting of the muscle. It has been hypothesised that the formaldehyde produced in this reaction cross-links the muscle proteins, contributing to toughening that occurs in gadoid fish during frozen storage (Mackie, 1993: Sikorski and Kolakowska, 1994; Foegeding *et al*., 1996). During salt ripening of cod previously frozen and thawed, the denaturation of the muscle proteins may increase due to the interaction effects from crosslinking of the muscle proteins and salting-out effects. By storage of the cod prior to salting, micro-organisms can utilise amino acids in the muscle, leading to a large number of breakdown products, such as hydrocarbons, aldehydes, ketons, sulfides, mercaptans and amines (Foegeding *et al*., 1996). These products may interact with the protein, carbohydrate and lipid fractions of the cod muscle tissue during salt ripening.

Lipid deterioration

By heavy curing, water activity is lowered in the cod muscle to inhibit bacterial growth and enzymatic spoilage. Labuza (1971a,b) studied food stability as a function of water activity (aW) and moisture content. The results are summarised in Figure 3. The aW value of heavily salted cod lies generally in the range of 0.7 to 0.75 (Lupin *et al.*, 1981; Gomez and Fernandez-Salguero, 1993; Fernandez-Salguero *et al.*, 1994). In this aW-range the non-enzymatic browning is maximal and enzymatic activity is at moderate level. One may therefore suggest that the main causes of changes in the stability of the cod flesh during ripening are influenced by non-enzymatic pathways.

Cod lipids are highly susceptible to oxidation because they contain high levels of polyunsaturated fatty acids, 50-60%, including 15-18% eicosapentaoic acid (EPA) and 35-38% docosahexaenoic acid (DHA) (Ackman, 1967). The cod muscle also contains prooxidants (ferritin, transferrin, NADPH/ATP, ascorbate, lipoxygenase) that generate lipid free radical indicators, and natural antioxidant (ascorbate, tocopherols, citrate, phosphates, amino acids) systems inhibiting oxidations. These pro- and antioxidant systems are affected by many factors, including proteolytic enzyme activity, storage temperature, oxygen concentration, water activity (aW), pH, metal contaminations, salt concentration, addition of anti-oxidants, UV-light, and processing causing cellular damage (Labuza, 1971a; Khayat and Schwall, 1983; Kanner *et al*., 1987; Pokornỳ, 1987; Hultin, 1994; Frankel, 1998).

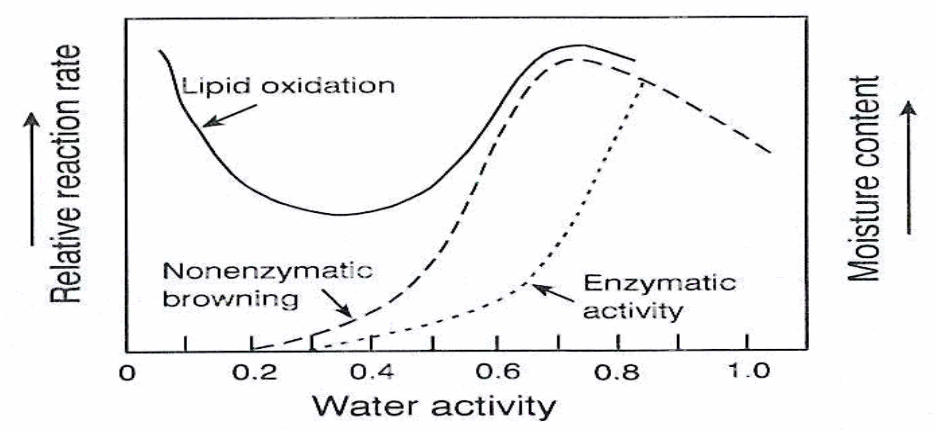

Figure 3. Relative reaction rate as a function of lipid oxidation, non-enzymatic browning and enzymatic activity. Adapted by Labuza (1971a).

Increased NaCl has been reported to increase the auto-oxidative processes and to reduce enzymatic oxidation in salted sardines (Takiguci, 1989). Kanner *et al*. (1991) found that NaCl increased rancidity in turkey meat due to an ionic exchange between Na and Fe ions in the proteins, increasing the catalytic activity by “free” iron ions.

Non-enzymatic, auto-oxidation of PUFAs may be divided into three steps; (1.) Initiation (induction), (2.) Propagation (chain-reactions) and (3.) Termination.

Direct initiation involves two types of mechanism:

- higher valance state iron (Fe^{3+}) which can extract hydrogen from an unsaturated fatty acid to produce an alkyl radical;
- lower valence iron (Fe^{2+}) which can form iron-oxygen and hypervalent iron complexes able to directly generate alkyl radicals or which produces reactive oxygen species through the Haber-Weiss reaction coupled with the Fenton reaction as outlined below:

1. $Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot-}$
2. $O_2^{\cdot-} + H^+ \rightarrow HO_2^{\cdot-}$
3. $O_2^{\cdot-} / HO_2^{\cdot-} \rightarrow H_2O_2 + O_2$ Haber –Weiss reactions (1-3)
4. $Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe^{3+}$ Fenton reaction (4)

The Fenton-type reaction is the main reaction involved in hydroxyl radical formation in biological systems (Gutterigde, 1984). It is probably the phospholipids (PL) of the muscle membranes that are most susceptible to lipid oxidation during ripening. There are primarily two reasons for this. Firstly, the PL contain long chain PUFAs which are very sensitive to oxidation. Secondly, PL are membrane components in close contact with catalysts of lipid oxidation located in the aqueous phase of the muscle cell. In the water fraction of the cod muscle cells we find metal catalysts such as iron and copper ions. Iron is implicated in several stages of the lipid oxidation chain. Both heme and non-heme iron present in fish muscle tissue catalyse lipid oxidation. Iron complexed metabolites, referred to as low-molecular weight iron, and iron complexed with myoglobin and haemoglobin, referred to as high–molecular weight iron, participate in the intiation and propagation of lipid oxidation in fish. Lipid auto-oxidation forms secondary end products such as aldehydes and ketones (4-heptenal, 2,4-heptadienal, 2-hexenal, 2,4,7-decatrienal, 1-octen-3-ol, 1,5-octadien-3-ol, 2,5-octadien-1-ol, 1,5-octadien-3-one, 2-nonenal, 2,6-nonadienal) which are strongly aromatic and flavourful compounds at very low concentrations (Hsieh and Kinsella, 1989). Volatiles with the highest smell impact have been reported to be 1,5-octadien-3-one, 2,6-nonedienal, 3-hexenal and 3,6-nonadienal (Milo and Grosch, 1993). The overall picture of the reactions involved in nonenzymatic lipid oxidation is outlined in Figure 4.

The endogenous enzymes called lipoxygenases which oxidise polyunsaturated fatty acids have been found particularly in the gills and skin of fish. The role of lipoxygenase in initiating lipid oxidation in meat systems is not clear yet. Salt cured skin-on products may be influenced by the enzymatic lipid oxidation processes (German and Kinsella, 1985), which probably occur at a moderate level according to Labuza (1971a). These enzymes produce

conjugated diene hydroperoxides that may degrade either by gluthatione peroxidase to produce stable hydroxyl fatty acids, or non-enzymatically by metal-heme catalysis to produce alkoxyl radicals that undergo cleavage into aldehydes and carbonyl compounds causing rancidity. The acid value of fat in salted chum salmon has been reported to increase from 3.6 to 22.5 during storage (Zaitsev *et al*., 1969). The relationship between enzymatic activity and flavour remains unclear however.

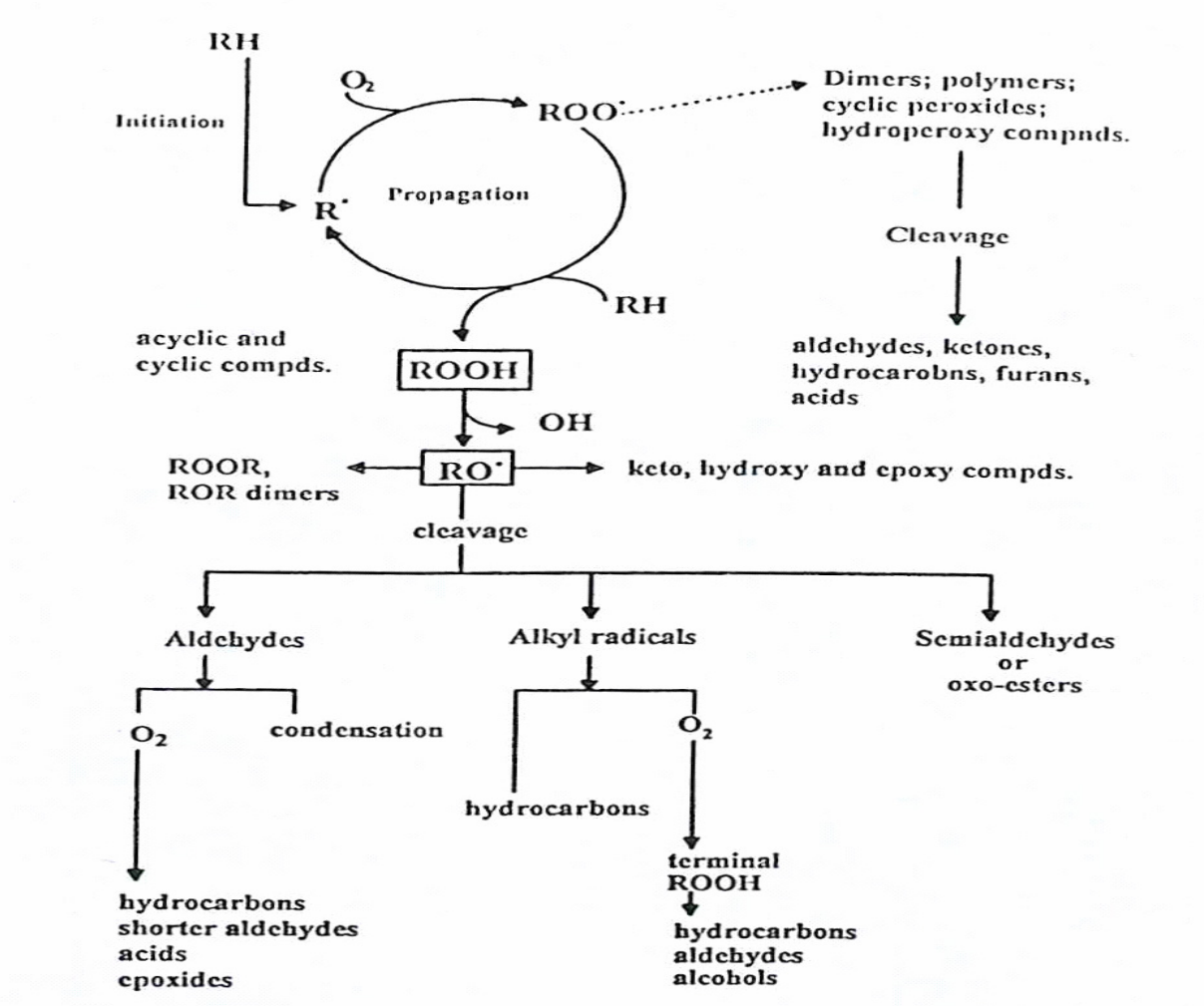

Figure 4. Overall picture of the non-enzymatic auto-oxidation of fatty acids (From Foegeding *et al.*, 1996).

Interactions in the fish muscle

We know that lipids affect the development of sensory properties such as the texture, colour, flavour and taste of salted fish (Shewan, 1955; Zaitsev *et al*., 1969; Sikorski and Ruiter, 1994). The lipid oxidation end products can interact both with carbohydrates and proteins and thereby affect the flavour deterioration, the colour and nutritive value of food proteins. Sugars and other carbonyl compounds interact with amino acids or proteins in a sequence of complex reactions known as the Maillard type reaction or as non-enzymatic browning. The browning

products from this reaction have a marked influence on lipid oxidation. They generally retard lipid oxidation in foods and contribute to flavours. Lipid oxidation products can also react with proteins and amino acids, leading to the loss of essential amino acids with impact on the oxidative stability and the nutritional quality of foods. The interaction of amino compounds with reducing sugars proceeds by addition of a carbonyl group to a primary amino acid, peptide or protein, by water elimination through an intermediate imine, cylizing to a glycosylamine (N-glycoside). Aldehydes, dialdehydes and epoxides derived from decomposition of hydroperoxides may react with amines to produce imino Schiff bases (R-CH=N-R`). Schiff bases polymerize by aldol condensation producing dimers and complex high-molecular weight brown macromolecules known as melanoids that are well characterised (Figure 5). These polymeric brown materials are unstable and generate new volatiles by scisson of the macromolecule or by dehydration, that affect the flavour characteristic of foods during cooking and processing. Hydroperoxides react with proteins to form protein free radicals which cause the polymerization of the peptide chain to form protein-protein cross-links (Pokornỳ, 1981). The polymerization of lipids and peptide chains lead to browning of food proteins and changes in food texture and rheological properties.

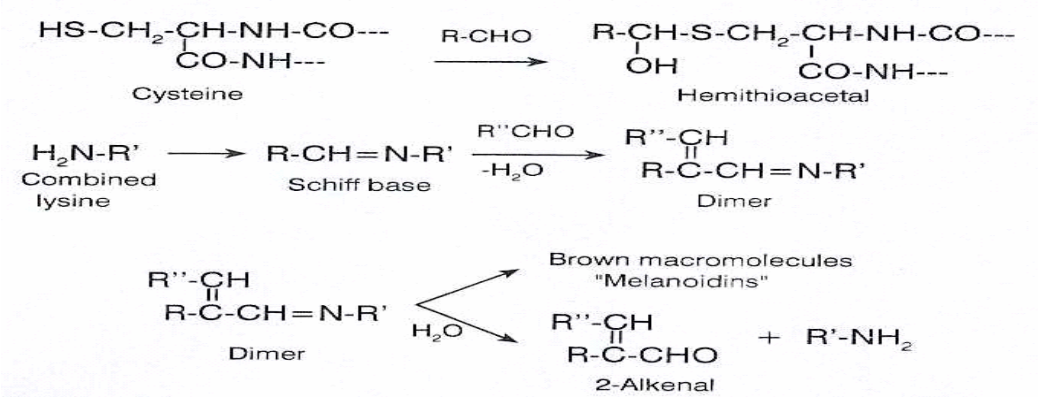

Figure 5. Interaction products between cysteine and aldhydes, and between a Schiff base and combined lysine. From Pokornỳ (1981).

Inhibition of lipid oxidation

Most of the antioxidant strategies that have been developed have been designed to inhibit iron-catalysed oxidation and different strategies may be required for copper. However, copper forms complexes with many of the same low molecular weight components of muscle tissue as does iron (Decker *et al.,* 1989).

Muscle tissue contains a multi-component antioxidant system consisting of lipid-soluble compounds (alpha-tocopherol, ubiquinone), water-soluble compounds (ascorbate, histidine-dipeptides) and enzymes (superoxide dismutase, catalase, glutathione peroxidase). Lipid oxidation is retarded in fish by synthetic antioxidants (butylhydroksianisol (BHA),

butylhydroksytoluen (BHT), TBHQ), natural antioxidants (tocopherols, flavenoids), metal chelators (EDTA, phosphate, citrate, carnosine) and by reducing agents (ascorbic acid, isoascorbic acid and their salts, sulphur dioxide) (Labuza, 1971a; Gordon, 1990; Hölmer, 1995).

In water solution, ascorbic acid is known by its multifunctional effects: as an antioxidant (>1 mM), a prooxidant (0.01mM), a metal chelator, a reducing agent or as an oxygen scavenger. Ascorbic acid retards the oxidation of herring during frozen storage (Thed and Erickson, 1994), but may promote the oxidation of cooked fish (Ramanathan and Das, 1993). A combination of these effects may predominate in many food applications. Metals such as Fe^{3+} reduced by ascorbic acid are much more catalytically active in their lower valance state (Fe^{2+}) in the homolytic decomposition of hydroperoxides. Ascorbate acid also interacts with Cu^{2+} to accelerate the decomposition of linoleate hydroperoxides (Kanner *et al.,* 1977; Deng *et al.,* 1978; Niki, 1991; Kanner, 1994; Hultin, 1994). Flavenoids are effective antioxidants in prolonging the shelf life of ground fish. EDTA and other antioxidants inhibit enzyme-catalysed lipid oxidation by removing iron and reducing hydrogen peroxide. Antioxidants are more effective in minced fish where they become more readily incorporated with oxidisable lipids than in whole fish (Löliger, 1991; Hölmer, 1995).

3 OBJECTIVES OF THE STUDY

The main aim was to investigate initial factors during salt curing of cod which have influence on the quality of the salted product.

Part issues:

- To investigate the influence of the raw material on the salt cured product
- To investigate ionic effects and their interaction effects on the fish muscle quality of salted cod

4 MAIN RESULTS AND DISCUSSION

The main results and discussion of this thesis are devided into three parts.
The three parts are:

(1) Intrinsic factors of the cod muscle which have influence on the salted cod.
(2) Denaturation of muscle proteins affecting the quality of heavily salted cod.
(3) Salt ripening processes affecting the quality of the heavily salted cod.

4.1 Intrinsic factors of the cod muscle which have influence on the salted cod. (Papers I-V)

Freshness is one of the most important parameters of quality in most markets. Fresh products often achieve a higher price based on a general attitude among consumers that "Fresh is better". For the processor it is desirable to preserve freshness by starting production as early as possible after slaughter in order to gain time for distribution. The influence of state of *rigor* and *post mortem* storage (cold or frozen) of cod prior to salting, on the salted products have been investigated in Papers I and II. In Paper I, cod fillets were lightly salted in different states of *rigor*. A significant higher weight yield (13%, w/w) was obtained by salting the fish *post-rigor* than by salting *pre-rigor*. All of the farmed fish used in these experiments came from fish that were fed similarly prior to processing and were sampled from the same net-pen. Differences in feeding regime among fish individuals should therefore not have affected the results. In Paper II, the influence of *rigor* state during heavy salting of cod was investigated and again a larger weight reduction was found for fish salted *pre-rigor* compared with fish salted *post-rigor*. Both light and heavy salting gave similar tendencies regarding the weight yield of the product, but the weight reductions were largest in the latter. Light salting of cod fillets (<5% NaCl, w/w) was expected to induce swelling of the fish muscle proteins, which usually occurs when increasing the NaCl concentration in meat muscle up to approximately 6% (Hamm, 1960). However, the fillets salted *pre-rigor* lost weight instead of gaining weight and seemed not to swell as the fillets salted *post-rigor* (Paper I). During the onset of *rigor mortis*, there is little change in the "bound" water, but the proportion of so-called "free" water in the extracellular region increases at the expense of the "free" intracellular water. Careful histological studies have shown that at least two extracellular environments develop in muscle during *rigor mortis* (Offer *et al*., 1983-1985). Prior to and during the onset of *rigor mortis*, there is in unsalted cod muscle a decrease in pH from ~7.0 (at death) to ~6.5-7.0 in wild caught cod and usually down to pH<6.4 in farmed cod *post mortem* (Love, 1988; Rustad, 1992; Lauritzsen, 1993; Ofstad *et al*., 1993; 1996a,b) and a concurrent decrease in WHC occurs (Hamm, 1975). The intermolecular cross-linking between myofilaments (actomyosin formation) caused by the *rigor* development, does not exert an additional effect on WHC in the absence of salt (Honikel *et al.*, 1981a,b). However, the various membranes and connectin and desmin, may be expected to exert constraints on swelling. It may be suggested that the

low WHC of cod muscle salted in *pre-rigor* state, was due to a low electrostatic repulsion between the dissociated myofibrillar proteins, myosin and actin, caused by the combined influence of a high ionic strength (at 26% NaCl concentration), low pH and low ATP (Papers I and II). Probably, the interfilamental cross-linkages during *rigor mortis* more than counteracted the electrostatic repulsion and loosening of the protein network induced by a moderate salt content (Paper I).

In Papers I and II, the influence of the NaCl concentration of the brine on the extent of muscle contractions in the fish salted *pre-rigor*, was not investigated. However, one may suggest that the saturated NaCl concentration of the brine and dry salt may have affected the nerve cells of the muscle tissue, more efficiently than a low brine concentration (<2% NaCl, w/w) would have. The reason might be the 100 x higher electro-potential between the muscle tissue (0.15-0.3 % NaCl, w/w, in fresh cod muscle *post mortem*) and the saturated brine (26%, w/w). If the nerve cells still function in *pre-rigor* muscle within 2 hours *post mortem*, they might induce muscle contractions immediately after the fish has been soaked into the brine or dry salt (personal communication with nerve physiolog Ellen Aasum with coworkers at the University of Tromsø). Electrically activated muscle contractions and *rigor* contractions may have worked synergistically and it may be suggested that this is why *pre-rigor* muscle lost such huge amounts of water and weight and absorbed less NaCl both by light and heavy salting (Papers I and II).

In Paper I, light salting of *pre-rigor* cod fillets also resulted in unfavourable changes in the texture, and appearance. However, in Paper II it was found that the fish should be heavily salted in *pre-rigor* state to reduce the waste of proteins from the raw material and to increase the instrumental lightness values (L*) of the salt ripened cod products. There seem to be contradicting effects from light and heavy salting on the lightness of the cured product. This may be due to the fact that different methods were used for colour measurements in Papers I and II respectively. It may also be due to that heavy salting denaturates muscle proteins more severely and results in a greater increase in lightness and firmness of the muscle tissue than light salting does (Hamm, 1960; von Hippel and Schleich, 1969; Asghar *et al.,* 1985).

Increased firmness was found in salted cod previously frozen compared with fish cold stored prior to salting. It is suggested to have been caused by protein denaturation occurring both during the freezing and salting processes (Paper II). The significant ($p<0.05$) lower water holding capacity (WHC) of the salted muscle that had been frozen, was probably due to protein denaturation and increased cross-linking of the polypeptide chains by the divalent cations; Ca and Mg (Hamm, 1960; Asghar *et al*., 1985; Shenouda, 1980). Processes dealing with denaturation of muscle proteins are discussed in detail below in subchapter 4.2.

In papers IV and V, the results demonstrated that lipid oxidation occurred in muscle samples brined with pure NaCl solution. The large proportions of EPA and DHA in the

cellular membranes are susceptible to non-enzymatic auto-oxidation processes caused by the pro-oxidative effect of NaCl (Smith *et al*., 1990; Castell *et al*., 1965; Koizumi *et al.,* 1981; Osinchak *et al*., 1992). The pro-oxidative effect of NaCl may be due to the reduced water activity in the muscle as reviewed by Labuza (1971a,b), the reduction in muscle-pH during the salting, and/or the ability of sodium ions to replace iron-ions of cellular complexes (Hultin, 1992; Kanner *et al*, 1991). In most food products, the development of rancid smell and taste is regarded negatively. One may suggest however, that the slight increase in TBARS during salt ripening of cod contributes to some positive sensory properties required in this type of ripened product.

In paper IV it was observed that the lowest ultimate muscle-pH prior to heavy salting of cod, gave the highest lipid oxidation. Castell *et al*. (1965) observed similar tendencies at moderate NaCl concentration (12%, w/w) in cod muscle blends. The mechanisms by which pH controls the lipid oxidation of the fish muscle, are unclear. However, it has been suggested, in studies on porcine and beef meat, that this may be the effect on the metal catalysts present (Liu, 1970; Owen and Lawrie, 1975; Yasosky *et al.,* 1984). It has been theorized that histidine residues (pKa<6.0) at low pH could alter the tertiary structure of proteins thus reducing their ability to sequester catalytic ions such as Mn, Cu, Co and Fe. The solubility of iron complexes of the cod muscle increases with lowering of the pH (Hultin, 1994). Enzymatic reactions producing the reduction products of molecular oxygen are pH dependent as is enzymatic reduction of ferric iron. Further the protonated form of superoxide (pKa 4.8) is not only more reactive than the anionic form but passes readily through the membranes. It thus allows transport of the potential catalyst to other areas in the muscle cell. Recent reports have shown that pH affected lipid oxidation of washed cod muscle when trout hemolysate was used as a catalyst (Richards and Hultin, 2000). The level of pro-oxidative deoxyhemoglobin was found to sharply increase with pH reduction from 7.6 to 6.0 (Richards *et al.,* 2002). However, in heavy curing of cod this mechanism may not be relevant since the level of hemoglobin is very low in light muscle of cod.

4.2 Denaturation of muscle proteins affecting the quality of heavily salted cod (Papers II- V)

Proteins can be denaturated by changes of various external parameters: temperature, pressure, denaturant concentration and pH. The main results from heavy salting experiments with cod discussed in Papers II - V are most probably explained by variations in the extent of crosslinking, precipitation, aggregation and denaturation of the proteins of the cured cod muscle. Denaturation of the salted cod proteins is probably caused by increased frozen storage prior to salting, increased salt concentration, lowering of the pH, and/or increased concentration of lipid oxidation end products and interactions of these processes (Hamm, 1960; Asghar *et al*., 1985; Mackie, 1993; Baadi and Howell, 2002).

Model studies of proteins in aqueous solutions may give relevant information about processes that may occur in fish muscle during heavy curing of cod (Arakawa *et al*., 1990). Proteins are of co-operative nature and their physical properties do not change gradually as the environments are changed (temperature, NaCl, pH), i.e. the native protein does not gradually loose its biological activity. There are instead small changes or no changes at all, until a certain point is reached where drastic alterations occur and protein denaturation takes place. The principle difference from the native state is that the unfolded or denaturated state is inhomogenous, i.e. a constant fluctuation between many different conformations. The folding intermediates, however, are in many cases very similar to the native structure. The thermodynamics of unfolding proteins have been described by Privalow and Makhatadze (1993) and Khechinashivili *et al*., (1995). The following description is confined to effects of changing pH and salt addition as denaturants.

Unfolding at extreme pH (<5 and >10) usually occurs because the folded protein has groups buried in nonionized form that can only ionize after unfolding, thus forcing the equilibrium towards the unfolded state. Histidine (pKa ca. 7) is often involved in pH induced unfolding at low pH and tyrosine (pKa ca. 10) at high pH (Creighton, 1993). Salt-bridges between ionising groups may be weakened or disrupted at extreme pH, at which one of the interacting groups is no longer ionized. If the salt-bridge is a very strong one, it would require a very low pH to protonate acidic groups and a high pH to deprotonate basic groups. For instance the protonation of aspartic (pKa 3.9-4.0) and glutamic (pKa 4.3-4.5) acids at low pH, would contribute to weaken internal electrostatic interactions like salt-bridges and hydrogen bonds. It may be suggested that acid induce more conformational changes of the cured cod muscle than alkali does, because there are more ionisable groups having their pKa values in the pH range of 2.5-7 than in the pH range 7-11. Glutamic acid and aspartic acid amount to ~25% of the amino acids in whole cod muscle (Dagbjartsson, 1975). Lysin (pKa 9.5-10.5), tyrosine (pKa 9.1-10.8) and cysteine (9.1-10.8) (Hamm, 1994) make together ~15% of the amino acids in whole cod muscle (Dagbjartsson, 1975). There is little evidence that repulsive charges on the surface of the protein are large enough to cause denaturation by themselves but they may have an influence on the process of denaturation. Kristinsson (2001) has studied the effects of variations in pH on some of the cod muscle proteins. Full unfolding of the myosin headgroup and partial unfolding of the rod part at pH 2.5 was found. However at pH 11, only the headgroup unfolded.

The effect of adding salts to proteins in aqueous solutions is dependant on whether the salt has preferential interactions with the aqueous interface or with the protein interface. Salts that increase stability of the native protein state, are excluded from the protein surface (higher concentration in bulk solvent), tending to decrease the protein solubility. This is called a negative binding of the stabilising additives, and the protein is said to be “preferentially hydrated”. Salts increase protein solubility and interact preferentially with the protein surface

and increase the solubilities of both polar and nonpolar amino acids in rough proportion to their accessible surface area. It decreases interaction between hydrophobic groups by one-third. It must be concluded that salts interact with both non-polar and polar surfaces more favourable than water. The physical nature of the interaction with non-polar surfaces is not known but it has been found to exist through crystallographic studies (Creighton, 1993). Thorarinsdottir *et al*. (2002) have studied changes in myofibrillar proteins during heavy salting of cod by electrophoresis and scanning calorimetry. They reported that the myosin heavy chain was cleaved into smaller sub fragments in the heavy salting of cod with the two heavy mero-myosin fractions (S1 and S2) and the light mero-myosin fraction being the most abundant. Actin was less affected than myosin.

4.3 Salt ripening processes affecting the quality of the heavily salted cod (Papers I-V)

Sensory properties of salt ripened cod are mainly determined by increased salt concentration, lowering of pH, enzymatic processes (proteolytic and lipolytic), non-enzymatic changes in the water, protein and lipid fractions of the flesh and by interactions between these processes. Pro- and antioxidant systems that are inherent components of the cod muscle are affected by many factors, including enzyme activity, storage temperature, oxygen concentration, water activity (aW), pH, metal contaminations, salt concentration, addition of anti-oxidants, UV-light, and processing causing cellular damage (Labuza, 1971a,b; Pokornỳ, 1987; Kanner, 1994; Hultin, 1994; Frankel, 1998). External factors affecting the activities of the proteolytic enzymes and lipid oxidation processes, have been investigated during salt curing of cod (Papers I-V).

Cold and frozen storage of the fish prior to salting

During the light salting experiments (Paper 1), the sensory evaluators found that fillets cured *pre-rigor* and *in-rigor* had a particularly stronger smell of ammoniac than fillets cured *post-rigor*. To check these statements, additional chemical analyses (the total volatile basic nitrogen content, TVBN) were performed on some samples. The results showed that fillets salted *in-rigor* had 35.8±2.9 and fillets salted *post-rigor* had 4.0±1.2 mg N/100 g homogenised muscle (results not shown earlier), which verified the observations made by the sensory panellists. The reasons for the higher TVBN values of the *in-rigor* salted fillets may have been that *post-rigor* fillets which were stored in ice prior to salting, had a lower fish temperature at salting than *pre-rigor* and *in-rigor* fillets. The enzyme activities of fillets salted *pre-rigor* or *in-rigor* may therefore have been higher (Castell *et al*., 1973; Mackie and Thomson, 1974).

Muscle cathepsins are localised in the lysosomes of the fish muscle. They are moderately active up to 2.5% NaCl concentrations and may therefore have been active in the

lightly salted cod products investigated in Paper I (Reddi *et al,* 1972; McLay, 1980; Gildberg, 1988). Proteolytic enzymes usually split proteins into end products such as; amino acids, dipeptides, and polypeptides which may have strong and characteristic tastes and smells. If the fish is cold and/or frozen stored before salting, the amines (NADPH, TMAO, TMA and DMA) and amino acids (cysteine and lysine) that are present in the flesh may interact with lipid oxidation end products either during the storage period prior to salting and/or during the salt ripening period. Sensory properties of the salted product may therby change. For instance, discolouration (Schiff base reactions) of the muscle tissue may occur as described earlier in the background subchapter 2.5 (Pokornỳ, 1981).

In Paper II, the effects of storage (cold and/or frozen) of the fish prior to salting on the salt cured product were investigated. Both instrumental lightness (L*) and shear force values were found to increase significantly by freezing compared with chilling of the fish prior to salting. When cod muscle proteins are exposed to lipid oxidation end products, high NaCl and traces of Ca and Mg ions during salt ripening (Papers II-V), increased cross-linking of the polypeptide chains, shrinkage and dehydration may have occurred (Hamm, 1960; Shenouda, 1980; Ragnarsson, 1987; Morrissey *et al*., 1987). Increased toughness of cod flesh after frozen storage has earlier been explained by reduced protein solubility and denaturation of muscle proteins (Shenouda, 1980; Ragnarsson, 1987; Mackie, 1993; Sikorski and Kolakowska, 1994). It has been hypothesised that the formaldehyde produced by enzymatic degradation of TMAO of the cod flesh, cross-links the proteins, contributing to increased toughening (Foegeding *et al.,* 1996). It is well known that lipid oxidation end products develop several rancid tastes and flavours in food products. The compound found to be responsible for off-flavours in frozen stored and thawed cod is cis-4-heptenal (McGill, 1974; McGill *et al.,* 1974) which might be found in heavily salted cod products. Brown macromolecules, aminocompounds and aldehydes are end products of Schiff base type reactions described in the background subchapter 2.5. Both formaldehyde and cis-4-heptenal are thus potential compounds that might influence the equilibrium of Schiff base type reactions (Pokornỳ, 1981). An increased content of aldehydes in the cod muscle prior to salting, might force the equilibrium of the reaction backwords during the salt ripening processes. Then the salted product would be more white coloured compared with fish chilled before salting. However, if both the amine and aldehyde concentrations increase due to cold and frozen storage respectively prior to salting, the equilibrium of the reaction might be forced both forwards and backwords during salt ripening.

Freezing of cod prior to heavy salting, might also influence the activities of the proteolytic enzymes during the salt ripening (Stefansson *et al*., 2000). If the muscle proteins are denaturated during frozen storage, one would expect the activities of the protelytic enzymes to be reduced. This relationship has not been investigated so far and should be focused in future studies on heavy curing of cod.

Pro-oxidants and pH in the brine

Quantitatively the principal transition metal in seafoods is iron, as it is in almost all foods. Most of the information that is available on the role of transition metals in seafood and related products is concerned with iron, although copper undergoes a similar redox cycling and make an important contribution to the activation of oxygen (Hultin, 1994).

In Papers IV and V, yellow/brown discolouring of the cured cod muscle surface was investigated in downgraded heavily salted cod fillets and in muscle cubes by a model system simulating heavy salting processes of cod. Both the results from the processing plant and from the model studies showed that discoloured areas were evenly distributed on the muscle surfaces and that lipid oxidation correlated with the copper and not the iron content in the muscle. The observation that the yellow colour penetrated 2-5 mm into the flesh was consistent with an oxygen dependant process (Lawrie, 1974). Ferrous iron seemed to be "inactivated" either by physical interaction (chelation) or by chemical oxidation as suggested earlier by Hultin (1992). It has been suggested that the oxidative damage catalysed by copper occurs at different target molecules than those attacked by iron. This may be principally due to the different binding properties of the two metals to various cellular components (Gelvan and Saltman, 1990). Copper has been reported to be as effective or even more effective in stimulating the decomposition of peroxides (Guttridge *et al*., 1984), causing for instance protein modification (Stadtman, 1990) and formation of fluorescent lipid complexes (Guttridge *et al*., 1984). A positive correlation between the copper content, lipid oxidation (TBARS) and instrumental yellow colour was clearly demonstrated using the model system in both Papers IV and V. Of the redox states, the reduced form of copper was most potent (Paper IV). This may be explained by direct participation of the reduced copper in a Fenton-type reaction where hydroperoxides are reduced to hydroxyl radicals or lipid alkoxy radicals. In addition, auto-oxidation of the metal may reduce oxygen to a superoxide radical anion, which can dismutate to hydrogen peroxide or oxidize to singlet oxygen. The latter can react directly with an unsaturated fatty acid to initiate lipid peroxidation. The oxidised metal may then react with fatty acid peroxides to form fatty acid radicals or fatty acid peroxy radicals (Background subchapter 2.5). Potential sources for copper contaminations with the fish muscle during heavy curing are the tap water, salt and fish processing equipments.

In Paper III, the pH of the salt was found to be positively correlated with the muscle-pH and negatively correlated with the Ca and Mg content of the heavily salted cod muscle. Increased calcium content of the cured muscle increased both the lightness and firmness of the cured product. Increased magnesium content of the cured muscle significantly increased the lightness of the product. The effects of the pH, Ca and Mg levels of the salt on lipid oxidation and enzyme activities of the cured cod muscle were not investigated (Paper III). However, preliminary results after 6 months of cold storage of the salted products at +4°C

with access to oxygen and light, showed slight indications of increased lipid oxidation in the products salted with a low pH (<5) in combination with high levels of Ca and Mg of the salt (results not shown). Small (0.1- 0.3 cm in diameter) yellow discoloured spots were noticed visually on the cured muscle surfaces but no particular acrid or rancid smells from the fillet surfaces were observed. These preliminary results are in accordance with the results discussed in subchapter 4.2 (Paper IV), showing that the lowest muscle pH *post-mortem* prior to salting, gave the most oxidised salted product. In salted cod muscle *post-mortem*, calcium ions absorbed from the salt/brine, may activate enzymes such as lipases, phospholipases and proteases, either directly or through modulators like calmodulin (Hultin, 1994). This may play an important role in lipid oxidation during salt ripening of cod. The storage stability of cod salted with a low pH and high levels of calcium and magnesium of the salt should therefore be investigated in future studies.

Adjustments of the pH of the brine may also affect the enzyme activities of the fish muscle tissue during heavy salting and thus affect the quality of the product. Digestive enzymes, such as exopeptidases from herring, are active up to 25%, w/w NaCl concentrations (Granroth *et al*., 1978; Gudmundsdottir, 1995). These enzymes are thought to have significantly influence on the salt ripening processes of herring. However, in cod muscle digestive enzymes probably are absent since the fish is gutted prior to salting. The complexity of the cod muscle matrix makes it difficult to predict how variations in NaCl concentration and pH values, would affect the enzyme activities during salt ripening. However, one may suggest from earlier reports that the activities of salt-tolerant enzymes would be highest at pH <5 in salted cod muscle (Reddi *et al,* 1972; McLay, 1980; Gildberg and Raa, 1978; Gildberg, 1988; Toldra and Flores, 1998). In order to manipulate enzymatic processes during salt ripening towards specified qualities of salted cod, further studies are needed.

Anti-oxidants in the brine

Inhibitors of lipid oxidation may directly or indirectly inhibit the initiation or propagation stages of the lipid oxidation reactions (Background subchapter 2.5). Some of these inhibitors can function to prevent both the initiation and propagation reactions. Oxidation of ferrous to ferric irons tends to be favoured in extracellular fluids while chelation is more likely intracellularly (Halliwell and Gutridge, 1986).

The model system simulating normal production of salted cod was used to investigate the interaction effects of ascorbate and copper ions in the brine and the effects of ascorbate alone on lipid oxidation in salt ripened products (Paper V). The results clearly showed that asorbate used during production of heavily salted cod might have pro-oxidative or anti-oxidative effects depending on the ascorbate and metal concentrations. Without added copper in the brine, ≤500 ppm concentrations of ascorbate had a prooxidative effect on the cured product. This pro-oxidative effect is often explained by the ability of ascorbate to reduce

endogenous transition metals (Haase and Dunkley, 1969a,b; Kanner *et al*., 1977; Frankel, 1998). One may suggest that at higher concentrations (≥1000 ppm), ascorbate are able to reduce hydroperoxide radicals formed by the reduced metals. In addition, it is known that ascorbate is able to chelate transition metals thereby inactivating them (Khan and Martell, 1967; Kanner *et al.,* 1977). The concentrations we found to be pro-oxidative and those found anti-oxidative, are in accordance with the findings of Ramanathan and Das (1993) studying the effect of ascorbate on steam-cooked ground fish and of Deng *et al.* (1978) studying the effect of ascorbate on cold stored mullet.

With 5 ppm copper added in the brine, increasing concentration of ascorbate resulted in different lipid oxidation levels. When no ascorbate was included in the brine, the salt ripened product had, as expected, a high oxidation level (Paper IV). Under such pro-oxidative conditions the presence of relatively low concentrations of ascorbate (≤50 ppm) inhibited the formation of malondialdehyd-bis(diethylacetal)-like substances in the cured product. The explanation for this may be that ascorbate functions mainly as a radical scavenger. At higher concentrations (100-200 ppm) the antioxidative properties are lost and pro-oxidative effects may even be present. One plausible explanation for this may be that the ascorbate concentration must increase to a critical level before it reduces the transition metals. The antioxidative properties of ascorbate at higher levels may be explained as described earlier. These results appear similar to those obtained by Haase and Dunkley (1969a,b) and Kanner *et al*. (1977) who studied oxidation in a potassiumlinoleate and in a â-carotene-linoleate model system, respectively, and found pro-oxidative effects with low copper and high ascorbic acid concentrations. As copper concentrations were raised in these investigations antioxidative effects were found. On the basis of the present results, it may be concluded that application of ascorbate as an antioxidant in salt curing of cod requires the use of high concentrations (≥1000 ppm) in the brine.

The effects of added EDTA, citrate and ascorbate in the brine on copper-induced lipid oxidation were studied by the model system (Paper V). At low concentrations used (0.5 mM), EDTA was the only effective antioxidant while citrate behaved as a pro-oxidant. EDTA is a chelator that forms thermodynamically stable complexes with transition metal ions inhibiting electron transfer and thus oxidation (Pribil 1972; Pokorný 1987). The spatial structure of the anion of EDTA, which has six donor atoms, allows it to satisfy the coordination number of six frequently encountered among metal ions. The effectiveness of EDTA in inhibiting copper-induced lipid oxidation has been shown in several studies. Examples are inhibition of the development of off-flavour in margarines during storage (Melniek, 1961), inhibition of copper induced rancidity in cod muscle blends at 12% NaCl concentrations (Castell *et al*., 1965) and inhibition of metal-induced lipid oxidation of fish oil enriched mayonnaise products (Jacobsen and Timm, 2001). The reason why citrate appeared to facilitate the uptake of copper is not known. One can speculate that the binding of copper to citrate neutralizes the

positive charge of the metal and thereby makes it easier to penetrate into the muscle (Goldstein and Czapski, 1986; Kanner, 1994). At the end of the curing the increased copper concentration observed in some of the samples could be explained by the loss of moisture during kench curing.

The most important biochemical mechanisms affecting cod muscle *post-mortem* during salting processes are illustrated in Figure 6. The quality of salt cured cod products can most probably be controlled by state of *rigor,* storage (time-temperature) of the fish prior to salting, salt concentration and composition, salting time-temperature, salting method, water activity (aW), pH, availability of oxygen and UV-light, presence of pro- and anti-oxidants of the muscle tissue, storage (time-temperature) of the salt cured product (Papers I-V).

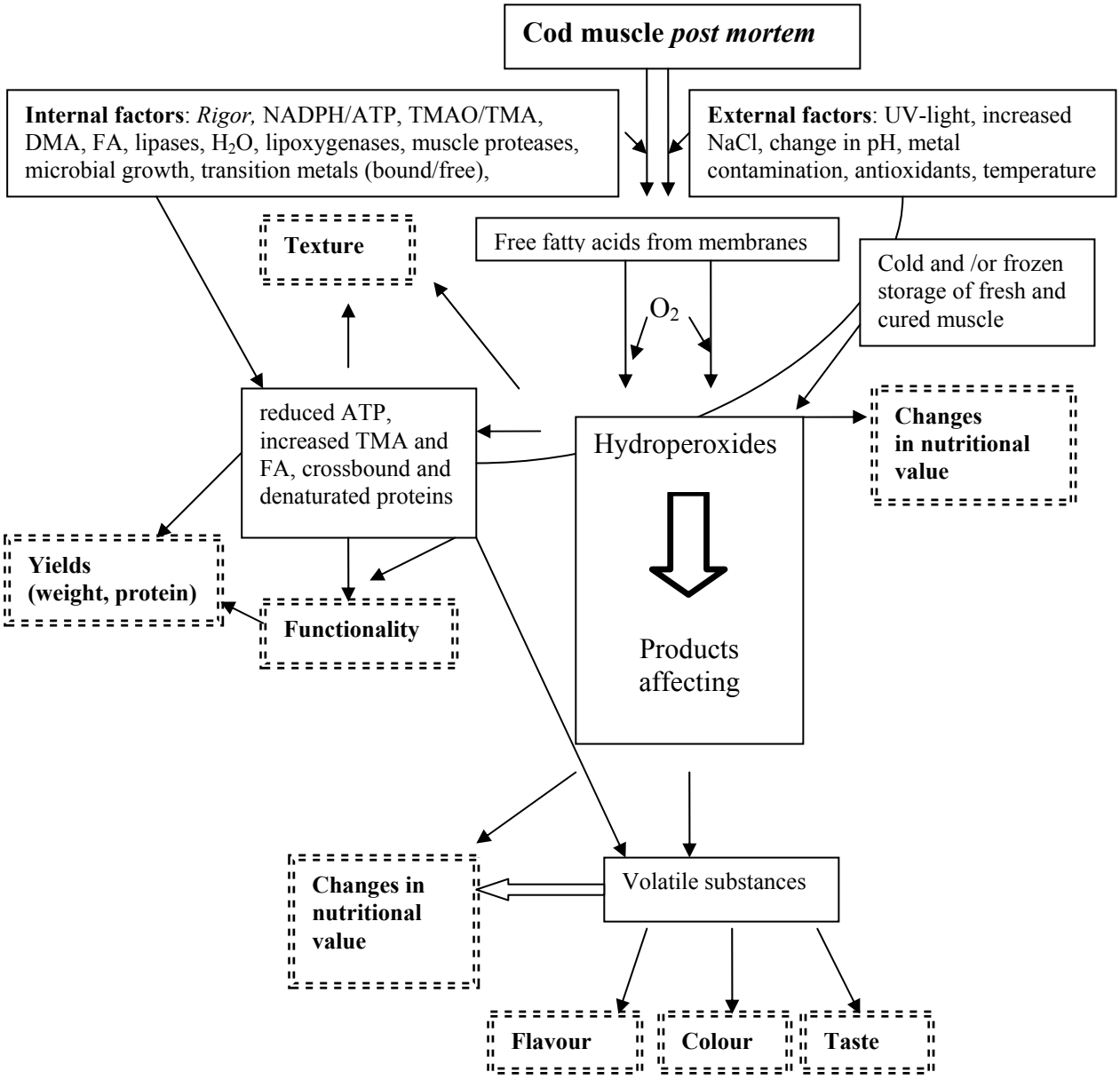

Figure 6. Biochemical processes in cod *post mortem* muscle which are affecting the quality of the salt cured product. Yields, changes in nutritional value, flavour, colour, taste, texture and functionality are used as quality indicators (Modified from Shenouda, 1980 and Martinsen, 1995).
NADPH = Nicotinamide adenine dinucleotide phosphate hydrate, ATP = Adenosine triphosphate,TMAO = Trimethylamine oxide,TMA=Trimethylamine, DMA=Dimethylamine, FA = Formaldehyde,UV-light = Ultra violet light.

5 CONCLUSIONS

- Muscle contractions simultaneously with a salt influx in the cod muscle probably cause larger reduction in weight, higher water loss and lower uptake of NaCl in muscle salted *pre-rigor* compared with muscle salted *post-rigor*.

- If the primary focus is on the weight yield of the cured product, the fish should be lightly or heavily salted in *post-rigor* state after chilling or freezing *pre-* or *post-rigor*.

- The fish should be heavily salted in *pre-rigor* state to reduce the loss of proteins from the raw material and to increase the instrumental lightness values (L*) of the salt ripened cod products.

- In order to obtain a salt cured product with a white coloured and firm texture and a minimal protein loss during the salting process, the calcium and magnesium contents of the salt should be high (800 and 400 mg Kg^{-1} respectively) and the pH of the salt/brine should be low (<6).

- Copper, particularly the reduced form, has a strong pro-oxidative effect on cod muscle lipids and reduces the storage stability of the product. Copper concentration should be kept as low as possible during salt curing of cod.

- Application of ascorbate as an antioxidant in salt curing of cod requires the use of high concentrations (≥1000 ppm) in the brine.

- At low (0.5 mM) concentrations used, EDTA was an effective antioxidant while citrate behaved as a pro-oxidant.

Further studies:

- Brine concentration affecting muscle contractions in cod muscle during *pre-rigor* salting.

- To investigate denaturation of cod muscle proteins as result of change in the isoelectric point (Ip) of the muscle proteins at different salt concentrations.

- To investigate the effects of pH at different concentrations and combinations of Cu, Fe, Ca, Mg, citric acid and EDTA in the brine on lipid oxidation of cod muscle during salting.

- To investigate the activites of cod muscle enzymes as influenced by increasing salt concentration, change in pH and after frozen storage.

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