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The role and fate of added phosphates in salted cod products

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Report summary

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Titill / Title	**The role and fate of added phosphates in salted cod products / Hlutverk og afdrif viðbætts fosfats í saltfiski**		
Höfundar / Authors	Kristín Anna Þórarinsdóttir, Sigurjón Arason, Guðjón Þorkelsson		
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Ágrip á íslensku:	Markmið verkefnisins var að meta afdrif viðbætts fosfats í saltfiski. Ljóst er að magn þess lækkar við verkun og útvötnun. Sama gildir um fosföt sem eru náttúrulega til staðar í fiskvöðva. Þess vegna er heildarmagn fosfats í útvötnuðum afurðum yfirleitt lægra en í ferskum fiski. Hins vegar hefur verið sýnt fram á að viðbætt fosföt (dí- og trífosföt) finnast bæði í verkuðum og útvötnum fiski. Það er þó háð magni viðbætts fosfats í afurðinni og hvaða söltunarferlum er beitt, þ.e. hvort fosfati var bætt í fiskinn með sprautun eða pæklun. Lítið eða ekkert greinist í útvötnuðum afurðum ef pæklun er beitt. Munur á milli ferla getur stafað af söltunaraðferð (sprautun/pæklun), gerð og upphaflegu magni viðbætts fosfats og verkunartíma. Frekari rannsókna er þörf til að meta áhrif af mismunandi söltunarferlum á afdrif fosfats í söltuðum þorskvöðva.		
Lykilorð á íslensku:	*Saltfiskur, viðbætt fosfat, niðurbrot, verkunarferlar, sprautun, pæklun*		
Summary in English:	The aim of this study was to investigate the fate of added phosphates in salted cod products. The content of both added phosphates and naturally occurring phosphates, decreases during salting and rehydration. The final content in rehydrated fish (approx. 1-2.5% NaCl) is usually below values in the raw fish. However, di- and triphosphates are present both in salted and rehydrated products. The amount depends on the quantity of added phosphates in the product and on the salting procedures applied. It seems that lower contents are present in brined products than in injected products. Differences may depend on the method used for adding phosphates (injection/brining), phosphate type and, initial content of added phosphates in the muscle after pre-salting and finally on the curing time. Further studies are needed to get accurate information on the effects of different salting procedures on the fate of phosphates in salted cod products.		
English keywords:	*Salted cod, added phosphates, degradation, salting procedures, injection, brining*		

ACKNOWLEDGEMENTS

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PREFACE

The aim of the project is to provide information based on analytical results of the role and presence of added phosphates that will hopefully clear uncertainties in the legal interpretation of the use of polyphosphates in the processing of salted cod. Import authorities in some EU countries state that use of polyphosphates is illegal and the imports of salted cod processed with polyphosphates should be banned. Some producers have at the same time been using polyphosphates in the belief that it is a processing aid that has no influence and is not present in the final product that is sold to consumers in South Europe.

Furthermore, the aim of the project is to bring authorities, producers/or their organizations and research institutes in Iceland, Denmark, Norway and Faroe Islands, that are all dealing with polyphosphates in salted cod, together for a seminar in order to exchange information and discuss the issues of using polyphosphates in salted cod with the aim of reaching a ”Nordic conclusion” that could be submitted to authorities in the EU. The scientific information and collaboration between producers of salted cod in the Nordic countries is a key to maintain the market in South Europe for salted cod produced by adapting traditional methods to the social changes of the last decades and the customer demands for milder taste and improved colour through higher water retentions in the first steps of processing.

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1 PROCESSING OF SALTED COD

Salting and curing of cod is a long process that can be divided into several steps (Figure 1). Salting of the fish starts after filleting or splitting (butterfly filleting) when salt and possibly other ingredients are added to the products. The processes used for bacalao production have changed rapidly over the past decades. Originally, the fish was stacked with alternating layers of coarse salt, often termed kench or pile salting. It was restacked several times to obtain an even pressure and curing of the fish. Finally, it was sun dried. In the late 20th century producers in Iceland started to pre-salt the fish before the pile salting step, using pickling (from ~1980) and brining (from ~1990). The main difference between pickling and pile salting is that pickling is carried out in closed tubs, in some cases with an addition of water/brine. On the other hand, the brine formed during pile salting is allowed to drain away. Exporters started to control the ambient temperature in storage, which made it possible to shorten the salting process and produce lightly cured products. The fish was only stacked for 10 to 12 days after pre-salting and packed as lightly/wet cured. This curing stage of the products is known in Iceland as "tandurfiskur" (Þórarinsdóttir, 2010). Today, different combinations of injection, brining, pickling and dry salting (also termed pile salting/stacking) are used, varying with producers and production countries. In brine injection and brining, a prepared brine with a controlled salt concentration is used. These steps are followed by dry salting.

In the late 20th century, brine salting became the main pre-salting method in Iceland. The use of brining as a pre-salting step before pile salting is believed to improve quality and weight yields of salted products (Þórarinsdóttir, 2010). Brining is carried out by immersing fillets into brine prepared from coarse salt and tap water. The diffusion of salt into the muscle depends on several factors, such as concentration and composition of the brine, the shape and thickness of the product, the ratio of brine to product and duration of brining. The temperature is kept low (2-4°C) to minimise bacterial growth. Several studies have been carried out in which the muscle is brined in a high brine-to-fish ratio to minimise dilution effects from liquid diffusing from the muscle to the brine (Andres and others 2002; Andres and others 2005; Barat and others 2002; Barat and others 2003). In praxis, common initial ratios are 1:1 to 2:1, meaning that significant changes are observed in the salt concentration of the brine during brining.

Now, in the early 21st century, injection is increasingly used in Iceland as the initial step in the salting procedure (Þórarinsdóttir, 2010). Injection results in a relatively homogenous salt concentration in the muscle in a short time, in comparison to other salting methods. Additionally, other ingredients such as proteins, can be added to the products, which would not be possible by brining alone. However, injection can only be used to add small quantities of salt to the fish because of limited amounts of brine pumped into the muscle. Injection volumes,

distribution of brine and retention of the brine in the muscle depend on the characteristics of the raw material, composition of the brine, the equipment and settings used: type of needles, needle density, needle-strokes per minute, release of the brine (in/out), dwelling time and pressure applied. The following treatments of the injected product and storage conditions also influence the retention of the injected brine. Disadvantages of injection are risk of microbial contamination and possible damage of the muscle structure due to the pressure applied and needle holes in the muscle (Bakowski and others 1970; Birkeland and others 2003; Birkeland and others 2007; Boles and Swan, 1997; Brunk, 1985).

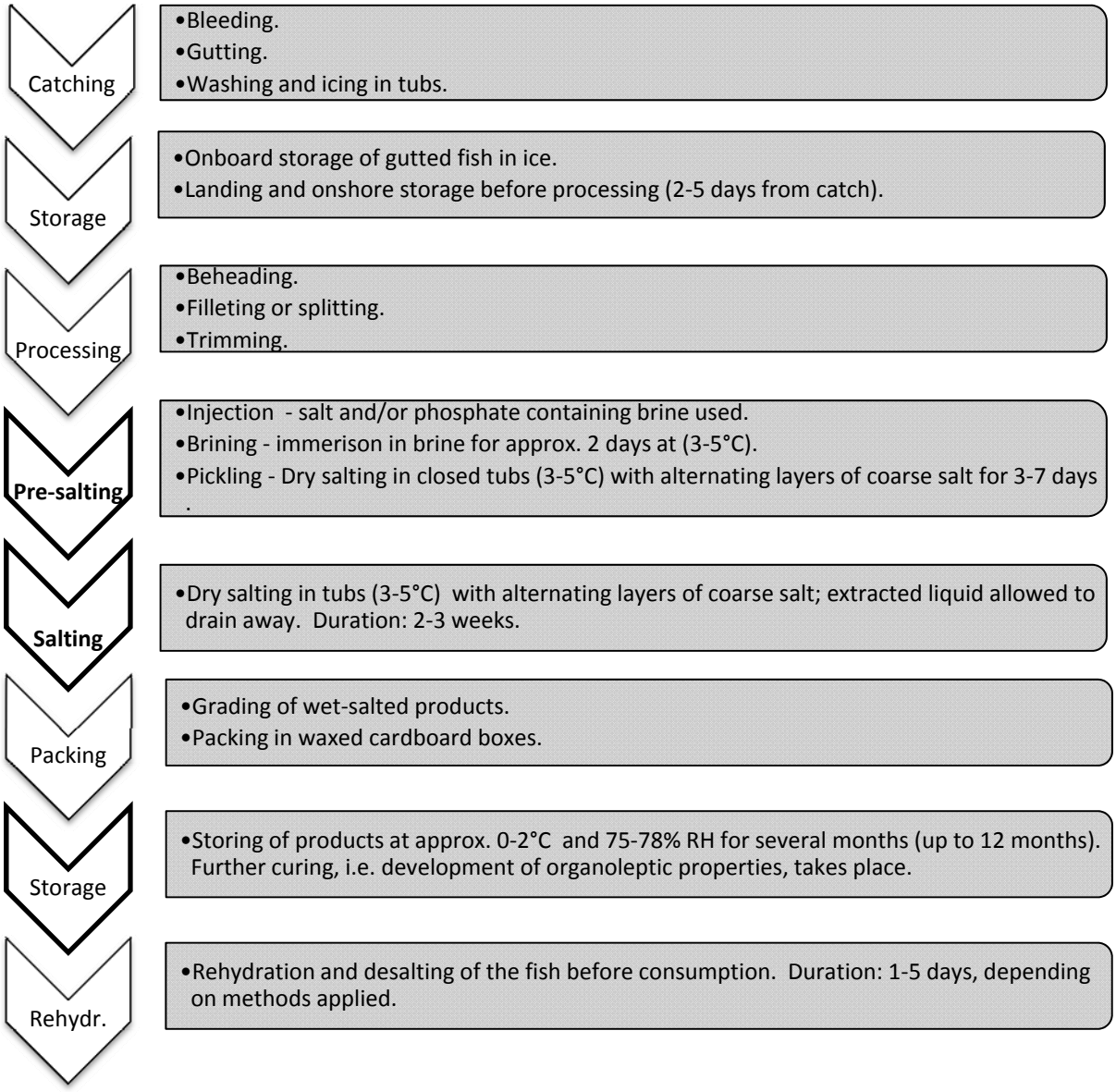

Figure 1. The process for the salted cod in the study, from catch to a product ready for cooking. The steps of interest in the study were the pre-salting and salting steps. Different pre-salting methods were used: injection and brining, brining only and pickling. (RH = relative humidity) (Þórarinsdóttir, 2010).

The changes in salting procedures and curing conditions have altered the characteristics of the products, increased weight yields and improved commercial quality (Lindkvist and others 2008). The shorter curing time and lower temperature during curing have resulted in milder curing flavours and a whiter appearance (Barat and others 2003; Lindkvist and others 2008). Secondly, the raw material is of better quality than before due to improvements in catching, handling and storage techniques. Weight yields and quality are highly important with regard to process return, market share and prices. Salted products are rated according to size and commercial quality (SPIG/PORT) at packaging. The appearance and colour of the fish are the key criteria. PORT refers to export of the products to Portugal and SPIG to Spain, Italy and Greece. The SPIG fish is of higher quality, it is supposed to be whiter and thicker than the PORT fish (Þórarinsdóttir, 2010).

2 PHOSPHATES IN SEAFOOD

Protein-rich foods, such as seafood and meat products contain phosphorous compounds such as nucleotides, phospholipids, together with naturally occurring orthophosphates. The large range of natural orthophosphate contents (0.11–4.8% (0.026–1.1% in terms of phosphorous content)), makes it hard to detect added phosphates by quantitative analysis alone ((Lawrie, 1998; Lee and others 1998) as cited by (Ünal and others 2004)). Phosphate content of fresh cod (*Gadus morhua* and *Gadus macrocephalus*) is approximately 4.4 g P_2O_5/kg of muscle (Schröder, 2010; Thorarinsdottir and others 2001).

The addition of phosphates is allowed in frozen products but not in salted or fresh products. The maximum quantity of added phosphates allowed in frozen products is 5 g P_2O_5/kg of the product (phosphorus pentaoxide P_2O_5 ≈ 2.29×P; orthophosphate PO_4^{3-} ≈ 3.06 P). Polyphosphates have also been used by some produces in the production of salted cod in the Nordic countries (Lindkvist and others 2008) in the belief that they are processing aid. The use depends on the export country, product but also on producer/customer. However, phosphates are not used in dry salted cod exported to Portugal.

2.1 The use of added phosphates in salted cod

The main reasons for adding phosphate to salted cod have been improved weight yields and quality of the products. However, effects of phosphates on weight yields are minor in comparison to application of injection alone, using brine containing salt only. The injection improves weight yields through both salting and rehydration, whereas addition of phosphates by brining, increases weight yields during salting but not after rehydration (Thorarinsdottir and others 2001).

Recent studies on the effects of phosphates on colour and quality of the salted products have shown both positive effects and no effects on these parameters (Þórarinsdóttir, 2010). Differences between studies may result from variation in the way of adding phosphates to the fish (injection/brining), phosphate type used, in the concentration of phosphate and salt in brine and in condition of the fresh fish. The positive effects of phosphates on colour and commercial quality of salted fish are due to reduced oxidation, which is mainly related to sequestering effects on metals present in the salt used. Oxidation of lipids and proteins in the muscle increase yellowness and results in darker colour of the products. These changes will result in lower commercial quality of the products since colour/appearance is the key criteria where whiter colour is preferred.

The function of the phosphates is based on maintaining the natural colour of the fish through salting and curing. The phosphates do not have a bleaching effect, i.e. do not improve the quality of raw material or products where oxidation or spoilage has already occurred. Furthermore, phosphates will not increase whiteness of the salted product during rehydration. On the other hand, sulphites which have been approved for use in salted cod products are in some case added to the rehydration water. Sulphites can serve as bleaching agent. Increases in whiteness of the products because of the bleaching could give the expression that the quality of the products was better than it was before bleaching. Another function of the sulphites can be longer shelf life of the products due to retarding effects on microbial growth.

Phosphates are added to salted cod products by injection and/or brining. The advantage of injection is that the added molecules are uniformly distributed in the muscle in a short time. In brining, the phosphates must diffuse from the brine into the muscle. The diffusion depends on several factors like concentration and composition of the brine, the thickness of the product, temperature and ratio of brine to product. The risk of injection is microbial contamination where the microbes are distributed quickly through the muscle by injection not only at the surface as in dipping. Another disadvantage is that the injection pressure and needles can lead the structural damages and loss of water binding capacity of the muscle. Especially, in fish muscle which is more sensitive than meat from land animals.

2.2 Diffusion of phosphates

Diffusion is an effective mechanism in transfer of phosphates from brine to muscle and within the muscle during salting. However, diffusion mechanisms for added and naturally occurring phosphates in the muscle have only been investigated to a limited extent. During immersion of muscle in phosphate solutions, the total phosphate content may decrease before it starts to increase. The reason is that naturally occurring orthophosphates diffuse out of the muscle (beef), i.e. in a counter-current direction to the added phosphate (Figure 2). Both diffusions result from concentration gradients between muscle and solution. Initially, the diffusion rate of orthophosphate from the muscle is faster or until a water-protein-phosphate (added) complex barrier is formed in the surface layer of the product (Ünal and others 2006, Ünal and others 2004). These effects depend on the composition and ionic strength in the immersion solution. In salting of pork ham, diffusion rates of salt decrease during hydration of proteins but increase again as the muscle fibres shrink >1-1.4M (Ockerman, 2003). The diffusion of phosphates is likely to be affected in similar way. Ockerman (2003) explained different diffusion rates by alterations of extracellular spaces. During swelling of the myofibrils, the diffusion rates in the

muscle decreased as the extracellular spaces/channels became narrower. The opposite was believed to occur when salting-out resulted in shrinkage of the muscle fibres.

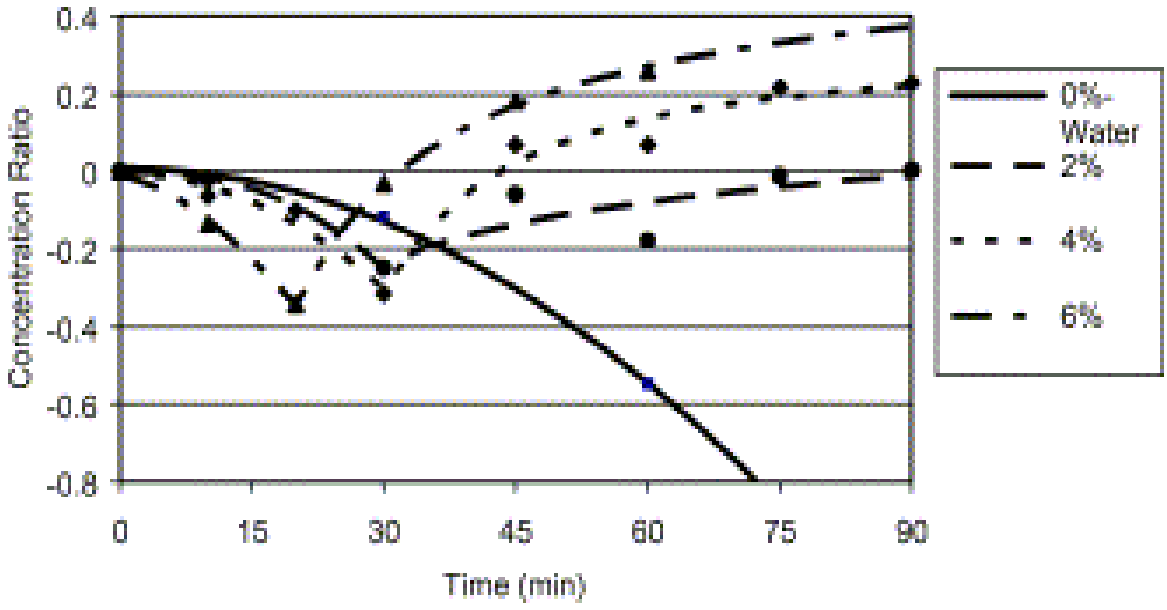

Figure 2. The change of phosphate concentration ratio $\left(\ln\left[\frac{C(t)-C_\infty}{C_i-C_\infty}\right]\right)$ *in meat samples dipped in water and 2, 4, and 6% (w/v) sodium triphosphate (STP) solutions (Ünal and others 2004).*

In the beginning, diffusion rates of added phosphates are mainly influenced by concentration gradients between the muscle and the immersion solution. The gradients increase with higher phosphate concentration in solution (Figure 3). The diffusion rates are soon reduced by the formation of a barrier in the surface layer of the muscle. Diffusion rates are reduced and differences between groups decrease with time. Thicker barrier is assumed to be formed in solutions of higher concentration (Ünal and others 2004). However, concentration gradients may override the effects of the barrier if they are strong enough. The penetration of phosphate (TPP) into shrimp muscle has shown that high concentrations (5-10%) could drive more equal distribution of the added phosphate in the muscle in comparison to 0,5% and 1% TPP (Tenhet and others 1981a).

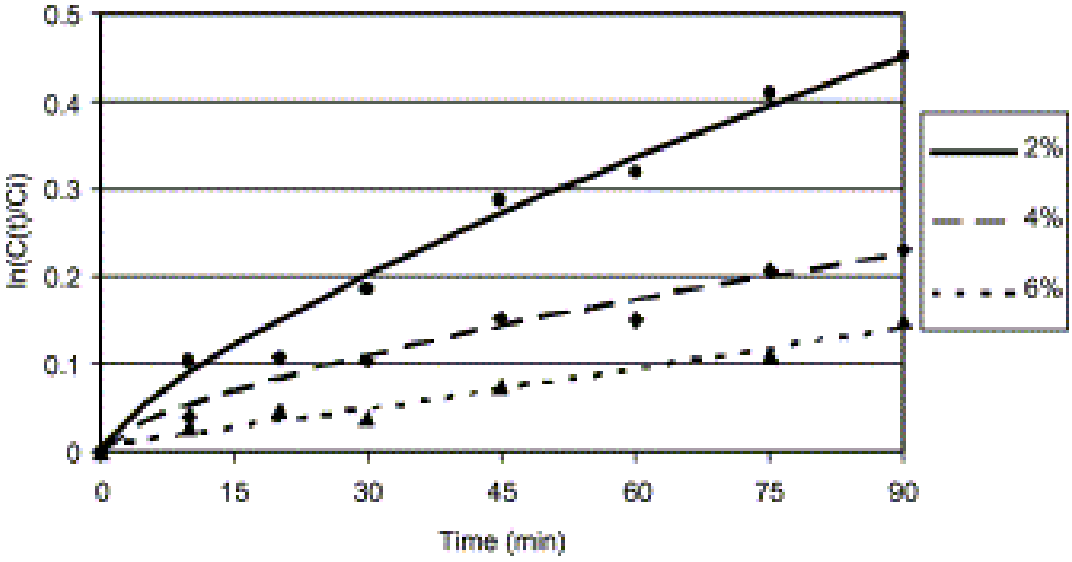

Figure 3. The change of phosphate concentration $\left(\ln\left[\frac{C_{(t)}}{C_i}\right]\right)$ *in 2, 4, and 6% (w/v) sodium triphosphate (STP) solutions versus dipping time (Ünal and others 2004).*

The effects of TPP, PP and HMP on marinade penetration (with and without salt) in chicken filets have also been studied. Marinade gradients formed depended on the phosphate type and concentration and presence/absence of salt (Figure 4).

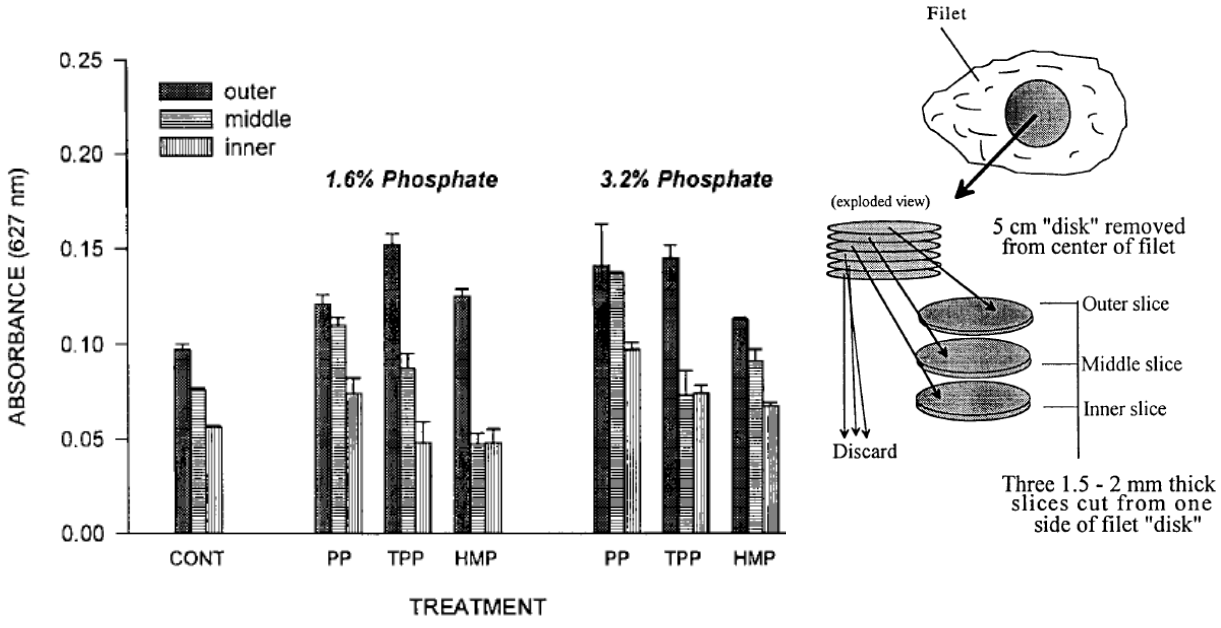

Figure 4. Penetration and relative concentration gradients of various salted (8% NaCl) phosphate solutions in three layers of the chicken filets after 30 min of tumble marination. Marinade pickup was traced by measuring the absorbance (627 nm) of a dye in the different filet layers (Xiong and Kupski, 1999).

The greatest gradient was formed with TPP, which enter the filets rapidly, but the diffusion became slower in the deeper layers. The results indicated that the diffusion rate depended on the size of the phosphate molecules, i.e. smaller molecules had higher diffusion rates. The phosphates had significant effect in salt free solutions. However, the effects diminished at high phosphate concentration (3.2%) or when salt (8%) was present. The overall conclusion was that low concentration (1.6%) of phosphate facilitated water penetration deep into the filets. High concentration (3.2%) and presence of salt improved water penetration in the surface layers of the filets (Xiong and Kupski, 1999). Differences were believed to result from salting-out effects at the higher concentration.

2.3 Calculation of phosphate uptake

The uptake of phosphate depends on the initial phosphate concentration in brine, the method used to add phosphates to the products, the concentration of brine absorbed by the products and the weight gain/uptake of brine. The uptake can be roughly calculated using the following equation:

$$\%\ added\ phosphate\ in\ product = \frac{\%\ in\ brine}{100 + \%\ weight\ gain} * \%\ weight\ gain$$

The concentration of phosphates in brine can in a similar way be calculated from the optimum phosphate content in the products:

$$\%\ in\ brine\ = \frac{100 + \%\ weight\ gain}{\%\ weight\ gain} * added\ phosphate\ in\ product$$

These formulas were presented in guidelines from the Chemische Fabrik Budenheim KG, which is a producer of phosphates for food processing. The equation above for phosphate uptake, gives higher value than the exact increase in P_2O_5 mg/g, which depends on the type(s) of phosphate applied. Usually, a combination of different phosphates is used. For each type, a conversion factor can be found, which is based on the chemical structure of each compound (Table 1). The P_2O_5 (phosphate) can be converted to P (phosphorus), by multiplying P_2O_5 by 0.4364. To change P to P_2O_5, P is multiplied by 2.2914.

Table 1. A list of different phosphate types and factors for conversion to P_2O_5 (Icelandic Food and Veterinary Authority)

Code	Name	Molecular formula	Molar mass (g/mole)	1 g material = g P_2O_5	1 g P_2O_5 = g material
E 338	Phosphoric acid	H_3PO_4	98.00	0.724	1.38
	Monophosphates				
E 339i	Monosodium phosphate	NaH_2PO_4	119.98	0.592	1.69
		NaH_2PO_4, H_2O	138.00	0.514	1.94
		NaH_2PO_4, $2H_2O$	156.01	0.455	2.20
E 339ii	Disodium phosphate	Na_2HPO_4	141.96	0.500	2.00
		Na_2HPO_4, $2H_2O$	177.99	0.399	2.50
		Na_2HPO_4, $7H_2O$	268.06	0.265	3.78
		Na_2HPO_4, $12H_2O$	358.14	0.198	5.05
E 339iii	Trisodium phosphate	Na_3PO_4	163.94	0.433	2.31
		Na_3PO_4, H_2O	181.96	0.390	2.56
		Na_3PO_4, $12H_2O$	380.12	0.187	5.36
E 340i	Monopotassium phosphate	KH_2PO_4	136.09	0.522	1.92
E 340ii	Dipotassium phosphate	K_2HPO_4	174.18	0.407	2.45
E 340iii	Tripotassium phosphate	K_3PO_4	212.28	0.334*)	2.99*)
E 341i	Monocalcium phosphate	$Ca(H_2PO_4)_2$	234.05	0.606	1.65
E 341ii	Dicalcium phosphate	$CaHPO_4$, $2H_2O$	172.09	0.412	2.43
E 341iii	Tricalcium phosphate	$10CaO$, $3P_2O_5$, H_2O	1004.67	0.424	2.36
E 343i	Monomagnesium phosphate	$Mg(H_2PO_4)_2$, $4H_2O$	290.34	0.489	2.05
E 343ii	Dimagnesium phosphate	$MgHPO_4$, $_nH_2O$	120.28*)	0.59*)	1.69*)
	Pyrophosphates (diphosphates)				
E 450i	Disodium diphosphate	$Na_2H_2P_2O_7$	221.94	0.640	1.56
E 450ii	Trisodium diphosphate	$Na_3HP_2O_7$	243.92	0.582	1.72
		$Na_3HP_2O_7$, H_2O	261.94	0.542	1.85
E 450iii	Tetrasodium Diphosphate	$Na_4P_2O_7$	265.90	0.534	1.87
		$Na_4P_2O_7$, $10H_2O$	446.05	0.318	3.14
E 450iv	Dipotassium diphosphate				
E 450v	Tetrapotassium diphosphate	$K_4P_2O_7$	330.34	0.430	2.33
		$K_4P_2O_7$, $3H_2O$	384.39	0.369	2.71
E 450vi	Dicalcium diphosphate	$Ca_2P_2O_7$	254.10	0.559	1.79
E 450vii	Monocalcium diphosphate	$CaH_2P_2O_7$	216.04	0.675	1.52
	Triphosphates				
E 451i	Pentasodium triphosphate	$Na_5P_3O_{10}$	367.86	0.579	1.73
		$Na_5P_3O_{10}$, $6H_2O$	475.95	0.447	2.24
E 451ii	Pentapotassium triphosphate	$K_5P_3O_{10}$	448.41	0.475*)	2.11
	Polyphosphates				
E 452i	Sodium polyphosphate	$(NaPO_3)_n$ (n>3)	101.97xn	0.696	1.44
E 452ii	Potassium polyphosphate	$(KPO_3)x$	118.08xX	0.601	1.66
E 452iii	Sodium calcium polyphosphate	$(NaPO_3)_nCaO$ (n is generally 5)			
E 452vi	Calsium polyphosphate	$(CaP_2O_6)_n$ (n x 2)	198xn	0.717	1.40

*) calculated on the anhydrous basis

2.4 Hydrolysis of the added phosphates

After addition to seafood and meat products, phosphates are degraded both chemically and enzymatically through action of muscle phosphatase, especially pyrophosphate and triphosphate. The degradation rate is influenced by different factors, phosphate type, muscle species, enzymes, other ingredients, processing methods and storage conditions (Belton and others 1987; Hamm and Neraal, 1977a; Hamm and Neraal, 1977c; Hamm and Neraal, 1977e; Hamm and Neraal, 1977b; Hamm and Neraal, 1977d; Neraal and Hamm, 1977f; Neraal and Hamm, 1977c; Neraal and Hamm, 1977g; Neraal and Hamm, 1977b; Neraal and Hamm, 1977e; Neraal and Hamm, 1977a; Neraal and Hamm, 1977d; Sutton, 1973). The hydrolysis is also influenced by the method of sample preparation. It is considerably slower when whole muscle is injected with phosphates in comparison to mixing of minced muscle with phosphates. Mincing increases the access of enzymes to the substrate (Belton and others 1987). Most of the studies referred to below have been carried out on minced muscle.

The hydrolysis of different phosphate compounds (pyrophosphate, triphosphate and trimetaphosphate) in pork meat, has been studied by the aid of capillary isotachophoresis (Jastrzebska and others 2008). The phosphates were added to the meat in the concentration of 0.5% of phosphorus by weight of meat. The accumulation of orthophosphate during 5 days depended on the type of phosphate added to the meat (Figure 5 and 6). The final concentration was similar in all samples, in the range of 401-432 mg P/100 g. The phosphorus content in meat without added phosphates was 150 mg P/100g used.

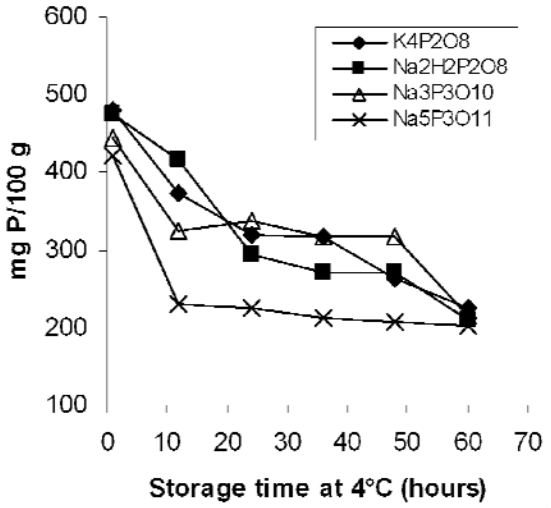

Figure 5. Degradation of different phosphates during a 5 day period (graphs plotted from data) (Jastrzebska and others 2008)

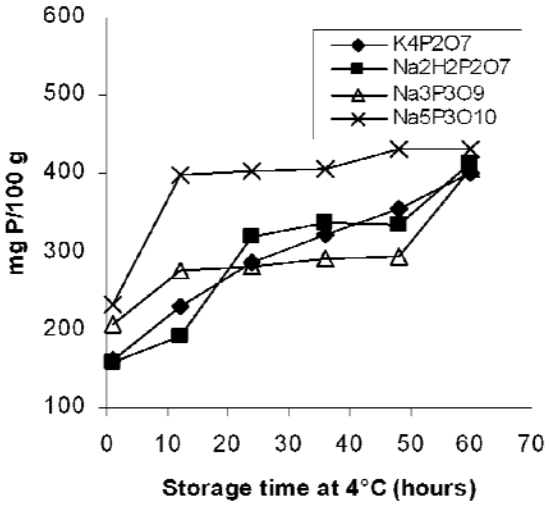

Figure 6. Accumulation of orhtophosphates origin from different phosphate species, during a 5 day period (graphs plotted from data) (Jastrzebska and others 2008)

Phosphate in meat has been studied much more than in seafood. It is known that the enzyme activity is different between these categories (Neraal and Hamm, 1977e; Sutton, 1973). Sutton (1973), who studied hydrolysis of triphosphate in cod and beef muscle, found that the degradation rate was higher in cod than in beef ($k_{2(beef)}<<k_{2(cod)}$). In meat, the degradation of triphosphates to diphosphates was faster than degradation of diphosphates to orthophosphate (beef, $k_1>>k_2$). In cod, the reaction rates for those two steps were similar ($k_1 \approx k_2$).

$$(P_3O_{10})^{5-} \xrightarrow{k_1} (P_2O_7)^{4-} \xrightarrow{k_2} (PO_{10})^{3-}$$

Storage conditions influence the enzyme activity in the muscle. Freezing slows down enzyme activity. After thawing, tripolyphosphatase (Tpase) activity seems to be similar as in unfrozen meat but the freezing and thawing process has reversible inhibition on dipolyphosphatase (Dpase) activity. The reactivation depends on the pH in the muscle (Belton and others 1987; Hamm and Neraal, 1977a). Hamm and Nerall 1977 reported that the optimum pH for Tpase activity of bovine muscle was 5.6 but in the range of 6.7-6.8 for Dpase. The maximum activity of Dpase isolated from big head carp was at pH 8 (Gao and others 2008).

Salt increases the activity of Tpase whereas the activity of Dpase is reduced (Hamm and Neraal, 1977a). It is strongest at 4-5% NaCl. The enzymes are also influenced by composition of the salt and type of divalent ions present. Tpase and Dpase are activated by high concentration of Mg^{2+}. Low concentration of Mg^{2+}, Ca^{2+}, EDTA reduces the hydrolysis of diphosphates. The activity of Tpase decreases with increasing concentration of Ca^{2+} and diphosphate (Neraal and Hamm, 1977b). Gao and others (2008) showed that $Co^{2+,}$ Mn^{2+} served as co-factors for Dpase whereas Fe^{2+}, Cu^{2+} and Ca^{2+} had no detectable effects. Information about effects of heavy salting on the enzymatic activity in cod muscle was not found (Gao and others 2008).

3 ANALYSIS OF ADDED PHOSPHATES

3.1 Quantification of phosphorus - spectrometry

Quantification of total phosphate content is usually carried out by spectroscopic analysis. The sampling preparation is based on a decomposition of polyphosphates to orthophosphate in the presence of sulphuric or trichloroacetic acid (as described by (Jastrzebska and others 2008)). The orthophosphates react with ammonium molybdate and ammonium vanadate in nitric acid (HNO_3) and a yellow precipitate is formed. The concentration of phosphovanadomolybdate is used to calculate to content of phosphate or phosphorus (Hanson, 1950; Sutton and Ogilvie, 1967).

Modifications of the standard method have led to higher sensitivity and precision, and a wider optimum detection wavelength. An example is a formation of an ionic-associate of molybdophosphate with malachite green (MG) in an acidic medium. However, the reaction is slow and the absorbance can continue to increase for many hours. Polyvinyl alcohol can be used to stop the reaction, but that is troublesome (Jastrzębska, 2009).

In the presence of reducing agents, molybdenum yellow is reduced to molybdenum blue complex which shows a stronger light absorption the standard method and the maximum absorption occurs at longer wavelengths (Jastrzębska, 2009). The advantage of the myolybdenum blue procedure is high sensitivity and smaller interferences from coexisting ions. The effects of three different reducing agents of the molybdenum blue method on detection limits and precision and accuracy of the quantification of phosphate have been tested. The reducing agents were ascorbic acid (AA), hydrazine sulphate (HS); hydroquinone and hydrazine sulphate (HHS). The use of HSS proved to be the most accurate procedure for determination of the total phosphorus content in biological materials. The results showed high recoveries, reasonable repeatability and accuracy in comparison with AA and HS (Jastrzębska, 2009).

3.2 P-value (Total P_2O_5/protein)

The quantification of phosphate alone cannot be used to verify the presence of added phosphates due to naturally occurring orthophosphates and other phosphorus compounds in the muscle. The biological variation in total phosphorus content between individuals is also large (Gibson and Murray, 1973). One way to estimate the quantity of added phosphates is to calculate the difference between the total phosphorus content and the protein bound phosphorus. A known ratio of phosphate to protein (0.0106 g P/g protein) is used to evaluate

protein bound phosphate based on the concentration of nitrogen (Kjeldahl method) in meat products. The ratio of total P_2O_5/protein is in the range of 21-25 mg/g protein in different meat species. If meat products contain other protein-rich material, for example, milk, egg, plant protein extracts, this will affect the results due to a different ratio of total phosphate/protein in these products than found in meat (Dusek and others 2003). Extraction of nitrogenous or phosphor-containing compounds may also influence the results obtained (Kruse and Bartelt, 2009b). A simulation of this factor is the ratio of P_2O_5 to salt free dry matter in lean muscle. The fat content of cod is <1% (Murrey and Burt, 1969). Therefore, the salt free dry matter consists mainly of proteins. The purpose of using salt free dry material is to prevent deviations due to increases in salt content during processing.

3.3 Thermo-differential-photometry (TDF)

The thermo-differential-photometry (TDF) is used to evaluate the presence and contents of added phosphate in fish products. It is a modified version of the standard method used for analysis of orthophosphates (Kruse and Bartelt, 2009a). The quantification of added phosphates is based on a time difference in orthophosphate content. The differences result from hydrolysis of added di-, tri-, and other polyphosphates to orthophosphates (
Figure *7*).

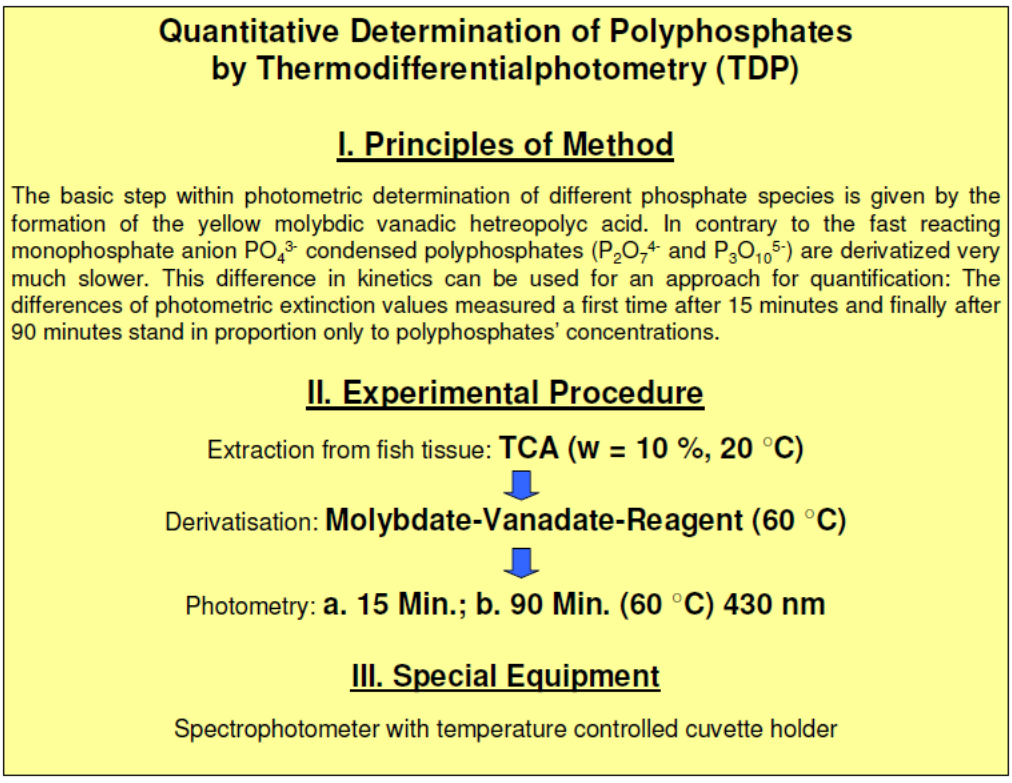

Figure 7. Thermo-differential-photometry (TDF) for detection of added phosphates (Kruse and Bartelt, 2009a)

The TDF has been tested on different fish products, for example, on heavy salted, rehydrated (pacific) cod. Results obtained have been verified by comparison to results from ion chromatography (coupled with electric conductivity detection (IC-ECD)). The method has been described as reliable and useful as a routine control method for products of animal origin (Kruse and Bartelt, 2009b).

The drawback is that this method cannot be used to differentiate between di-, tri- or other polyphosphates. Differences in the kinetics of phosphate degradation must also be considered. The kinetics are influenced by muscle species, processing methods, other ingredients and storage conditions (Belton and others 1987; Hamm and Neraal, 1977a; Hamm and Neraal, 1977c; Hamm and Neraal, 1977e; Hamm and Neraal, 1977b; Hamm and Neraal, 1977d; Neraal and Hamm, 1977f; Neraal and Hamm, 1977c; Neraal and Hamm, 1977g; Neraal and Hamm, 1977b; Neraal and Hamm, 1977e; Neraal and Hamm, 1977a; Neraal and Hamm, 1977d; Sutton, 1973).

3.4 Thin layer chromatography (TLC)

The use of Thin Layer Chromatography (TLC) can be used to detect different types of phosphate (Tenhet and others 1981a; Tenhet and others 1981b). The drawback is that hydrolysis of phosphates during sample preparation and analysis may influence the results obtained. Hydrolysis of one mole of triphosphate leads to a formation of one mole of an orthophosphate and of one mole of pyrophosphate. One mole of pyrophosphate is hydrolyzed to two moles of orthophosphate (as described by (Heitkemper and others). Hydrolysis of linear polyphosphates occurs at a higher rate in acetic solutions but these compounds are reasonable stable in neutral or alkaline solutions at room temperature (Jastrzebska, 2006).

3.5 High performance thin layer chromatography (HPTLC)

The High Performance Thin Layer Chromatography (HPTLC) is a sophisticated and automated form of the traditional TLC method. The main difference between these methods is that in HPTLC quantitative analyses are possible with the aid of advanced type of densitometer. Other important differences are particle and pore size of the sorbents (Table 2) (Meyyanathan, 2010).

Table 2. Difference of HPTLC and TLC (Meyyanathan, 2010)

	HPTLC	TLC
Layer of Sorbent	*100µm*	250µm
Efficiency	High due to smaller particle size generated	Less
Separations	3 - 5 cm	10 - 15 cm
Analysis Time	Shorter migration distance and the analysis time is greatly reduced	Slower
Solid support	Wide choice of stationary phases like silica gel for normal phase and C8, C18 for reversed phase modes	Silica gel , Alumina & Kiesulguhr
Development chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual spotting
Scanning	Use of UV/ Visible/ Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively. The scanner is an advanced type of densitometer	Not possible

3.6 Chromatographic methods

Chromatographic methods have lower detection limits than TLC that can be used to detect and quantify small fractions of added phosphate food products (Kaufmann and others 2005). Several methods have been tested that involve separation and determination of different phosphates, i.e. di-, tri- and higher polyphosphates. For example, ion chromatography, high-pressure liquid chromatography (HPLC), modified end group titration method and ion chromatography and capillary electrophoresis (Baluyot and G. Hartford, 1996; Cui and others 2000; Sekiguchi and others 2000). These methods are often too complex to apply in standard laboratory conditions for routine control of food samples (Jastrzebska, 2006). However, capillary isotachophoresis (CITP) is promising. It is one mode of capillary electrophoresis requiring minimal sample pre-treatment and short time of separation. Different phosphate species can be detected with good precision and accuracy. Furthermore, it has been described as a method that can be used for quality control and assurance laboratories (Jastrzebska and others 2008).

4 PHOSPHATE CONTENT IN SALTED COD PRODUCTS

Handling and storage methods influence the phosphate content in fresh fish. Naturally occurring phosphate is partly extracted as liquid drains from the muscle, for example, during thawing (Schröder, 2010). Further changes in phosphate content during salting and rehydration depend on the procedures applied.

It is obvious that both naturally occurring orthophosphates and added phosphates diffuse from the muscle during the process. Previous findings indicate that the orthophosphates present in the raw muscle are partly lost already during the brine salting step (Schröder, 2010; Thorarinsdottir and others 2001). During dry salting, phosphate content decreases as muscle liquid is extracted and squeezed out of the muscle due to concentrations gradients and shrinkage of muscle cells (Þórarinsdóttir, 2010).

During rehydration, the content of both naturally occurring and added phosphates is largely reduced in comparison to the phosphate content in fresh muscle. The main transfer mechanism is diffusion, driven by concentration gradients between the muscle and the water used for soaking. However, added polyphosphates have been found after rehydration of injected salted cod products (Figure 8) (Kruse and Bartelt, 2009a; Kruse and others 2009), in spite of the reduction in total phosphate content and assumed hydrolysis of di- and triphosphates to orthophosphates.

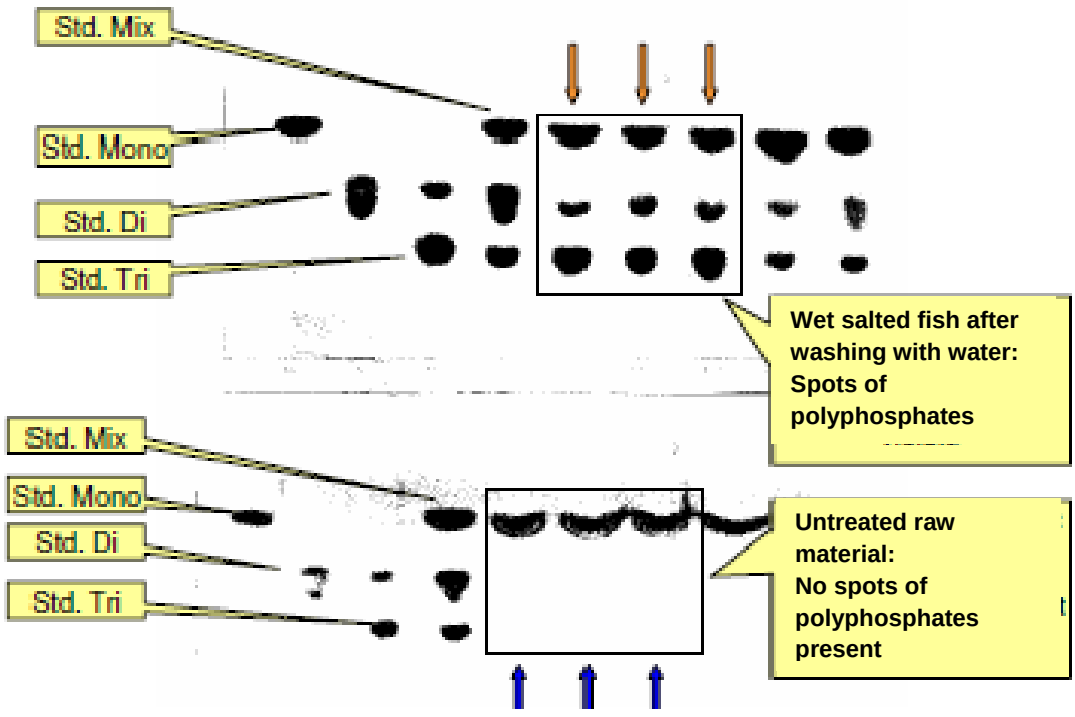

Figure 8. Detection of polyphosphate species (after rehydration) in samples of wet salted cod by TLC (Kruse and others 2009).

Added phosphates have not been found in brined fillets but only a limited number of analysis has been carried out. Differences in total content of phosphates (P) between P-treated and untreated fillets after rehydration vary with pre-salting methods. They are minor when phosphates are added through brining only, but 2-3 fold when phosphates have been added by injection. In brining, concentration gradients within the muscle and between the product and the brine are determinant for the phosphate uptake. Injection spreads the phosphate relatively homogeneously through the muscle and eliminates the effects of muscle thickness and presence of skin on phosphate uptake. In addition, injection leads to stronger swelling of the myofibrils (Þórarinsdóttir, 2010) and that may also reduce the outward diffusion of naturally occurring orthophosphates, i.e. leading to higher total contents of phosphates. During dry salting and rehydration, phosphates may be more easily extracted from the muscle in brined products since a larger portion is located in surface layer of the muscle in comparison to injected fillets.
More details from different studies are given in the following sub-chapters, where phosphates were added to raw cod fillets in the pre-salting step, by injection or brining.

4.1 Pre-salting –phosphates added by brining

During brining, phosphates are absorbed but during dry-salting, they are extracted to some extent, in a counter-current direction to diffusion of the salt ions (Table 3). After rehydration, similar average phosphate contents were obtained for phosphate added and untreated fillets. However, the phosphate content in phosphate treated fillets varied between different parts of the muscle, being lower (1.2 mg P_2O_5/g) in thinner parts (tails) compared with thicker parts (loins) of the fillets containing 1.7 mg P_2O_5/g (data not shown). Differences in phosphate content are also influenced by rehydration methods and factors like duration and ratio of water to fish.
Unpublished studies on Pacific cod and Atlantic cod show similar levels of phosphates in salted stage as in previous findings. Di- and triphosphates are present, but only orthophosphates were detected in rehydrated or cooked fillets. The phosphate content was higher in the cooked than rehydrated stage, due to cooking loss and total weight reduction.

Table 3. Chemical content of cod (*Gadus morhua*) fillets (n=3) with (PP) and without added phosphates (Control) at different stages of salt fish production (Thorarinsdottir and others 2001).

	Group	Raw material	Brined (17.5%S, 2%P)	Salted (14d) and stored (21d)	Rehydrated
mg P_2O_5/g)*	PP-test		6.0 ± 1.0	4.2 ± 0.4	1.0 ± 0.3
	Control	4.4 ± 0.4	3.3 ± 0.9	3.4 ± 0.5	1.0 ± 0.2
Water (%)	PP-test		74.3 ± 0.7	56.1 ± 0.3	83.3 ± 0.6
	Control	81.8 ± 0.4	75.9 ± 3.3	56.6 ± 0.4	84.1 ± 0.7
Salt (%)	PP-test		8.1 ± 0.6	21.0 ± 0.4	0.8 ± 0.0
	Control	0.4 ± 0.0	7.2 ± 3.9	20.3 ± 0.1	0.8 ± 0.0
Dry matter (%)**	PP-test		17.6	22.9	15.9
	Control	17.8	16.9	23.1	15.1
P_2O_5/DM (mg/g)***	PP-test		34.0	18.2	6.4
	Control	24.8	19.7	14.7	6.6

PP: Brifisol B512 (BK Giulini Chemie, Germany) a blend of sodium polyphosphates with different chain lengths, pH 9,0.
* Phosphorus was estimated colorimetrically as phosphovanadomolybdate by the spectrometric method (vanadum phosphomolybdate) which is based on the reaction of orthophosphate in an acidic solution with ammonium molybdate and ammonium vanadate in nitric acid.
** salt free dry-matter
***Ratio of phosphates to salt-free dry matter (P/DM) were calculated from the published results.

4.2 Pre-salting – phosphates added by injection, following step: brining

Injection leads to higher increases in phosphate content than brining since thickness and diffusion time are not limiting factors like in brining. When comparing the results presented in Table 4 and Figure 9 to previous findings (Table 3), it should be kept in mind that different phosphate (P) blends and concentrations were used. The results show that phosphates diffuse out of the injected muscle during brining, both in P-treated and untreated fillets. The phosphates lost from P-treated fillets, may have originated from the fresh fish as well as from phosphates added by injection.

After brining, the ratio of phosphate to salt-free dry matter was 39 mg/g in injected and brined fillets (Table 4) in comparison to 34 mg/g in fillets that were only brined in phosphate containing brine (Table 3). The main difference between these treatments was that the reduction during dry salting and rehydration was less than in injected fillets. That was probably due to more even distribution of brine through the muscle after injection in comparison to brining. In brined fillets, relatively higher ratios were expected to be located in surface layer of fillets in comparison to inner parts. Therefore, the phosphates would be more easily extracted from the muscle.

Table 4. Chemical characteristics of Pacific cod (*Gadus macrocephalus*) fillets at different stages of salting and desalting process (fish to water: 1:10; time 48h, 3 x water changes) (Schröder, 2010).

Variable analysed	Group	Frozen fish	Thawed fish	Injected (24%S, 4%P)	Brined (20%S)	Dry salted (15d)	Rehydrated "I" (48 h)
Water (%)	S	81.6	80.3	80.1	75.9	58.1	84.1
	S+P			78.7	74.6	58.9	84.7
Salt (%)	S	0.3	0.3	5.9	5.8	22.1	1.0
	S+P			6.1	11.5	21.6	1.0
Dry matter (%)*	S	18.1	19.4	14.0	18.3	19.8	14.9
	S+P			15.2	13.9	19.5	14.3
P_2O_5/DM (mg/g)**	S	24.0	18.4	19.2	9.3	7.8	4.7
	S+P			53.2	39.1	31.5	20.4

*Phosphorus was estimated colorimetrically as phosphovanadomolybdate by the spectrometric method (vanadum phosphomolybdate) which is based on the reaction of orthophosphate in an acidic solution with ammonium molybdate and ammonium vanadate in nitric acid.

* salt free dry-matter

**Ratio of phosphates to saltfree dry matter (P/DM) were calculated from the published results.

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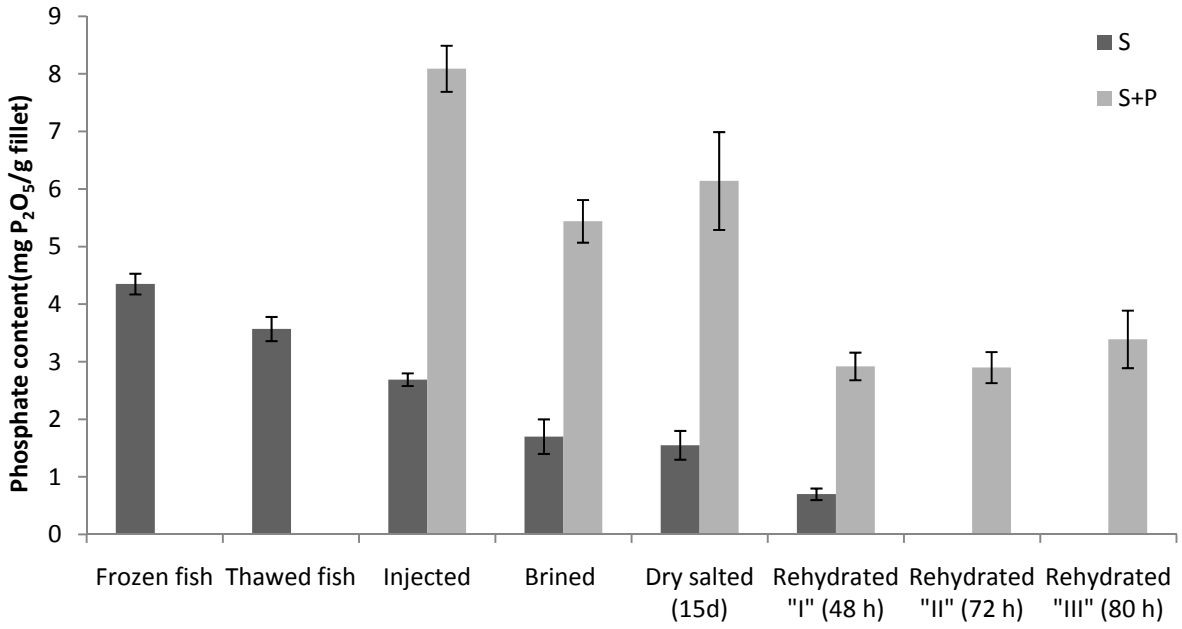

Figure 9. Phosphate content in Pacific cod (Gadus macrocephalus) fillets at different stages during salt fish production (S=salt, P=added phosphate). Different soaking methods were used for rehydration of the fillets (I: fish to water 1:1, 48h, 3 x water changes; II: fish to water 1:15, 72h, water change after 24h; III: fish to water 1:5, 80h, water change after 30h) (Schröder, 2010).

Kruse and Bartlet (2009) published results of a study on phosphate species in Pacific cod fillets. After thawing, the fillets were injected with brine consisting of homogenised fish mince (optional), salt and triphosphate. After injection, fillets were immersed in brine containing no phosphate. Dry salted fillets were rehydrated by different procedures, varying fish to water ratio and water changes (Table 5). Thermo-differential-photometry confirmed the presence of added phosphates in the rehydrated fillets, showing that they were not fully hydrolysed or extracted during the process.

Table 5. Speciation analysis* of phosphates in rehydrated Pacific cod fillets (n=5) (Kruse and Bartelt, 2009a)

Duration of rehydration (h)	**50**	**65**	**80**
Fish to water ratio	1:10	1:15	1:10
Water changes	3	2	2
Acid**-soluble phosphate (mg P_2O_5/g)	2.55 ± 0.22	2.70 ± 0.41	2.67 ± 0.63
Mono-phosphate (mg P_2O_5/g)	1.39 ± 0.29	1.44 ± 0.28	1.29 ± 0.15
Poly-phosphate (mg P_2O_5/g)	1.16 ± 0.16	1.26 ± 0.21	1.38 ± 0.18

*Thermo-differential-photometry, **Trichloroacetic acid (TCA) (Kruse and Bartelt, 2009a)

4.3 Pre-salting – phosphates added by injection, following step: dry salting

In Denmark, study on the phosphate content in Pacific and Atlantic cod fillets after salting, rehydration and cooking has been carried out (Table 6 and Table 7). The fillets were pre-salted with injection alone, which was followed by dry salting. The average weight gain by injection was in the range of 18-20%. A rough estimation of % added phosphates in the products after injection based on the equation below is 5.8%. However, the actual value is lower, as discussed in chapter 2.3 Calculation of phosphate uptake.

$$\%\ added\ phosphate\ in\ product = \frac{\%\ in\ brine}{100 + \%\ weight\ gain} * \%\ weight\ gain = \frac{3.5}{120} * 20$$

The estimated content of added orthophosphates was 2.9 mg P_2O_5/g of the injected fillets, assuming that the conversion factor was 0.5 (Table 1. A list of different phosphate types and factors for conversion to P2O5 (Icelandic Food and Veterinary Authority) That would mean that

the total content of orthophosphates was 5.2 mg P_2O_5/g, if the content of naturally occurring phosphates had remained unchanged during pre-salting. Further estimations are based on the results from chemical analysis of salted cod and the assumption that the water and salt content in the fresh muscle was 82% and 0.3% respectively:

- the raw fillets ~15 mg P_2O_5/g salt free dry material (estimated content)
- the injected fillets ~34 mg P_2O_5/g salt free dry material (estimated content)
- the salted fillets ~21 mg P_2O_5/g salt free dry material (estimated content)
- the rehydrated ~10-12 mg P_2O_5/g salt free dry material (estimated content)

It should be kept in mind that these are only rough estimations, base on the information presented in Table 6. The salt content indicates that the rehydration and/or water changes may have been extreme since salt content was <0.5% but is often in the range of 1 to 2% in rehydration. Therefore, the phosphate may have been more extensive washed out, leading to lower phosphate content in the rehydrated products than in other studies on injected and brined fillets (Table 4). Another explanation, may be related to differences in salting procedures, i.e. the injection was followed directly followed by dry salting but not brining. A strong concentrations gradient was probably formed, leading to strong extraction of water/brine from the muscle and losses of phosphates soluble in the liquid phase of the muscle.

Table 6. Chemical analysis of raw Pacific cod fillets and after salting, soaking (fish to water: 1:3; 48 hours: water changes every 12 hours) and cooking (in plastic bag soaked in water bath at 80°C for 20 min). Salting was carried out by injection (brine: 3.5% phosphate and 17.5% salt) and dry salting.

Variable measured	*Gadus macrocephalus* Raw	*Gadus macrocephalus* Salted	*Gadus macrocephalus* Soaked	*Gadus macrocephalus* Cooked
Humidity (gravity)		58		
NaCl (%) Volhard	<0.5	21.3	<0.5	2.1
HPTLC (mg/g)	[1]	[1]	[1]	[1]
P_2O_5 – Orthophosphate [2]	2.7	4.3	1.2	1.8
P_2O_5 - Diphosphate	<0,9	1.7	<0.8	<0.8
P_2O_5 - Triphosphate	<1.1	2.6	<0.8	<0.8

[1] Presence of diphosphate, triphosphate in minor amounts to the quantification limits are detected (HPTLC = High Perfomance Thin Layer Chromatography). [2] Total phosphate content

The results for phosphate content in Pacific and Atlantic cod fillets were similar during salting, rehydration and cooking. These results show that the content of di- and triphosphates were below the detection limits of the analysing method used (HPTLC), both after rehydration and cooking. The increases in phosphate content in cooked samples compared to rehydrated samples, indicated that the phosphates were only partly extracted by the liquid diffusing from the muscle during cooking. The effects of cooking must, though be influenced by the cooking method, for example, baked or cooked in water.

Table 7. Chemical analysis of Atlantic cod fillets after salting, soaking (fish to water: 1:3; 48 hours: water changes every 12 hours) and cooking (in plastic bag soaked in water bath at 80°C for 20 min). Salting was carried out by injection (brine: 3.5% phosphate and 17.5% salt) and dry salting.

Variable measured	***Gadus morhua*** Salted	***Gadus morhua*** Soaked	***Gadus morhua*** Cooked
Humidity (gravity)	59.0		
NaCl (%) Volhard	20.2	1.1	1.9
HPTLC (mg/g)	[1]	[1]	[1]
P_2O_5 – Orthophosphate [2]	4.0	1.4	1.7
P_2O_5 - Diphosphate	1.1	<0.8	<0.8
P_2O_5 - Triphosphate	2.1	<0.8	<0.8

[1] Presence of diphosphate, triphosphate in minor amounts to the quantification limits are detected (HPTLC = High Perfomance Thin Layer Chromatography). [2] Total phosphate content

4.4 Pre-salting – phosphates added by both injection and brining

In table 8, further values for phosphate content in cod fillets at different stages in the process can be found. The fish was processed by the same producer in Denmark as the supplied the samples for the analysis presented in table 6 and 7. However, the salting procedures were different, phosphates were added to the fish by both injection and brining. The range of phosphate content in salted fillets was relatively high (3.0-7.5 mg/g), i.e. 2-5 times higher than in fish produced without phosphate addition. The large range in phosphate content may be related to variation in the volume of brine injected to the fillets, i.e. increases in green weight and possible to condition of the raw material. Higher uptake of phosphates during pre-salting are presumed to have led to higher contents at later stages of the process, wet salted, rehydrated and cooked. Addition by both injection brining, probably increased the phosphate content compared to only injection. The phosphates may also have been better retained in the muscle when brining was carried out between injection and dry-salting, in comparison to direct dry salting of the fish after injection.

Table 8. Phosphate content (mg P_2O_5/g) of raw cod fillets after salting, soaking (fish to water: 1:3; 48 hours: water changes every 12 hours) and cooking (in plastic bag soaked in water bath at 80°C for 20 min). Salting was carried out by injection (brine: 3.5-4% phosphate and 17.5% salt), brining (1% phosphate and 13.5% salt) and dry salting.

Specie	Use of phosphates (P)	Raw	Wet salted	Soaked	Cooked
Fresh Atlantic cod		3,0			
Thawed Atlantic cod		2,0			
Pacific cod (thawed)		3,9			
Pacific cod (thawed)	With P		7,5	4,7	4,9
Pacific cod (thawed)	With P		4,8	1,2	
	Without P		1,6	0,7	
Pacific cod (thawed)	With P		5,8	3,8	
Atlantic cod	With P		3,0	1,3	

The phosphates were partly lost during rehydration, but the range remained large and the phosphate content was different from the untreated fish. After cooking the phosphate content was similar as in the uncooked product.

5 CONCLUDING REMARKS

Quantitative analysis show that the content of both added phosphates and naturally occurring phosphates, decreases during salting and rehydration. The phosphates soluble in the muscle liquid diffuse out of the muscle as the muscle is dehydrated at high salt contents. During rehydration, they are washed/extracted out of the muscle like the salt. The final content in rehydrated fish (approx. 1-2.5% NaCl) is usually below values in the raw fish. Losses during cooking were not significant, but further studies are needed to confirm these results and to evaluate effects of different cooking methods.

Qualitative analyses have shown that both di- and triphosphate are present in products in salted and rehydrated stage. However, the results depend on salting procedures applied and the content of added phosphate after injection/brining. It seems that the lower contents of added phosphates are present in brined products in comparison to injected products, in both salted and rehydrated stage of the muscle. Differences may depend on the application of injection, phosphate species, initial content of added phosphates in the muscle after pre-salting and finally on the curing time. Further studies are needed to evaluate the effects of different salting procedures on the fate of phosphates in salted products. The parameters of interest must be studied at experimental conditions that minimise differences in raw material, phosphates species and concentrations used and processing conditions. Finally, detection limits depend on the analysing technique applied.

It may be argued that the phosphates are sometimes a processing aid that cannot be detected in the final consumer products and therefore legally used and in some cases food additives and therefore not permitted according to regulations and directives.

Addition of phosphates should perhaps only be carried out by brining. The presence of phosphate in brine used for injection does not improve the weight yields compared to use of salt only. Therefore, the main benefit is related in colour and appearance of the fillets, i.e. the retarding effects of phosphate on oxidation and the maintenance of the natural colour of the fish muscle. Brining could be an effective way to improve these parameters and at the same time lead to lower increases in phosphate content. Another perspective is that phosphates added by brining may not be detected in rehydrated stage and they may be regarded as processing aid. The reason is presumed to be stronger extraction during salting and rehydration.

Added phosphates are still present in rehydrated salted cod pre-salted by multi-needle injection. So producers of salted cod must submit requests to relevant authorities, asking them to allow the use of polyphosphates in salted fish due to the recent development in processing methods. In the old kench salting procedure, addition of phosphates was impossible but this has changed due to recent developments in salting procedures. It should be emphasized that use of

phosphate must be declared and that producers must label their products in accordance to food legislations. Phosphate could be permitted in salted as in frozen products, provided that injected/absorbed phosphates do not exceed the limits of 5 mg P_2O_5/g after injection/brining. Since the muscle undergoes extreme changes in chemical content, it is hard to set limits for the cured and rehydrated products. The limits could be based on the ratio of P_2O_5 to salt free dry material, due to variation in water content in products processed by different procedures. The limits for added phosphates products in salted stage could be 30 mg/g salt free dry material, or total content of phosphates 40 mg/g salt free dry material. In rehydrated stage, the limits could be 25 mg/g salt free dry material (total content P 30 mg/g salt free dry material). However, this subject must further discussed by the different parties; producers, buyers, and food authorities.

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