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Effect of brine salting at different pHs on the functional properties of cod muscle proteins after subsequent dry salting

O. Martínez-Alvarez *, M.C. Gómez-Guillén

Instituto del Frío, CSIC, José Antonio Novais, No. 10, 28040 Madrid, Spain

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Abstract

Fillets of Atlantic cod (*Gadus morhua*) were wet-salted in brines of pH 6.5 and 8.5 containing different combinations of NaCl, KCl, $MgCl_2$ and $CaCl_2$, then dry-salted in NaCl. Proximate analyses, functional properties (water holding capacity and protein solubility) and hardness were determined in the dry-salted cod. The compositions of the protein fractions soluble in water and in 0.86 M sodium chloride were determined by SDS–PAGE. The composition and pH of the brines slightly affected the protein composition of the major extract constituents and the functional properties of the muscle after dry-salting. Brining at alkaline pH produced a larger variety of water-soluble proteins, particularly actin, than at pH 6.5. Furthermore, the compositions of the protein fractions extracted with 0.86 M NaCl were very similar for both pHs, irrespective of the composition of the brine; in this case, myosin heavy chains were absent in both extracts due to aggregation caused by a massive uptake of salt by the muscle.

Keywords: Salted cod; Divalent salts; Protein solubility; pH; Brines

1. Introduction

Salting is one of the oldest known means of preserving fish. Despite the development of other means of preservation and falling catches, salted cod today is a highly popular product, thanks to low production costs, simplicity of processing, high demand and the sensory characteristics imparted by salting (Gallart-Jornet, Rodríguez-Barona, Barat, Andrés, & Fito, 2003).

Fish is typically salted by dry or "kench" salting, a process in which the fish is filleted or "butterfly-split" and stacked with alternating layers of salt (Thorarinsdottir, Arason, Bogason, & Kristbergsson, 2004). It is advantageous to soak the fish in brine first, long enough for the muscle to absorb a significant amount of salt (Gallart-Jornet et al., 2003; Madrid, Madrid, & Madrid, 1994), as this both shortens the time and increases the yield of the salting process (Bogason, 1987; Thorarinsdottir et al., 2004).

Salting entails loss of water and uptake of salt. This affects the conformation of the muscle proteins, causing changes in water-holding capacity (WHC) and subsequent protein denaturation. As salt is taken up, and hence ionic strength increases, the anions bind to the filaments; this raises the negative charge and hence the repellent forces, thus increasing the space between filaments (Offer & Trinick, 1983). In salted fish, where the salt concentration reaches ≈20% (Thorarinsdottir, Arason, Geirsdottir, Bogason, & Kristbergsson, 2002), high ionic strength causes contraction of the myofibrils (Offer & Knight, 1988) and dehydration of the proteins in a process known as "salting out" (Dyer, French, & Snow, 1950; Kelleher & Hultin, 1991; Stefansson & Hultin, 1994). Also, the pH of the medium and the type of salts

* Corresponding author. Fax: +34 91 549 36 27.
E-mail addresses: oscarm@if.csic.es (O. Martínez-Alvarez), cgomez@if.csic.es (M.C. Gómez-Guillén).

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used for salting can influence the degree of protein denaturation (Kinsella, 1982; Morrissey, Mulvihill, & O'Neill, 1987), thus affecting protein functionality to a greater or lesser degree. $CaCl_2$ and $MgCl_2$ at certain concentrations, may reduce the solubility of muscle proteins (Stefansson & Hultin, 1994), whereas NaCl and KCl increase solubility at concentrations of up to 5% and 6%, respectively (Kelleher & Hultin, 1991). However, these concentrations vary, depending on the pH of the medium (Munasinghe & Takai, 2003). The degree of denaturation attained as a consequence of salting can also influence the protein quality of the desalted product.

The object of this work was to determine how different combinations of NaCl, KCl and divalent salts ($MgCl_2$ and $CaCl_2$) in brines at different pHs (6.5 and 8.5) influence the functional quality of muscle proteins after dry salting, using Atlantic cod (*Gadus morhua*), a commercially valuable species, as raw material. A second objective was to determine (by sodium dodecylsulphate–polyacrylamide gel electrophoresis [SDS–PAGE]) how brining conditions might affect the composition of the soluble protein fraction in the final product.

2. Materials and methods

2.1. Fish and salting

Following capture off the coast of Iceland by a commercial fishing boat, cod (*Gadus morhua)* specimens were headed, gutted, washed and placed in bins, covered with ice. The bins were immediately transported to the Icelandic Fish Processing School. Cod specimens were cut lengthwise into two parts, each weighing between 500 and 900 g, and salted by immersion in brine for 36 h: fish/brine ratio 1/1.4, temperature 4 °C. The salt concentration in all brines was the same (18% w/v). Brines consisted of distilled water and a mixture of salts containing variable quantities of sodium chloride, potassium chloride, calcium chloride and magnesium chloride (Table 1). The initial pH of each brine was adjusted to the level shown in Table 1 by addition of 0.1 M citric acid or sodium hydroxide. Sodium chloride (NaCl) was supplied by Supreme Salt Co., Ltd.; potassium chloride (KCl) was supplied by Saltkaup Ltd.; magnesium chloride ($MgCl_2$) hexahydrate and calcium chloride ($CaCl_2$) dihydrate were supplied by Merck.

The fillets were then dry-salted for 25 days by covering in Torrevieja salt (99.4% NaCl, max. 0.1% Ca, max. 0.09% Mg, max. 0.45% sulphate) from Unión salinera de España (Barcelona, Spain). The temperature throughout the process was 4 °C. The fillets were then shipped to Spain by refrigerated transport. The dorsal part of each fillet was chopped into 150–200 g portions (approximate dimensions: length 9 ± 2 cm, width 5 ± 1 cm, and thickness 3 ± 1 cm). All these portions were then mixed together.

Table 1
Composition and pH of brines used in cod brining

Brine compositions	NaCl (%)	$CaCl_2$ (%)	$MgCl_2$ (%)	KCl (%)	Brine PH
NaCl	100	0.0	0.0	0.0	6.5
NaCl	100	0.0	0.0	0.0	8.5
NaCl + KCl	50.0	0.0	0.0	50.0	6.5
NaCl + KCl	50.0	0.0	0.0	50.0	8.5
NaCl + $CaCl_2$	99.2	0.8	0.0	0.0	6.5
NaCl + $CaCl_2$	99.2	0.8	0.0	0.0	8.5
NaCl + $MgCl_2$	99.6	0.0	0.4	0.0	6.5
NaCl + $MgCl_2$	99.6	0.0	0.4	0.0	8.5
NaCl + KCl + $CaCl_2$	50.0	0.8	0.0	49.2	6.5
NaCl + KCl + $CaCl_2$	50.0	0.8	0.0	49.2	8.5

2.2. Determination of moisture

Moisture was determined on ≈5 g of minced muscle, by oven-drying at 110 °C to constant weight, following technique 950.46 (AOAC, 2000). Results were means of three determinations and were expressed as g of water/100 g of muscle.

2.3. Determination of ash

A sample of minced muscle (≈5 g) was taken and heated at 500 °C in a Lenton muffle furnace (Leicestershire, England), following AOAC 923.03 (AOAC, 2000). Results were means of five determinations and were expressed as g of ash/100 g of muscle.

2.4. Determination of protein

Samples of minced muscle (1.5 g) and brine (5 ml) were heated to 1050 °C following AOAC 992.15 (AOAC, 2000) in a LECO model FP-2000 protein/nitrogen analyser calibrated with EDTA (Dumas method). The nitrogen-to-protein conversion factor considered was 6.25. Results were means of four determinations and were expressed as g of protein/100 g of muscle or per 100 ml of brine.

2.5. Water holding capacity

Water holding capacity was determined as described by Montero, Gómez-Guillén, and Borderías (1996): 2 g of chopped cod muscle was placed in a centrifuge tube with 3 dried pipette filters (Gilson, Viliers-le bel, France) to act as absorbents. Each sample was centrifuged for 10 min at 6000*g* (Sorvall, RT6000B; Du-Pont Co., Wilmington, Del., USA) at room temperature. WHC was expressed as g of water retained per g of muscle protein, and was the result of at least 3 determinations.

2.6. Extractable protein

Extractable protein was obtained by a variation on the method used by Stefansson and Hultin (1994). Soluble protein was extracted in distilled water (low ionic strength), making it possible to see which proteins were solubilised as a result of adding salt to the muscle, and in 0.86 M NaCl solution (high ionic strength).

Two grammes of minced muscle were homogenised at low temperature for 1 min in 50 ml of distilled water in an Omnimixer-Homogenizer (model 17106, OMNI International, Waterbury, USA), setting 6. The homogenates of these solutions were stirred constantly for 30 min at 2 °C, then centrifuged (6000*g*) for 30 min in a Sorvall model RT 6000B centrifuge (Du Pont Co., Delaware, USA) at 3 °C. Protein concentration was determined in the supernatant by the colorimetric method of Lowry, Rosebrough, Farr, and Randall (1951). Optical density was measured at 750 nm in a Perkin–Elmer UV/Vis spectrophotometer (model Lambda 15, Massachusetts, USA). The standard curve was determined with various known concentrations of bovine serum albumen.

2.7. Sodium dodecylsulphate–polyacrylamide gel electrophoresis

The protein concentration in the resulting supernatants was adjusted to 0.20 mg/ml. The supernatants were treated with a denaturing solution composed of 5% 2-β-mercaptoethanol, 2.5% sodium dodecyl sulphate (SDS), 10 mM Tris–HCl, 1 mM ethylenediamine tetra acetic acid (EDTA) and 0.002% bromophenol blue, as described by Hames (1985).

Electrophoresis was performed by the method of Laemmli (1970), on a Mighty Small II SE250 unit (Hoefer Pharmacia Biotech. Inc., San Francisco, USA), using 10% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS), at 20 mA per gel and 250 V at 2 °C.

Protein bands were stained with Coomassie Brilliant Blue tablets (Phastgel Blue R., Pharmacia). Destaining was performed in an aqueous solution of 30% methanol and 10% acetic acid. Samples were conserved in a solution of 5% glycerol and 10% acetic acid.

The standard was a high molecular weight calibration kit (Amersham Pharmacia Biotech) consisting of: one sub-unit of ferritin (220 kDa), albumen (67 kDa), one sub-unit of catalase (60 kDa), one sub-unit of dehydrogenase lactate (36 kDa) and one sub-unit of ferritin (18.5 kDa). The molecular weight of each band was determined by the 1-D Manager (version 2.0) image analysis and quantification tool (Tecnología para diagnóstico e Investigación, S.A., Spain).

2.8. Shear strength

Shear strength was determined on a bone-free muscle sample, 4 cm long and 2 cm wide. This was divided in half lengthwise and spread on a Kramer cell (Kramer, Burkhardt, & Rogers, 1951) with the myotomes perpendicular to the cell. A computer-controlled Instron Universal texturometer model 4501 was used (Instron Engineering Corp., Canton, MA, USA), with a cell load of 5 kN at a setting of 100 mm/min. Results are the means of three determinations and are expressed as Newtons per g of muscle at the point of maximum load before sample breaking.

2.9. Statistical analyses

The significance of differences between mean value pairs was evaluated using one-way ANOVA. Tukey HSD test was used to identify significant differences among main effects. Statistical processing was done by the SPSS computer programme™ (SPSS Inc., Chicago, Ill., USA). The level of significance setting was $P \leqslant 0.05$.

3. Results and discussion

Cod fillets were salted in two consecutive stages: first, they were soaked in brines of varying composition and pH (Table 1) for 36 h, in which time the mean salt content reached around 5% and the moisture content reached between 75% and 80%. All fillets were then salted for a further 25 days with dry salt, composed basically of NaCl (99%). Table 2 shows the moisture, crude protein and total ash contents of the dry-salted fillets at the end of the two-stage salting treatment.

Dry-salting produced considerable loss of constituent water due to heavy uptake of salt (Sikorski, 1990) and raised the ash and protein contents by 13–15% and 5–6%, respectively, with respect to the brined cod. The final salt concentration in the different dry salted samples, expressed chiefly as % ash (Table 2), did not generally correlate with the concentration in the corresponding brined samples (Martínez-Alvarez, 2003). In no case were there significant differences in ash content that could be attributed to the initial pH of the brine in the previous stage. Ash contents were lower in dry-salted cod fillets initially brined with added $MgCl_2$ at pH 6.5 than in any other samples. These differences were significant ($P \leqslant 0.05$) with respect to all the samples initially salted in brines with KCl, where the salt levels were highest. In the sample salted with $MgCl_2$ at slightly acidic pH, the protein content was significantly higher than in most of the other samples. Only slight differences in protein content were observed among the rest of samples, mean values ranging from around 21–23%. The same was true of the moisture content, indicating that

Table 2
Proximate analyses of salted cod

Brines composition	Brines pH	Moisture (%)	Protein (%)	Ash (%)
NaCl	6.5	56.88 ± 0.03^{a}	23.1 ± 0.44^{abc}	20.54 ± 0.72^{abcd}
NaCl	8.5	56.81 ± 0.28^{ab}	21.4 ± 0.16^{fgh}	20.88 ± 0.38^{abcdef}
NaCl + KCl	6.5	55.55 ± 0.18^{abc}	21.90 ± 0.26^{defgh}	21.49 ± 0.43^{cdefg}
NaCl + KCl	8.5	55.48 ± 0.30^{abc}	22.0 ± 0.46^{cdefgh}	21.61 ± 0.48^{defg}
NaCl + $CaCl_2$	6.5	57.46 ± 0.31^{a}	21.9 ± 0.41^{defgh}	20.57 ± 0.48^{abcd}
NaCl + $CaCl_2$	8.5	56.75 ± 0.05^{ab}	22.0 ± 0.33^{cdefgh}	20.63 ± 0.54^{abcde}
NaCl + $MgCl_2$	6.5	55.77 ± 0.10^{abc}	23.7 ± 0.55^{a}	19.68 ± 0.51^{a}
NaCl + $MgCl_2$	8.5	56.59 ± 0.08^{ab}	22.9 ± 0.55^{abcd}	20.77 ± 0.31^{abcdef}
NaCl + KCl + $CaCl_2$ + $MgCl_2$	6.5	53.82 ± 0.11^{c}	22.0 ± 0.26^{cdefg}	21.03 ± 0.61^{bcdefg}
NaCl + KCl + $CaCl_2$ + $MgCl_2$	8.5	57.01 ± 0.91^{ab}	21.0 ± 0.33^{gh}	21.46 ± 0.24^{cdefg}

Different letters (a, b, c,...) indicate significant differences ($p \leqslant 0.05$) between samples.

the degree of osmotic dehydration produced by dry salting was scarcely influenced by the composition and pH of the initial brine. Nevertheless, it was noted that, in the cod fillets brined with the most complex mixture of salts with an initial pH of 6.5, the moisture level was significantly lower than with pH 8.5. Although higher than 3%, these differences did not correlate with any significant variations in protein or salt content as a function of pH.

Dry-salting reduced the water-holding capacity (WHC) of the muscle, from 3.32 g water retained/g protein in unsalted cod to final values ranging from 1.95 to 2.33 g water retained/g protein in dry salted fillets (Fig. 1). This is explained by the large uptake of salt (chiefly NaCl) by the muscle, resulting in competition with muscle proteins for water molecules, and denaturation and aggregation of these proteins by a process of "salting out" (Dyer et al., 1950; Kelleher & Hultin, 1991; Stefansson & Hultin, 1994) resulting in reduced hydration capacity (Hamm, 1975, 1982; Offer & Knight, 1988). No significant differences were observed in the WHC of dry-salted samples, as mediated by the pH or the salt composition of the brine used in the first stage. The only case where there was a significant ($P \leqslant 0.05$) slight reduction of WHC in dry salted fillets was the sample to which a small amount of $MgCl_2$ was added during brine salting at pH 8.5. No such effect was observed, however, when $MgCl_2$ was added along with $CaCl_2$ and KCl.

A certain amount of muscle protein can be extracted with distilled water. Variations in the solubility and type of water-soluble proteins due to the salting process may reflect differences in the susceptibility of these proteins to release during subsequent desalting prior to consumption. Bearing in mind that the dry salted fillets contained very high amounts of salt, the final salt concentration, in the homogenate of muscle with distilled water, ranged from 0.70% to 0.79%.

Fig. 2(a) shows the percent protein extractability in distilled water of dry salted cod fillets previously brine-salted using different combinations of salts and pH. Protein solubility under these conditions tended to be low (around 10–15%), as expected, given the extracting agent used (distilled water) and the degree of protein denaturation resulting from the high salt concentration

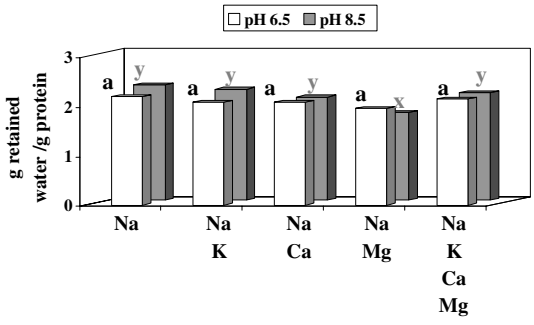

Fig. 1. Water-holding capacity (WHC) of myofibrillar proteins in brined and salted cod. Different letters (a, b, c,...) indicate significant differences among samples brined at pH 6.5. x, y, z,... indicate significant differences among samples brined at pH 8.5.

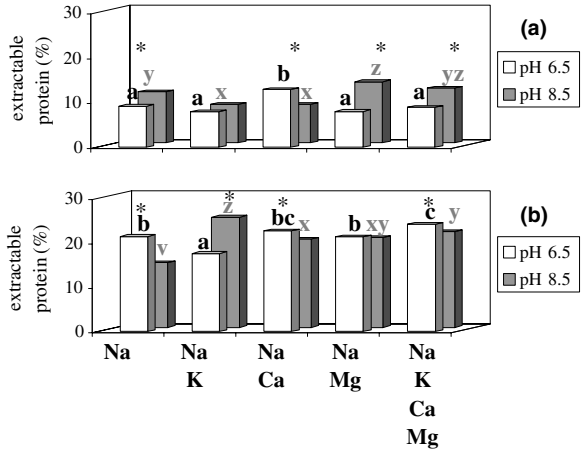

Fig. 2. (a) Soluble protein extracted in distilled water (low ionic strength). (b) Soluble protein extracted in 0.86 M NaCl (high ionic strength). a, b, c,... Indicate significant differences among samples brined at pH 6.5. x, y, z,... indicate significant differences among samples brined at pH 8.5. (*) Indicate significant differences between each salted sample brined at pH 6.5 and the corresponding brined at pH 8.5.

in the muscle (Kelleher & Hultin, 1991; Stefansson & Hultin, 1994). Water-solubility was higher in most of the samples previously brined at pH 8.5; the exception was the sample soaked in brines containing NaCl and $CaCl_2$, where solubility was higher with a brine pH of 6.5.

Differences in muscle salt content had no definite effect on solubility. Here again, the level of protein aggregation was so high that solubility ceased to depend on the concentration of salts in the muscle. As reported by Dyer et al. (1950), protein solubility ceases to vary at salt concentrations in excess of 1 M.

As reported by other authors (Hultin, Feng, & Stanley, 1995; Lee, 1984, 1986; Shimizu, 1985; Stefansson & Hultin, 1994; Suzuki, 1981), extractability of protein was higher with a 5% solution of sodium chloride (0.86 M) than with distilled water (Fig. 2(b)). Nevertheless, the extractability values (around 20%) indicate a high degree of protein aggregation as a result of salting/drying. In most cases, protein extractability was significantly higher in samples brined at pH 6.5 than at pH 8.5. In pH 8.5 brines, the final soluble protein concentration was significantly higher when 50% of the NaCl was replaced by KCl.

Following low and high ionic strength extractions, the constituent proteins of the soluble fractions were separated for identification by SDS–PAGE in denaturing conditions. Results are shown in Figs. 3 and 4.

Profiles of proteins extracted in distilled water varied according to the pH of the solution used prior to dry salting (Fig. 3). In cod brined at pH 6.5, except in the samples salted with the mixture NaCl, KCl, $CaCl_2$, $MgCl_2$, the main constituents of the soluble fraction were proteins of 35, 30 and 20 kDa. The 35 kDa protein

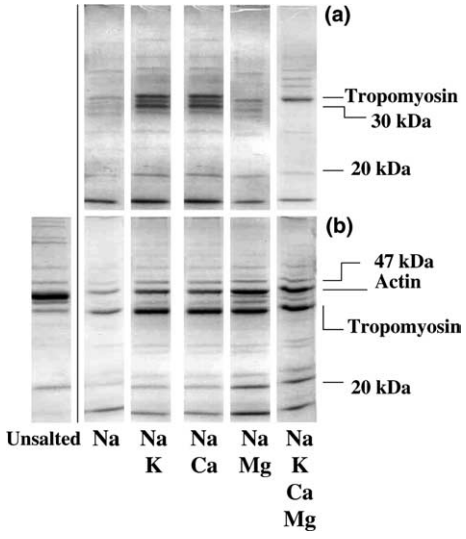

Fig. 3. SDS–PAGE banding pattern of: (a) protein in salted muscle, previously brined at pH 6.5, extracted by distilled water; (b) protein in salted muscle, previously brined at pH 8.5, extracted by distilled water.

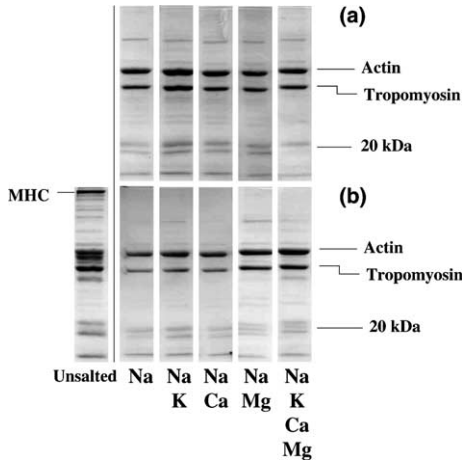

Fig. 4. SDS–PAGE banding pattern of: (a) protein in salted muscle, previously brined at pH 6.5, extracted by 0.86 M NaCl; (b) protein in salted muscle, previously brined at pH 8.5, extracted by 0.86 M NaCl.

was tentatively identified as tropomyosin subunits (Squire, 1994); the 30 kDa protein was tentatively identified as soluble products from degradation of higher-MW proteins such as troponin T (Ho, Stromer, & Robson, 1994; Mestre, 2001; Negishi, Yamamoto, & Kuwata, 1996). The band around 20 kDa could be composed of low-MW compounds such as troponin C, troponin J and myosin light chains (MLC) (Thorarinsdottir et al., 2002). In any event, there was a notable absence of actin and myosin heavy chains (MHC). There are several possible explanations for the absence of MHCs in addition to their poor solubility in water (Morrissey et al., 1987), including partial release into the brine (Martínez-Alvarez, 2003), aggregation in the early stages of salting (Ito, Kitada, Yamada, Seki, & Arai, 1990; Tambo, Yamada, & Kitada, 1992) and actual degradation during salting (Thorarinsdottir et al., 2002).

The water-soluble fraction of cod brined at pH 8.5, then dry-salted, was composed chiefly of actin, tropomyosin subunits and compounds of around 20 kDa. Electrophoresis showed slight traces of bands around 47 kDa (except in brine with NaCl) and 38 kDa (possibly troponin T), the latter only where brines contained $MgCl_2$. The water-soluble fraction of the final product was therefore generally more complex when the pH of the brine was 8.5 than when it was pH 6.5. Also, the bands were more intense after separation by electrophoresis. This could be due to more unfolding of proteins at alkaline pH, which would promote the formation of saline bonds between water molecules and charged groups of inward-oriented proteins.

The water-soluble fraction thus differed according to the prior treatment at both pHs. With pH 8.5, there was a greater predisposition to release proteins of slightly higher molecular weight, including part of the actin, during subsequent desalting.

The outcome of increased ionic strength of the extracting medium (0.86 M NaCl) was a soluble fraction composed mainly of actin and subunits of tropomyosin, irrespective of the pH or composition of the brines used in the first stage (Fig. 4). The absence of myosin in the soluble fraction of salted cod is indicative of the importance of the salting process in myosin aggregation. Electrophoresis also showed, if less intensely, bands corresponding to proteins of around 70 kDa (possibly light meromyosin [LMM], a product of myosin degradation [Wright & Wilding, 1984]), and others of about 23 and 20 kDa.

Unlike the case of the fraction extracted with distilled water, when a solution with a higher salt concentration (0.86 M) was used, the type of soluble proteins in the medium was unaffected by the mixture of salts used in brining or by the pH of the brine, because of the high degree of aggregation produced by intense salting. As a result, variations in the degree of protein aggregation in the dry-salted product as a consequence of different brining treatments did not correlate with qualitative changes in the composition of the salt-extractable muscle protein fraction.

As regards firmness of the dry-salted product (Fig. 5), the reduction of water content and the aggregating effect of the excess salt in the muscle protein considerably increased muscle firmness, from around 19 N/g in unsalted cod and 10–15 N/g in brined cod to 100–150 N/g in dry-salted fillets. Bligh and Duclos-Rendell (1986) confirmed the role of appreciable amounts of salt in hardening of cod. The pH of the solution used for wet-salting produced no significant differences in any case. Partial substitution of NaCl by KCl in the brine produced an appreciable increase in firmness of the muscle after dry-salting, as did the complex mixture of divalent salts and KCl. The hardening effect of KCl was reported by Frye, Hand, Calkins, and Mandigo (1986) in ham, and the effect of $CaCl_2$, in excess of 0.3%, has been reported by several authors (Beatty & Fougére, 1957; Lauritzen & Akse, 1995; Van Klaveren & Legendre, 1965). Lower firmness after salting with NaCl alone has been confirmed by Madrid et al. (1994) and by Wheaton and Lawson (1985).

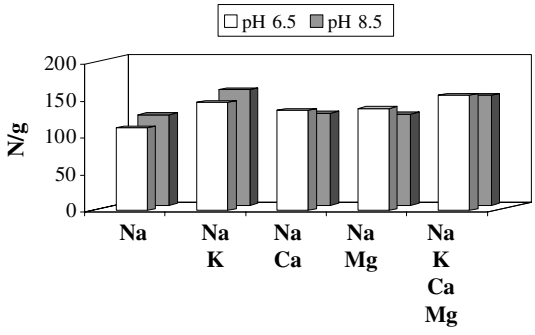

Fig. 5. Hardness of brined cod muscle at pH 6.5 or 8.5 after dry salting.

4. Conclusions

Differences in composition and pH of brines used prior to dry salting of Atlantic cod (*Gadus morhua*) slightly affected the composition of the muscle as regards the major constituents and the functional quality of the muscle protein. In most cases, the functional quality of the final product was lower with brining at pH 8.5 than at pH 6.5; this was reflected by salt extractable protein, indicating more intense aggregation of the myofibrillar protein as a consequence of the overall salting process. Protein extractability in water showed that the muscle brined at pH 8.5 was more predisposed to release proteins of higher molecular weight, particularly actin. This may have some importance when the dry-salted product is subsequently soaked, as a previous step before consumption. These pH-mediated differences were not rheologically appreciable; however, partial substitution of NaCl by KCl led to a noticeable increase in hardness.

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