SUMMARY

The purpose of this study was to prepare minces from hake (*Merluccius merluccius*); a dimethylamin (DMA) and formaldehyde (FA) forming species and Sardine (*Sardine pilchardus* W.) a fatty species with high lipid content. A corresponding mixtures of different ratios (3:1, 1:1 and 1:3) were prepared in order to study the stability of these products during frozen storage at -20°C for 12 months.

The main following points were considered:-

Part I: Chemical and physical properties:

* chemical composition and pH value.
* Formation of dimethylamine and formaledehyde during frozen storage.
* Texture and color measurements.

Part II: Changes occuring in functional properties of protein in relation to apparent viscosity, protein solubility, emulsion capacity and emulsion stability of the tested samples stored at -20°C for 12 months.

Part III: Isolation of natural actomyosin (NAM); and the tested parameters were:

- Extractability of NAM in 0.6 M NaCl.
- Electrophoretic pattern of NAM .
- Emulsifying properties: emulsion activity index (EAI) and emulsion stability index (ESI) of the extracted NAM
- Solubility (in different agents) of the residue obtained from the extractable natural actomyosin and electrophoresis pattern of the supernatant.

Part IV: Electron microscpy of minces, isolated actomyosin and protein aggregates of the same samples.

The obtained results could be summarized in the following points:

Part I- Chemical and physical properties of minced fish during frozen storage:-

* **Chemical composition :**

Variations in the chemical composition of the inves_ tigated fish samples; hake (Lot A), sardine (Lot E) and the prepraed mixtures (B, C, and D) were observed. These variation depending on species and to the different ratios used in forming the mixture between minces hake and sardine. However, the available data proved the stability of moisture of the tested samples during storage at-20°C for 12 months.

* **pH value:**

The highest pH value was noticed in hake (Lot A), while the pH value of minced sardine (Lot E) was the lowest one when compared with other lots. With respect to lots B, C, and D, their pH values were loed between lot A and E depending on the ratios of minced hake and minced sardine. However, during frozen storage at -20°C, there is a noticeable increase in pH values of all the investigated lots.

*** Dimethylamine (DMA) and Formaldehyde (FA):**

Prolonging the frozen storage period of minced hake (lot A), the DMA concentration and FA progressively increased. Such increment is attributed to the decompo_ sition of trimethylamine oxide (TMAO) to dimethylamine and formaldehyde by trimethylamine oxidase.

The lowest values of DMA and FA that obtained in minced sardine during storage, are matching with the fact that sardine fish is considered to be non DMA and FA forming

species. When more minced sardine was added to minced hake (lot C and D), a reduction in the concentration of DMA and FA formation was noticed during frozen storage.

* Texture:

The obtained results indicated that in non fatty fish, where textural changes are the main cause of deterioration due to the enzymatic breakdown of trimethylamine oxide to DMA and FA, the produced FA is then react with fish proteins to accelerate the undesirable texture changes. Subsequently it could be concluded that there is a real increase in the texture of investigated minced fish samples during storage at -20°c. Furthermore, in a formaldehyde forming species (lot A) the obtained values were higher than in non-formaldehyde species (lot E). On contrary, addition of minced sardine to minced hake minimize the changes which took place in texture during frozen storage and the highest reduction in toughening was observed in lot D. Variations in texture is usually started from the second month where a significant difference was found between different lots up to the end of storage period.

* color:

the obtained results proved the presence of significant changes in the L*, a* and b* values between lot A (100 % minced hake) and lot E (100 % minced sardine). In other words sardine minces had less lightness, more redness and yellowness, while hake mince is lighter, less red and yellow. Subsequently, it could be concluded that addition of minced sardine (dark muscle) to minced hake lead to a noticeable reduction in the lightness(L*), and samples turned redder (a* increased) and more yellow (b* increased). These changes are more evident in lots C and D. During frozen storage and thawing, changes in L*, a*, and b* values still occur within the tested samples.

From the available data it could be observed that both surface and internal color of lot A (100 % minced hake) exhibiting higher color differences (ΔE) and lower chromaticity differences (ΔC) at the end of storage period.

Part II: Effect of frozen storage at -20°C on the functional properties of minced fish:

One of the consequences of protein denaturation is the change in their functional properties which usually primary factors determing their utility in food products. Some of the most important functional properties in fish muscle are protein solubility, viscosity, emulsifying capacity and emulsion stability.

* **Changes in protein solubility:**

Data proved that minced sardine was characterized by high level of soluble protein. On prolonging of the frozen storage period the downward trend in protein solubility was found in all samples, while the declin that was more pronounced in lot A was mainly extended until the fifth month. With respect to the addition of minced sardine containing neutral lipids to minced hake; it causes more stability of proteins; i.e. lower level of denaturation and protein aggregation and so, contain the large amounts of soluble protein. The highest reduction in protein solubility was observed in lot B. The noticeable variation in protein solubility of lots B, C and D could be related to the different ratios of minced sardine which added to minced hake.

*** Changes in the total protein content of the mince homogenate:**

Data indicated that after freezing the homogenate of minced hake (lot A) contains lower level of total protein

when compared with other lots. With respect to lot E; the highest value of protein was found in its homogenate and the significant differences were recorded among lot E and other lots at any given time of storage at -20°C.

It is worth to note that the total protein in the filtrated homogenate which used to measure apparent viscocity and emulsifying capacity changed slightly during storage of all lots except lot A and B. In lot A the total protein decrease slightly up to the fifth month and then decline sharply up to the end of storage. With respect to lot E the experiments proved that the total protein in the homogenate remained nearly constant throughout storage at -20°C. This may be due to the fact that sardine is more stable during storage under frozen condition and do not form condenced aggregate. When 25 % of minced sardine was added to 75 % minced hake (lot B), the total protein of homogenate increased; but during frozen storage the total protein was slightly decreased with a lower rate. The total protein is approximately stable during storage at -20°C by increasing the ratios of minced sardine to 50 % in lot C and to 75 % in lot D.

* <u>Changes in apparent viscosity:</u>

A significant difference was found between the investigated lots and such trend was more pronounced in most of storage period at -20°C for 12 months. The highest initial value of apparent viscosity was noticed for lot A.

The obtained results indicate a real inverse relation between apparent viscosity and storage period at -20°C. Such trend was more evident in lot A. On the other hand, increment the ratio of minced sardine caused a reduction in the initial value of the apparent viscosity, in spite of the decrement which occured during frozen storage, the samples still showed higher values than lot A.

* Changes in emulsifying capacity:

The minced hake initially showed the lowest value of emulsifying capacity and no significant differences were found among lots B, C and D. However, the EC is reversibly correlated with frozen storage, a pattern which was more pronounced in minced hake

The available data also proved that the higher EC was recorded in minced sardine, and it maintained the largest value of emulsified oil per g mince when compared with the other lots. The loss in EC values was more pronounced in minced hake (lot A) within the 8th month of storage at -20°C due to lower solubility of protein in the homogent than that recorded for minced sardine. On the other hand, adding minced sardine to minced hake reduced the loss in EC value as showen in lot B, C, and D.

* Changes in emulsion stability:

Results showed that initially in lot E the emulsion is less stable when compared with lot A. On prolonging the frozen storage period of the different investigated lots a lower thermal emulsion stability was noticed. For instance the results showed a gradual decrease in emulsion stability of lot A during frozen storage, while in the eighth month the emulsion was less stable as indicated by much increase in total exudate, water and oil separation. At the end of storage period, the emulsion was not formed at 80 % oil. With respect to lot E, the emulsion is more stable during frozen storage when compared with lot A.

The increment of the added ratios of minced sardine to minced hake increase the total protein in the homogenate with a very slight changes during frozen storage which lead to the noticeable increment in emulsion stability as shown in lots B, C and D.

Part III: Effect of frozen storage at -20°C on the extractability of natural actomyosin (NAM):-

A) **Extractability of supernatant (S1 Fractions):**

The highest amount of extractable actomyosin was found in lot E (100 % minced sardine) when compared with the other lots. During frozen storage (in each sample) the extractibility of actomyosin in 0.6 M NaCl decreased indicating that denaturation and aggregation occurs. The lowest extractibility of actomyosin was found in lot A (100 % minced hake), and no NAM was extracted from lot A by 0.6M NaCL from the fifth month, while NAM extracted from sardine muscle had fallen to half of its value by the end of storage.

In the mixed hake/sardine minces, extractability was improved when higher precentage of sardine mince was found in the mixture (D>C>B).

*** Changes in the electrophoretic pattern of supernatant NAM (S1 Fraction):**

The SDS-polyacrylamide gel electrophoresis was performed on the natural actomyosin soluble in 0.6 M NaCl isolated from the different lots during frozen storage.

The electrophoretic patterns of the investigated lots differed considerably according to fish species and ratios of mixture. The results also indicated that changes in myofibrillar proteins were mainly in myosin, since it is considered to be the most sensitive fish myofibrillar protein with respect to freeze denaturation. As the times of frozen storage extended the amount of aggregated proteins at the top of the gel increased indicating the accumulation of very high molecular weight components.

Comparison among lots showed that fractions extracted from hake and sardine with 0.6M NaCl differed from the initial control. In hake, the majority peaks were for myosin heavy chain (MHC) and actin (Ac), while in sardine much of the extracted protein was joined by covalent bonds to form soluble aggregates of high molecular weight, so that the relative amounts of MHC and Ac in the extract were smaller.

As frozen storage progressed soluble aggregates in lot A (100 % hake) increased. In lot E (100 % sardine), peak 1 augmented gradually, indicating an increase in 0.6M NaCl-soluble high molecular weigh (micro-aggregates) which were retained in the stacking gel because of their size. At the outset, the electrophoretic patterns of the protein in mixed muscle lots revealed the presence of aggregates and showed majority bands of MHC and Ac. At the end of storage, the electrophoretic profile showed a decrease in MHC in the extracts, while the actin peak tended to decline further when the percentage of sardine in the lot was increased.

*** Emulsifying properties of NAM (S1 Fraction):**

All of the investigated lots showed high emulsifying activities at zero time of storage. The emulsion stability was higher at protein concentration of 5 mg/ml with higher values in lots containing from 100 to 50 % hake. In general lot C showed a good emulsifying functionality, since the values of the emulsifying properties (EAI, ESI) are much higher than the other lots. In addition, lot C retained higher emulsifying properties even at the end of storage period. Both lots D and E which showed lower emulsifying properties during the end of frozen storage.

B) Extractability of precipitated aggregates (P1 Fractions)

- For breaking down The secondary interactions, the following treatments were used:

* Treatment with 2 % SDS:

In all lots the amount of NAM extracted from aggregate P1 upon breakdown of secondary interactions with 2 % SDS declined as storage progressed. By the end of storage, practically no protein was extracted from the aggregate.

This fraction (S2) was studied by Polyacrylamide Gel Electrophoresis (SDS-PAGE), obtained results indicated:

Lot A: At 5 months' of storage at -20°C the protein extracted upon breakdown of secondary interactions consisted mainly of myosin heavy chain (MHC) and actin (Ac). As storage progressed the amount of MHC extraction declined, and by the end of 12 months the Ac peak was the largest.

Lot E: In this lot the protein extracted at fifth months was composed largely of aggregates which did not enter the stacking gel. MHC appeared and there was also an actin peak, although relatively smaller than in other lots. At 12 months' of storage at -20°C the majority band consisted of aggregates of intermediate molecular weight.

Values for mixed-species lots (B-D) were intermediate between lots A and E. At the outset there were protein aggregates which did not enter the gel, and majority bands of MHC and Ac. By the end of storage the majority band was actin, although a MHC peak was visible in all cases.

* **Treatment with 2 % SDS + 8M Urea:**

When the P1 aggregates from the different lots were treated with 2 % SDS + 8M urea, the amount of NAM extracted from precipitate P1 declined as storage progressed. But although both SDS and urea breakdown secondary interactions, the amount of protein extracted with these agents and with 2 % SDS was differed.

Similar amounts of NAM were extracted with 2 % SDS + 8M urea from the aggregates in lots C and A (100 % hake), which would indicate that more of the protein in lots A and C was bound by secondary interactions up to the 8th month of storage, whereas the pattern in lots B and D was more akin to that of lot E (100 % sardine), with greater initial involvement of covalent bonds.

This fraction (S2) was studied by (SDS-PAGE), and the obtained results indicated that at 5 months a majority high-MW band which did not enter the gel was detected in lots A and C. As storage progressed, peak 1 declined in all lots. The electrophoretic profile in lots B and D was similar to the profile for 2 % SDS. There was practically no variation after 12 months' storage. The similarity of electrophoretic profiles for lot E to the profiles of extraction with 2% SDS only, indicating that there was covalent bonding in the extracted protein.

- For breaking down secondary interactions and disulfide bonds the following treatment was applied:

- **Treatment with 2 % SDS + 5 % β-ME:**

1) Lot A: When the aggregate P1 was treated with 2 % SDS + 5 % β-ME, at 8 months' of storage at -20°C, although all the NAM was aggregated 98 % of the protein in fraction P1 from

lot A was solubilized. The soluble protein had dropped to 20 % by the end of 12 months storage.

2) Lot E: The amount of protein extracted from the aggregate with 2 % SDS + 5 % β-mercaptoethanol at 8 months was much smaller in sardine than in lot A. This suggests that covalent bonding occurred earlier in the sardine than in the hake aggregate.

3) In the mixtures of the two species, the amount of protein extracted remained practically unchanged at around 67 % in lots C and D and 38 % in lot B. In all lots breakdown of secondary and disulfide bonds with this treatment increased the amount of protein extracted from aggregate P1 as compared to the other solubilization treatments, at both 8 and 12 months' of storage under similar condition.

From (SDS-PAGE), the obtained results indicated that in lot A, there was practically no change in the electrophoretic profile between 8 and 12 months of storage at -20°C. The MHC appeared joined to peak 2, while the actin peak was clearly visible at both 8 and 12 months.

The behaviour pattern in lot E was similar at both 8 and 12 months. No clear trend was apparent in the electrophoretic profiles of the fractions extracted upon breakdown of secondary interactions and disulfide bonds in the aggregates from lots containing a mix of the two species.

Part IV: Transmission electron microscopy (TEM):

*** Muscle morphology:**

Initially the muscle structure was intact in both of the investigated fish species, with Z line clearly visible. As storage progressed, Z line in the hake became increasingly disorganized, whereas in the sardine there was scarcely any change.

* morphology of Supernatant NAM (S1 fractions):

Lot A: The protein in the supernatant that was in globular form, displaying numerous short filaments in association with mutually-interconnected globules. Also visible were numerous micro-aggregates with which these formed associations.

Lot B: At 2 months of storage at -20°C the protein in the supernatant exhibited filamentous zones linked by aggregates which were larger than in lot A. As storage progressed ring-shaped structures and micro-aggregates appeared which have been associated with the formation of covalent bonds.

Lot C: At 2 months of storage under similar condition there were few filamentous zones, the protein forming large agglomerations. As storage progressed, aggregated zones also appeared, in which ring-shaped structures were apparent. At 12 months of storage at -20°C most of the proteins appeared to form a tangled network with a less dense structure than in lot B.

Lot D: At 2 months' of storage at -20°C the extracted protein appeared as small aggregates which formed globular subunits with no sign of filamentous structures, giving an appearance quite different from the other lots. As storage progressed (5 months), protein aggregates containing filaments formed in the supernatant and with the passage of time ring-shaped structures became ever more clearly visible.

Lot E: At 2 months' storage, the extracted protein appeared as small aggregates similar to the ones observed in lot D. Globular aggregates were apparent from the outset but developed differently from those in the lots with mixtures of

hake muscle. As frozen storage progress, the protein in the supernatant adhered to the tube walls leaving no protein to be observed in the supernatant under TEM.

* Morphology of precipitate residual of NAM (P1 fractions):

Initially, the muscle structure is generally better retained in the sardine precipitates and in the lots containing a high percentage of sardine (lot D and lot C), while less so where the percentage of hake was higher (lot B). In hake (lot A), scarcely any of Z line was visible.

As storage progressed (8 months), the insoluble P1 aggregates exhibited denser structures in parallel alignment, which could be associated with the formation of aggregates among the actin and myosin myofilaments.

By the end of storage (12 months), the structure in the P1 insoluble aggregates from hake was more organized than at the outset and traces of Z line were visible. In sardine (lot E) at 12 months of storage at -20°C, the structure displayed a clearly defined Z line and was very similar to that of the original sarcomere. The mixed lots of the investigated samples exhibited structures half-way between the hake and the sardine lots.