ASSESSING THE EFFECTIVENESS OF A COLD CHAIN FOR FRESH FISH SALMON (*Salmo salar*) AND SARDINE (*Sardina pilchardus*) IN A FOOD PROCESSING PLANT.

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- Sensory evaluation established a high degree of freshness (90%) in salmon and sardine.

- The brightness of the skin was the best parameter evaluated.

- The principal component analysis (PCA) built showed a great susceptibility of the cold chain.

- Two conditions, a uniform process for salmon and other very heterogeneous for sardine were detected.

- Different origins of the raw material could be change the quality final products.

ABSTRACT

In recent years, consumer attention has centered on the acquisition of very fresh food. Therefore the food industry has focused not only on meeting the safety regulations in this field, but also in keeping customers by providing safe and healthy products. Considering this reality, the present work evaluates the inherent quality issues in a company located in the city of Zaragoza, Spain, dedicated to fresh fish processing and marketing. In order to do this, the cold chain process of the plant was evaluated through physical analysis (including pH and determination of surface temperature), chemical, microbiological and sensory changes in two species of fish (*Salmo salar* and *Sardina pilchardus*). In all cases the temperature was higher in the finished product than the raw material. Microbiological count were satisfactory, according to the Community Regulation EC 2073/2005. Sensory evaluation conducted by an expert panel established a high degree of freshness (90%) in both species, and the brightness of skin was the best evaluated parameter. The principal component analysis (PCA) built from the parameters evaluated showed a great susceptibility of these to the cold chain, being able to discriminate a uniform process for salmon and other highly heterogeneous for sardines, these results could be attributed to their different origins, farmed and captured respectively. However, both processes were effective in terms of quality and safety of the cold chain concerned.

Quality and Safety
Fish
Cold Chain
Seafood´s Industry
Salmon
Sardine

1. Introduction

According to ISO 9000:2005 the quality is defined as the degree to which a set of inherent characteristics fulfils requirements**.** A company which aims to offer quality products should be managed and controlled in a systematic way, as this set of characteristics is required by consumers, explicitly or implicitly in flow in its acceptability. In this context, the aspects that should be covered with this evaluation would be product safety, their genuineness, their nutritional status and other commercial aspects, also psychosocial, but this is less important (Nader, 2000).

The quality is the result of planning but also the outcome of management within the organization. Because of this, study and control are necessary especially in the case of food, taking into account the sensory, physicochemical, commercial and psychosocial aspects.

Sensory analysis is widely used in the industry to evaluate freshness. In Europe, this method is usually employed by the inspection services processing fishery products, this is based on Regulation 2406/96 which establishes common marketing standards for certain fishery products and whose validity has been much questioned. In this scale, the classification is based on changes that occur in the organoleptic characteristics which are, in the opinion of many, few specific and only allow classifying as 'Extra', A, B and unacceptable (Hernandez, 2005).

The physical and chemical parameters more commonly used for determining the quality of fish are volatile nitrogenous bases and trimethylamine (only in sea species). These are important in assessing the deterioration caused by bacteria, which can also be performed by microbiological count and identification. Prior to bacterial spoilage, there is a stage of autolytic changes that can not be measured by these indices. The methods generally used in the evaluation of the autolytic changes are the determination of Hypoxanthine and the K value. But it is the sensory evaluation index that can measure the loss of freshness and autolytic spoilage of bacterial origin. The potential of hydrogen ions (H^+) is also a physical parameter for evaluating the development of metabolic reactions, especially those that occur after the death of fish where ATP and glycogen degradation are of particular importance (Huss 1999; Yeannes, 2011).

One of the methods used in data mining is Principal Component Analysis (PCA) which was originally introduced by Pearson in 1901, and then it was developed independently by Hotelling in 1933. It is an exploratory technique in data analysis and synthesizing information, or reducing the number of variables. These new variables or principal components are calculated as a linear combination of the original variables and they are lineally independent (Dallas, 2000). In this way, many things, are synthesized in a small, but crucial group of variables. This would allow the discrimination of a set of parameters obtained from a process such as the cold chain of a particular food, and to determine the aspects that best describe it and its condition, allowing optimization in both, planning and quality control of food.

The fishing industry in Spain is one of the most important in the country because fish and aquatic *per capita* consumption are consistently high, because of their low prices and their health implications (Martin, 2010). This is the reason why it is necessary to go further in the study of quality issues to achieve the full satisfaction of the costumer. Special attention should be paid to the quality requirements and specifications for the fishing industry, the way they operate, whether it is developing in the right way, to look for improvements in their management processes and achieve greater benefits and ensure that operating within the framework of compliance with applicable laws. Within this scope, this work is a research whose results may be a scientific and technological contribution in the field of fresh fish and quality implications.

This work studied the effectiveness of the cold chain to ensure the safety and quality of raw materials and their respective finished products in a company engaged in the processing and marketing of fresh fish from the city of Zaragoza in Spain.

2. Materials and Methods

2.2. Species studied

Two fish species were selected for this research: 1) Common Name: Salmon, Family: *Salmonidae*, Scientific name: *Salmo salar*, Origin: Norway, Source:

Aquaculture (Hatchery), presentation of Raw material: Whole and gutted. 2) Common name: Sardine, Family: *Clupeidae*, Scientific name: *Sardina pilchardus*, Origin: Spanish Fleet (Northwest Atlantic, FAO area 21), Origin: Capture, Raw Material Presentation: Fillets (butterfly).

2.3. Sampling Plans

The choice of sampling plan used for the analysis of physical and chemical parameters (Total Volatile Basic Nitrogen -TVB-N-, pH and temperature) both in raw material and finished product was carried out taking into consideration the provisions of the Statement of *Codex Alimentarius* (CAC / GL 50-2004) "Guidelines on Sampling Plans for Sample Standard Deviation considering." The determination of the parameters mentioned was conducted in 2 batches of raw material that each corresponded to different days of reception and 2 batches of finished product from distinct days of production. For the salmon were taken five whole fish for each batch to raw material and five trays for each batch of finished product. In both, trays and whole fish, the physical and chemical analyses were carried out by triplicate. In the sardine, both raw materials and finished products ten fillets were taken and the chemical and physical analyses were executed in duplicate. The sampling were executed in the factory, the whole salmon, trays' salmon and sardines' trays were packed into polystyrene boxes, covered with melting ice and transported to the laboratory for further analysis.

2.4. Physical and Chemical parameters

2.4.1 TVB-N:

TVBN determination took place in a Kjeltec unit by direct steam distillation over boric acid, following the protocol described in the Community Regulation 2074/2005, Chapter III, "Determination of the Concentration TVB-N in Fish and Fish Products

2.4.2 pH:

This physical parameter was evaluated in 2 different ways. The pH in whole salmon was measurement employed a digital pH-meter with puncture electrode, (Crison, model PH25). The measurements were performed in duplicate on both sides of the head and fresh whole fish, just at the top of the back where the muscle has been exposed after the decapitation of the fish in the cutting machine. In the case of sardine fillets and salmon fillets determining pH was adjusted to the protocol described in AOAC (1997) for the determination of pH in food by the method of homogenization, using a pH-meter (Crison Instruments, SA, Alella, Barcelona, Spain).

2.4.3. Temperature Measurement:

Temperature (°C) was measured in the raw material and finished product. In the case of whole fish, an additional method of determining internal temperature was used, while the rest of the fish measurements were made only on the surface. Ten observations were made in duplicate obtaining an average values for each species. The surface temperature (TS), in all cases, was measured on the skin in raw material and finished products. In whole salmon, measurements were made in the head, trunk and tail. The others samples were evaluated taking into account the representativeness and avoiding areas with ice.

In the analysis, an infrared thermometer (Infrared Sensor FR260MV AMR) coupled to a console (ALMENO 2450-1L) designed to measure (without contact) was used. It was addressed directly to the skin of the product, at a distance less than 15 cm, recording and presenting the measures in a digital display. To determine the internal temperature (IT), a previously calibrated, sterile probe (TESTO 926) was used and introduced by the animal's mouth parallel to the dorsal spine to register a constant value on the screen steady digitally for at least 3 sec. Direct puncture is avoided on the body of the fish in order to minimize the negative economic impact for the company.

2.5. Microbiological Count

Total mesophilic viable count (MVC), *Enterobacteriaceae*, *Pseudomonas sp. Listeria sp.* investigations (presence/absence) were studied in samples of whole fish and fillets. A piece of fish muscle (10 g) was taken from the dorsal region of each fillet, transferred aseptically into a stomacher bag (Seward Medical, UK), mixed with 90 ml of 0.1 % peptone water containing 1 % NaCl and homogenised for 60 s using a Stomacher (Lab Blender 400, Barcelona, Spain). MVC was determined by pour plate methods in Plate Count Agar (Merck, Darmstadt, Germany) using conventional dilution procedures. Plates were incubated 72 h at 30°C for MVC (ISO 4833:2003). For *Enterobacteriaceae,* violet red bile dextrose agar (VRBD, Scharlab, Barcelona, Spain) with double layer was used and plates were incubated 48 h at 30°C (ISO 21528-2:2004). For enumeration of pseudomonas, samples (0.1 ml) of serial decimal dilutions (0.1% w/v peptone and 0.85% w/v NaCl) of fish homogenates were spread on the surface of the appropriate CFC medium (Oxoid code CM 559, complemented with selective supplement SR 103 consisting of cetrimide, fucidin and cephaloridine), Petri dishes were incubated at 25 ºC for 2 days in accordance with Tryfinopoulou, Drosinos & Nychas (2001). Twenty five g were taken to determine the presence or absence of *Listeria monocytogenes.* This was carried out following the ISO 11290-2:1998 Enumeration method, modified by ISO 11290-2:1998/AM 1:2004.

2.6. Sensory Analysis

Both raw material and finished product of both species were evaluated by a panel of 6 trained sensory assessors. For evaluations, the sensory quality format for fish products (Real Decreto 331/1999 "de normalización y tipificación de los productos de la pesca, frescos, refrigerados o cocidos") was used. Once collected the judgments made by the panel, there was a categorical data conversion for a range of numbers which was attributed as much to the quality defined as Extra (4) and reduced the so-called rejected (1) of according to legislation, in every aspect of quality assessed. The overall results were weighted for each treatment and presented as % of freshness.

2.7. Statistical analysis

TVB-N values, pH and temperature measured for the raw material and finished products were analyzed by applying SPSS 17.0. The program XLSTAT was used to establish a general profile of the effect of "cold chain" on the quality and safety of fish carrying out a principal component analysis (PCA). Experimental temperature measurements (°C) were expressed in the Fahrenheit scale in order to avoid the use of negative numbers.

3. Results and Discussion

3.1. TVB-N

Table 1 shows the values obtained of TVB-N for salmon and sardine. In most cases, both species showed an increase in the value of non- protein N_2 in the finished product, while those in the raw material were lower. However, the TVB-N values never exceeded the legal limit (35 mg.100 g^{-1}) set for *Salmo salar* muscle in accordance to the Regulation (EC) 1022/2008.

The results were subjected to analysis of variance (ANOVA) in both species resulting in a highly significant interaction ($p < 0.001$), therefore the study was performed again to consider the effect of the interaction. In the case of salmon, there was not established significance between the 2 batches (IN1 and IN2) for raw material (IN). The batches of finished product (OUT1) were not different on the first day of sampling. However, OUT2 presented highly significant differences ($p < 0.001$) showing the highest values. This demonstrated that the time between the reception of raw material and finished product are key to product quality and the batch was processed OUT1 before completing 24 h of receipt (IN1), while the batch OUT2 corresponded to specimens with a timeout greater than 24 h but less than 48 h.

Regarding TVB-N content in sardines, there was highly significant difference ($p <0.001$) which showed 3 different groups, probably attributed to the characteristics of the raw material as the result of previous industrial processes where the production of nitrogenous trimethylamine and other metabolites could be increased. The rate of deterioration depends on many factors, including fish species, physiological state, environmental conditions and after the capture, handling and subsequent storage conditions (Olafsdóttir, Martinsdóttir, Oehlenschlager, et al., 1997). The spoilage of fish is mainly due to the growth of bacteria which cause a quick increase in the content of nitrogen compounds (Riebroy, Benjakul, Visessanguan, et al., 2007). TVB-N values in the finished product did not differ for sardines of similar outputs (OUT1 = OUT2). The batch IN2 was immediately processed after arrival at the plant, and this condition resulted in a high homogeneity which is reflected in batch OUT2 values.

Karim, Kennedy, Linton et al. (2011) in a study with fresh herring (*Clupea harengus*), vacuum packed, kept at 2 + / - 0.5 ° C, they obtained an initial content (time 0) of 22.4 mg N_2.100 g^{-1}, this amount increased gradually to overcome the limit of 35 mg N_2.100 g^{-1} in approximately 5.5 days. This period considered as the shell-life for this product. In this work, the averages TVB-N in the finished products were 24.41 and 26.72 mg N_2.100g^{-1} for salmon and sardine, respectively. These values, considering the peculiarities of each species, are accepted as normal for such fish species.

Anderson (2008) in a study of fresh fish from 3 sea species sold in a market in Kuwait, found an increase from an initial amount of 28.05 to 36.05 mgN_2 .100g^{-1} about 8 mgN_2 .100g^{-1} of variation due to the temperature change for a period of public display on ice 6 to 8 h. These results evidentated the importance of a proper cold chain to ensure quality and safety of seafood. Our research has shown an average increase of 4 mgN_2 .100g^{-1} from raw material to finished products in both species, suggesting that level of TVB-N in fish could be a reliable estimate of the temperature drop effect in the manufacturing process.

3.2. pH

Table 2 shows the pH measures for raw material and finished products in Salmon. The results showed no significant differences between batches of raw material, regardless the method of measurement. However, for the different methods (insertion and homogenization) there were established highly significant differences ($p < 0.001$) being the insertion technique which provided a lower average value. The advantage of using an insertion kit makes possible to determine the exact value of the measure in the fish muscle, and decreases the possible interference that the use of solvents may cause; in contrast to the homogenization technique, this requires that the used specimen have to be of a considerable size, having enough muscular area to apply the electrode. Because of that, the homogenization constitutes a more versatile technique that just needs small portions of the food, one of its parts or even a small amount of a mix. The portions needed for the analysis must be taken from different areas, in that way, the values would be reliable and representative. Another important aspect is that the fish muscle has an specific morphology that it is damaged by the puncture and affects its presentation.

There were highly significant differences between raw material and finished product for salmon related to pH values measured by the homogenization procedure. Whole salmon had an average value of 6.9 + / - 0.2 while the modified atmosphere packed fresh slices had an average of 6.4 + / - 0.1. The latter value agrees with that obtained by Salam (2007) in slices of fresh salmon (*Salmo salar*), using the method of homogenization, where they found values of 6.45. The fact of finding lower pH values in the finished product is predictable, because the mechanisms that occur when the fish has been packed in a modified atmosphere with high carbon dioxide content, can cause a decrease in pH, because of the formation of carbonic acid.

The variability found between batches of sardines was probably due to the characteristics of the species and post-harvest treatment. Unlike salmon, sardines come from capture fisheries and also fillets have been subjected to previous industrial handling. As seen previously, the N_2 protein content of raw material batch IN1 presented very low values compared with those of IN2 and the difference was statistically significant ($p<0.05$). The presence of these compounds tend to raise the pH and such behavior is observed in IN1 (5.97 + / - 0.05) was statistically different and lower than IN2 (6.11 + / - 0.10).

In contrast, Salmon pH values were lower than those of sardine. It might be, because salmon is not fed for 24 hours before harvesting, in order to control pH fall that occurred after death and to maintain the functional characteristics of muscle as well as possible. Meanwhile, sardine is caught in the wild, without knowing if it have recently fed or been exposed to conditions of stress. It should be noted that sardine is an industrial product that has undergone treatment before arrival at the company, including refrigerated transport. According to Campos, Rodriguez, Losada, et al. (2004), one of the factors limiting the commercial use of the sardine is the difficulty of its storage at low temperature. Therefore, its lifetime is limited by the rapid bacterial degradation and lipid oxidation mechanisms, which cause a bad taste and discoloration of the flesh. Also variations in the initial pH may be due to the species, the annual season, diet, activity level or stress during capture, as well as muscle fiber type (Ocaño-Higuera, Marquez-Ríos, Cañizales-Dávila, et al., 2009).

The batch of raw material IN1 differed very significantly ($p < 0.01$) from all the rest of the batches of finished product and the reason for this behavior could be attributed to the effect of filleting, which may influence such aspects as waiting time for processing, irregularities in the cold chain, generation of hot spots, and of course, all those involved in food handling and hygienic characteristics of the plant.

Campos, Rodríguez, Losada, et al. (2004) obtained a pH value of 6.41 for fresh whole sardine (*Sardina pilchardus*) kept at low temperature by using flake ice. In this test, the values obtained for sardine were lower than those described in the above literature for whole fish. However, it should be taken into account that there are several factors that cause pH variability, especially in wild fish. In contrast, salmon pH values were similar to those described in the literature.

3.3. Control of Temperature

Table 3 shows the temperatures measured in the study both in raw material and finished product for both species. Because, raw salmon was made up of whole gutted specimens, weighing approximately 22 lb (9.9 kg) and with a length of about 50 cm., it was advisable to make a comparison of temperature measurements from the surface of the animal and the inside of its body. The values in ° C were converted to ° F to avoid the use of negative values in the statistical analysis.

There were highly significant differences ($p < 0.001$) for the temperature measurements on the surface (IN1 and IN2) and inside (IN1I and IN2I). In the same way, the results obtained by both methods, for batches with the same treatment (IN and OUT), also were highly significant different ($p < 0.001$). When comparing the inside temperature of the specimens IN1 I (-0.20 ° C) with that of IN2 I (0.4 ° C), it is clearly shown that the temperature of the last batch was higher. The same behavior was observed with surface temperature, but with higher values (= -0.52 ° C IN1 and IN2 = 1, 07 ° C). This was predictable, because the heat transfer takes place from the center of the animal to the surface by direct contact with ice; it is clear that skin is always cooler than the inside of the fish. Changes in the cold chain are more easily detectable on the surface of the fish than inside them.

From the perspective of quality control, the internal temperature measurement of the fish is an invasive technique that could break skin and muscle, and could become a vector for cross-contamination if hygienic conditions are not properly managed. However, it is useful as a confirmative method when there is suspicion of eventual rupture of the cold chain, or when there are highly variable values on the surface. The surface temperature measurement is fast, clean, non-invasive and allows distance measures. The effectiveness of this method depends on the proper use of the equipment and a suitable training of the analyst.

According to Table 3, the temperature of the finished product, in both studied species, exceeded the temperature established for the respective raw product. The highest recorded temperature for the salmon slices was 5.62 ° C, and for the filleted sardines reached 4.32 ° C. It is known that both the microbiological and enzymatic activities are highly influenced by temperature. However, in the temperature range from zero to 25 ° C, microbiological activity is relatively more important, and changes in temperature have a greater impact on microbiological growth than in the enzymatic activity (Huss, 1999).

Cann, Houston, Taylor, et al. (1984) established a decrease of 32.20% in the lifetime of salmon when the temperature increases by 3.8 ° C in the range of 0 to 5 ° C. If this value is compared with the average increment of 3.62 ° and 3.9 ° C for salmon and sardines respectively established in this case of study, it could be argued that variations in temperature within the company regarding technical, logistical, environmental reasons or other nature, are detrimental to the optimal quality of their finished products.

For a better understanding of the effect of storage conditions (temperature and atmosphere) in the microbiological quality of packed fish, it is necessary to evaluate different temperatures, this includes rigorous refrigeration and those temperatures present in a break of the cold chain. (Corbo, Altieri, Bevilacqua et al., 2005). Measuring the surface temperature could regulate the process and allow its characterization and control. That information could be used as well to apply the concept of Deterioration Relative Speed (DRS) that quantifies and mathematically describes the effect of temperature in the spoilage of fish product.(Huss, 1999).

3.4. Microbiological Assay

Table 4 shows the microbial count for both species, in raw material and finished product, which are within the specifications set by European legislation (Regulation-EC-2073 / 2005). The microbiological load of live fish is a reflection of the microflora in the environment at the time of the fishing, but is modified according to the ability of different microorganisms to grow in subenvironments such as the surface of the skin, gills and digestive tract. (ICSMF, 2001).

In the study, MTV count is a good indicator of the quality of the raw material in both species (see Fig.1). The low values found in whole and gutted salmon are in the order of 10^2 CFU.g^{-1} evidencing of good practice which was submitted at the farm, and the effectiveness of the cold chain in transport from the farm to the plant. These results match those obtained by Auburg, Quitral, Larrain, et al. (2007) in Coho salmon (*Oncorhynchus kisutch*), headless and gutted (HG) presentation, from fish farms, stored at ≤ 2 ° C, which did not show a significant increase in the count of MTV after 10 days of storage, exhibiting values around 10^2 CFU. g^{-1}.

In the case of the sardine, the raw material showed values of MTV (1.13×10^5 CFU.g^{-1}) much higher than those found in salmon probable due to filleting process of fish. Highlighting, that this species comes from sea fishing. In this way, the fish caught with nets, hooks and lines die quickly and the bacteria can penetrate into the tissues by puncture wounds and even eroded areas (scratches) produced during predeceased seizures; bacterial growth is rapid in these places increasing the initial count (ICSMF, 2001). The high counts of MTV for the raw material of sardine corresponded to the highest values of TVB-N detected in the study (29.6 mg. 100 g^{-1}) and this fact confirms the existence of a prominent microbiological activity. However, all counts never exceed a level of 10^7-10^8 CFU. g^{-1} when the fish was spoilt (Huss, 1999). This would correspond to the maximum limit of 5 log CFU. g^{-1} suggested by Department of Health and Children guidelines (Ireland) in 1992 (Fagan, Ronan & Mhuircheartaigh, 2002).

MTV count for the finished product of both fish were always much lower than initial counts of the raw material. It was observed a very marked decline in the sardine with a reduction of 3 log cycles, while the salmon was only down the number of microorganisms remaining in the same cycle (10^2). This decrease may be the result of effects such as temperature drop between 0 and -4 ° C caused by thermal shock, application of a modified atmosphere packaging, which leads to a bacterial transition using a sanitation agent like ice and / or ozonated water, which is effective suppressing part of the flora after application. In this sense, Campos, Rodríguez, Losada, et. al. (2004) showed that storage of sardine in an ice slurry on its own or in combination with ozone, would improve sensory, microbiological and biochemical quality.

The behavior of the *Enterobacteriaceae* (ET) was different for each species (Fig.1). Salmon count was low in the raw material ($2, 43 \times 10^2$) and decreased about 1 log cycle for the finished product showing values of 5×10 CFU. g^{-1} which are not very important. In the sardine, the ET value in the raw material was similar to the one obtained for salmon (2.63×10^2 CFU. g^{-1}), but the behavior was the opposite in the finished product, it means that the number of microorganisms increased. All these factors may increase health risks and lead to loss of quality. For these reasons, and as a security measure, a range between 10^2 and 10^3 CFU. g^{-1} of coliforms or enterobacteriaea should be considered as a limit of acceptance for fish (Corbo, Altieri, Bevilacqua, et al., 2005).

According to the above mentioned, sardines packed in modified atmosphere are between that interval, but they showed an increase of (3.8 x 10^2 CFU. $g^{-1)}$ from the input to output. This variation should be viewed with caution as fluctuations in temperature and atmospheric composition of the packed product, (if the fish is kept at a temperature below 4 °C), cause a prolonged latent phase which does not exceed 24 hours; it seems that the microorganisms do not multiply during this time and it can be seen that the best organoleptic conditions, creating a false impression that the microbiological risk is insignificant (Simeonidou, 1998; Corbo, Altieri, Bevilacqua, et al., 2005).

The behavior of *Pseudomonas spp.* (Ps) was very similar to MTV. In all cases, a decrease in the final count of finished products could be seen. The reason for this decline is also consistent with the factors previously considered for MTV. Finally the study for established presence/ausence of *Listeria monocyotgenes* was developed. In all species studied, regardless of being raw materials or finished products, indicated the absence in 25 g (<100 CFU.g^{-1}) of fish. This demonstrates the effectiveness of the appropriate "Standard Operating Procedures for Cleaning and Sanitation" in the plant.

Whereas the most common sources of contamination for fish products come from the environment; the microbiological quality of ice that came with the raw material was evaluated (sardine boxes and whole salmon). The salmon ice presented agglomeration and traces of blood, and the MTV counts were about 2.6 x 10^3 CFU.g^{-1} on both days of sampling. Meanwhile, flake ice used in the sardine protected by a plastic film, the MTV values were 1.5 x 10^2 CFU /g^{-1}. Based on the foregoing, the microbiological quality of ice in the raw materials was satisfactory. On the other hand, it was also evaluated in 2 different days, the quality of flake ice produced in the plant, which is used to cool both raw materials and finished product. In this case, the count was 2 x 10 CFU.g^{-1} resulting a satisfactory value. Both *Pseudomonas* and *Listeria* count in the ice cubes tested were <100 CFU.g^{-1}.

3.5. Sensory Evaluation

The results obtained for the sensory evaluation of the expert panel are shown in Table 5. The sensory method is widely used in the fishing industry to assess fish freshness. In Europe, it is employed by the services of inspection and the aquaculture product processing industry. It is based on Regulation (EC) 2406/96 that controls the changes taking place in the organoleptic characteristics of the various fish products. However, this assessment is based on very general parameters which have been questioned by its their lack of specificity, making it only possible to classify the degree of fish freshness in the categories of Extra, A, B and Rejected. This does not allow the discrimination of levels of quality within each classification.

All evaluations of raw materials and finished products with a freshness index greater than 88% are considered as very satisfactory (Fig.2), but the scale used in this research (Real Decreto 331/1999) is unable to discriminate. According to the latter, the values in all cases correspond to a category A, because they all exceeded 75% in the estimation of freshness. However, by assigning numerical values to each attribute of freshness, more flexible and real data can be obtained which would make possible to classify fish as of Extra quality. Fig.2b shows how the assessments exceed considerably the value assigned to the quality of A, approaching in most cases to 90%, such product would correspond to extra quality. In this sense, the worst score of the group was the raw material for salmon on the first day (IN1), while for sardine, it was the raw material on day 2 (IN2). Both cases had in common a time of waiting before processing > 24 h but ≤ 48 hours from the time of arrival at the plant. The rest of the raw materials were evaluated just when arriving at the plant (<24 hours of reception).

Simeonidou, Govaris & Vareltzis (1998) used the European regulation freshness index to evaluate sardines, mackerel and hake. These authors adjusted the classification scheme of the EC describing the product among the following criteria:

excellent quality (perfect condition), high quality (slight loss of excellent characteristics), good quality (some deterioration, but suitable for sale) and not suitable for sale; giving the names of the letters E, A, B and C respectively; and the values 4, 3, 2 and 1 degrees to the same scale. In the study, the whole sardine (*Sardine mediterraneus*) kept on ice (1 - 3 ° C) had a freshness index of 85% at day 0 and 40% after 3 days. In comparison to the values mentioned in the previous study, batches of raw material and finished products showed excellent sensory quality.

Some researchers emphasize the importance of using all the categories when applying the scale of the EC, because the omission of any could be interpreted as a form of manipulation. However, the EC scale was devised to assess whole fish, mainly in the first selling, and if some of the standards are not included (because of the nature of the evaluated material), this does not mean an improper handling of data. In this sense, Tzicas, Ambrosiadis, Soultos, et al. (2006) evaluated the freshness of yellow jack kept on ice, using a panel of experts and applying the EC scale, but omitting an aspect concerning the presence of mucus on the surface of the fish skin, because it is known that the frequent ice renewal reduces it, without compromising the quality.

The best freshness estimated in finished products (salmon and sardines OUT1) matched the values attributed to raw material (salmon and sardines IN1), making clear, once again, the close relationship between quality of raw material and quality of the finished product under standard and control conditions of manufacturing.

The allocation assigned by the assessors for each category varied depending on the food tested. The Fig. 2 shows the graphs of the various sensory attributes evaluated for the different treatments. The best aspect was always the brightness of the skin (alive and iridescent pigmentation). The panel used, consisting of experts trained in the use of CE scale, proved consistency (coefficient of variation-CV-0.4 and 0.2 salmon sardines). Taking into account that the physico-chemical values obtained and microbiological count matched and confirmed the weighing for the sensory quality of the fish analyzed; so it can be said that the company's products have a high degree of freshness which in some cases could be considered as optimal.

3.6. Global Analysis of the Cold Chain

The Figure 3 shows the plotter made by PCA for salmon that explained 84.78% of the variability of the results within the first two axes. The variables pH, Ps and ET display a similar behavior, taking into account that their highest values were in the batches of raw materials. Presumably, *Pseudomonas* are present in greater amounts, because they make up an important part of the fresh fish flora. In the same way, a pH near neutral (pH = 7), normal value in a live fish, represents a better freshness, in this case for both batches of raw material (IN1 and IN2) was around a value of 6.9. Fish handling, even under the best sanitary conditions, leads to an increase in the microbiological contamination from the environment, materials and / or handlers. Considering this, ET moderate count is predictable and should not cause any alert. The PCA highlights the homogeneity of the raw material for salmon perhaps as a result of the production method (fish farming), and the direct chain supply.

Regarding the salmon fillets, the model indicated a clear difference between both studied groups; OUT2 presented the poorest conditions, with the highest temperatures detected in the research, and therefore a greater amount of non-protein N_2 that comes from volatile bases. The bacteria are responsible for the high increase of TVB-N but to a large extent, they did not exhibit higher counts. Nevertheless, this could be a result of packing of the slices using an agent of sanitation during the manufacturing process. Finally, the best rated batch was OUT2 by the trained panel, presenting moderate values of TVB-N and pH, and a surface product temperature of nearly 2 °C.

In summary, the PCA for salmon showed a strong influence of the cold chain in the plant over the values of TVB-N and freshness of the finished products. Establishing a high homogeneity for the raw material with satisfactory microbiological and physicochemical values and a high degree of freshness.

The analysis of PCA to sardine (Figure 4) was unlike salmon, this analysis explained 91.11% of the variability presented a considerable heterogeneity between treatments. Related to the raw material it can be seen that the batches IN1 and IN2 are clearly differentiated. In the first case (IN1), it could be noticed a high MTV and Ps count. However, it was the best qualified in terms of freshness. All sardine fillets kept in boxes with ice (IN1 and IN2), had a higher microbiological count than those found in salmon, but it was still below the established limit. Any unpleasant characteristics were seen in fish that could restrict the sale, and it also showed the lowest surface temperature (-1.04 ° C). The high count could be a consequence of fresh fish characteristic flora (*Pseudomonas spp*.) and also other microorganisms associated with pollution from the environment and transport to the plant, which according to the data of temperature registered, is done properly.

It was different in the case of IN2, since pH was the variable with the highest value (6.11) for all the treatments. The microbial count and quantities of non- protein N_2 were lower than IN1. Despite this, freshness proved to be the lowest of all batches. The reason could be the absence of mucus on the surface (see Fig. 2) due storing fish with a surplus of ice and replenishing melted ice daily has been observed to result in a lack of slime on the skin. (Tzicas, Ambrosiadis, Soultos, et al., 2006), the presence of an odor associated with oxidative rancidness (autolytic decomposition) was a parameter not considered in this study and finally to physical damage suffered by the sardines probably because of the mechanical action of the filleting process.

The finished product of sardines showed heterogeneity. In the treatment OUT1, the highest count of ET was detected in the research, and a high content of TVB-N (28.58 $mg.100g^{-1}$) too. However, the evaluation of freshness was one of the best (91. 07%), giving important relevance to the skin brightness and the presence of superficial mucus. The high values of ET count could come from an inefficient fish evisceration. OUT2 showed a very high surface temperature (7. 21 °C) and one of the highest values of pH (6.08) but the microbial count were low. PCA always represented batches of raw materials such as those with lower temperatures but higher count as far as MTV is concerned. On the other hand, the finished product batches were associated with low MTV, but very high temperatures and pH. In this case, the freshness sensory evaluation showed questionable results; and the rest of microbiological counts (PS and TS) presented very similar behavior for each treatment.

Overall, the PCA applied to sardines showed an evident heterogeneity for all batches studied both raw material and finished product; and illustrated significant differences between batches of different sampling days corresponding to the same treatment (IN / OUT). Then, the fact that the raw material used in the case of sardine is an industrial product, provided by suppliers, is a factor that affects the quality of final manufactured product by the company. That goes beyond a proper cold chain, depending on other external and internal factors that require further analysis.

4. Conclusions

After obtaining the results of TVB-N, it could be stated that raw material and finished products are below the limits set by European legislation. The amount of non-protein nitrogen is influenced by the quality of the raw material, the speed with the process is carried out and the uniformity of temperature in the cold chain. Satisfactory pH values were detected in raw material and finished product for both species of fish. The study shows that the final value of pH in the finished product of sardine depends more on the quality of the raw material than the effect of processing in the company as long as they have a good manufacturing practice and adequate cold chain. In all species studied, the surface temperature of the finished product exceeded the set temperature for the respective commodity. In this sense the temperature range of variation for the process is estimated in 6.1 °C (from -0.52 ° C to 5.6 ° C). All microbiological count established for both fish species complied with the legal specifications set and had a high degree of freshness (about 90%) The PCA for salmon evidenced an enormous influence of the cold chain in the company on the values of TVB-N and freshness of the finished products. It was also established a great homogeneity in the raw material with consistent and satisfactory microbiological and physical-chemical values, and a high degree of freshness. The PCA for sardine showed a clear heterogeneity for all batches tested both raw material and finished product. This variation may be a result of the raw material manufacturing process prior to being submitted to an adequate cold chain. However, it is important to note that the values of physic-chemical and microbiological analysis were satisfactory and freshness quite good.

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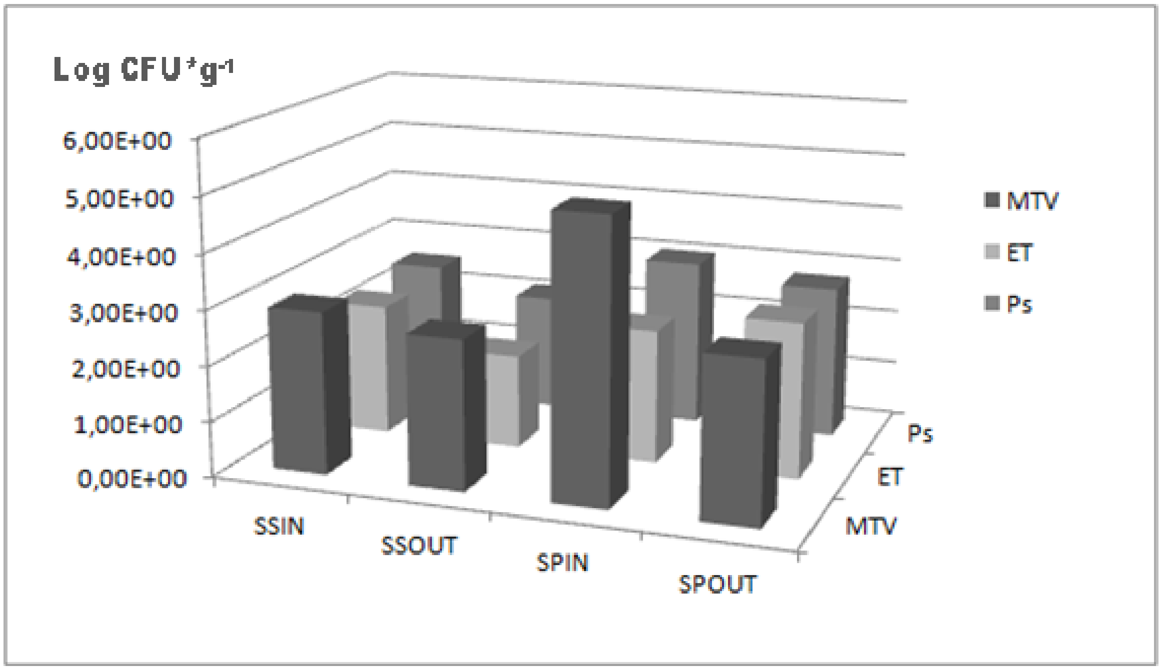

SSIN: RAW MATERIAL (SALMON) SSOUT: PRODUCT (SALMON) SPIN: RAW MATERIAL (SARDINE) SPOUT: PRODUCT (SARDINE) MTV: Total Mesophilic ET: Enterobacteria Ps: Pseudomonas

Figure 1

Logarithms of the general count obtained for salmon (Salmo salar) and sardine (Sardina pilchardus) in raw material and finished product.

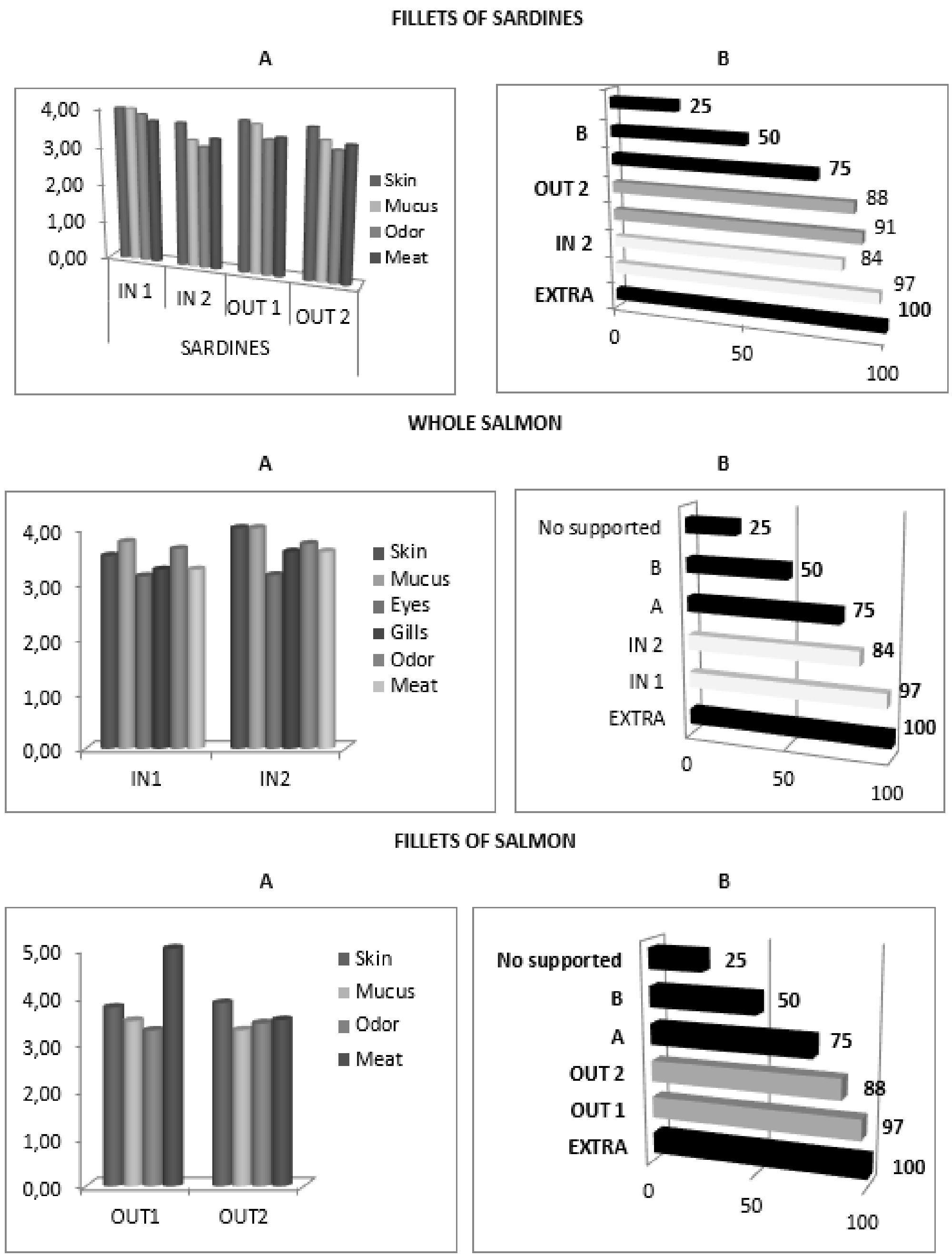

Figure 2

Sensory evaluation made for expert panel to sardine (*Sardina pilchardus*) and salmon (*Salmo salar*).

A: weighting aspect evaluated and B: % of freshness for treatment

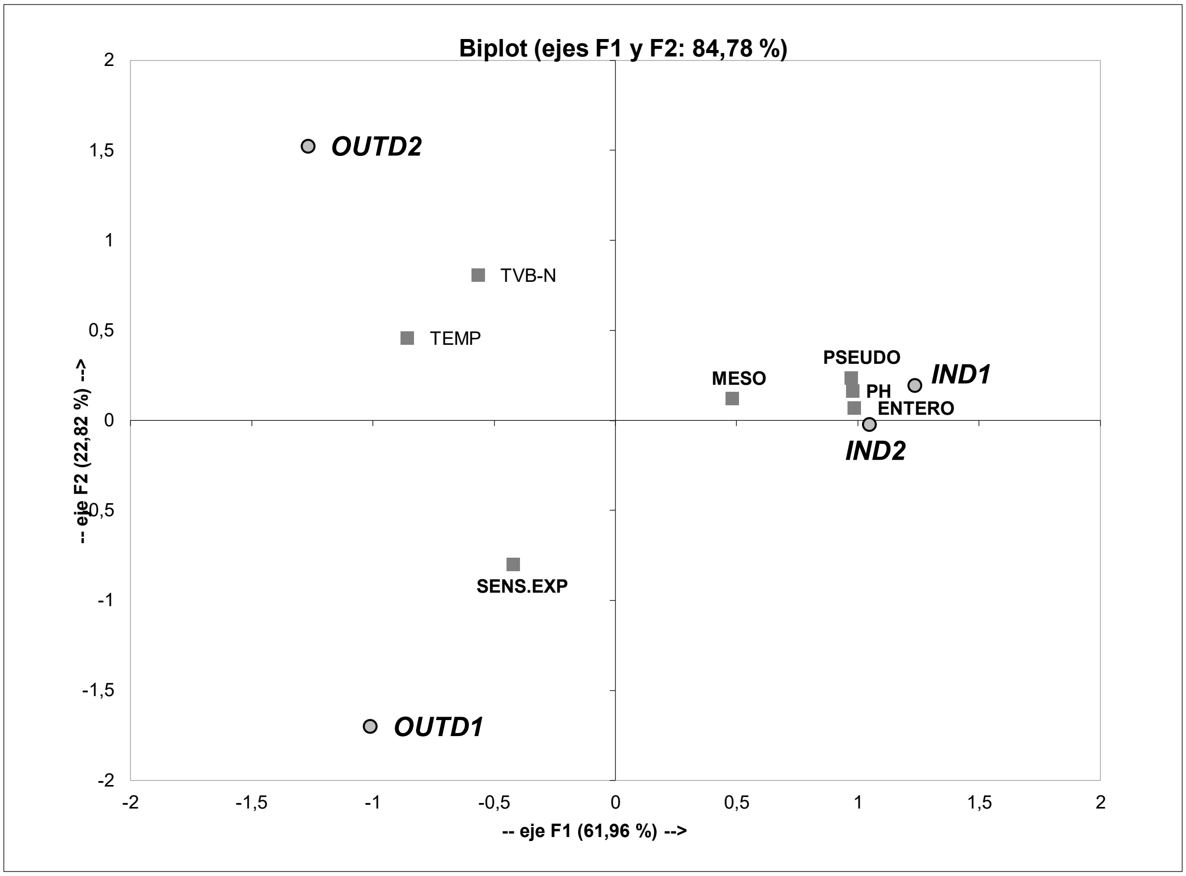

Figure 3

Graphical representation (plotter) of variables for the salmon (*Salmo salar*) showed by PCA

TVB-N = N_2 non protein, TEMP= internal temperature, MESO = mesophiles, PSEUDO = *pseudomonas sp.,* ENTERO = *enterobacteriaceae*, PH = pH and SENS. EXP = Sensorial analysis

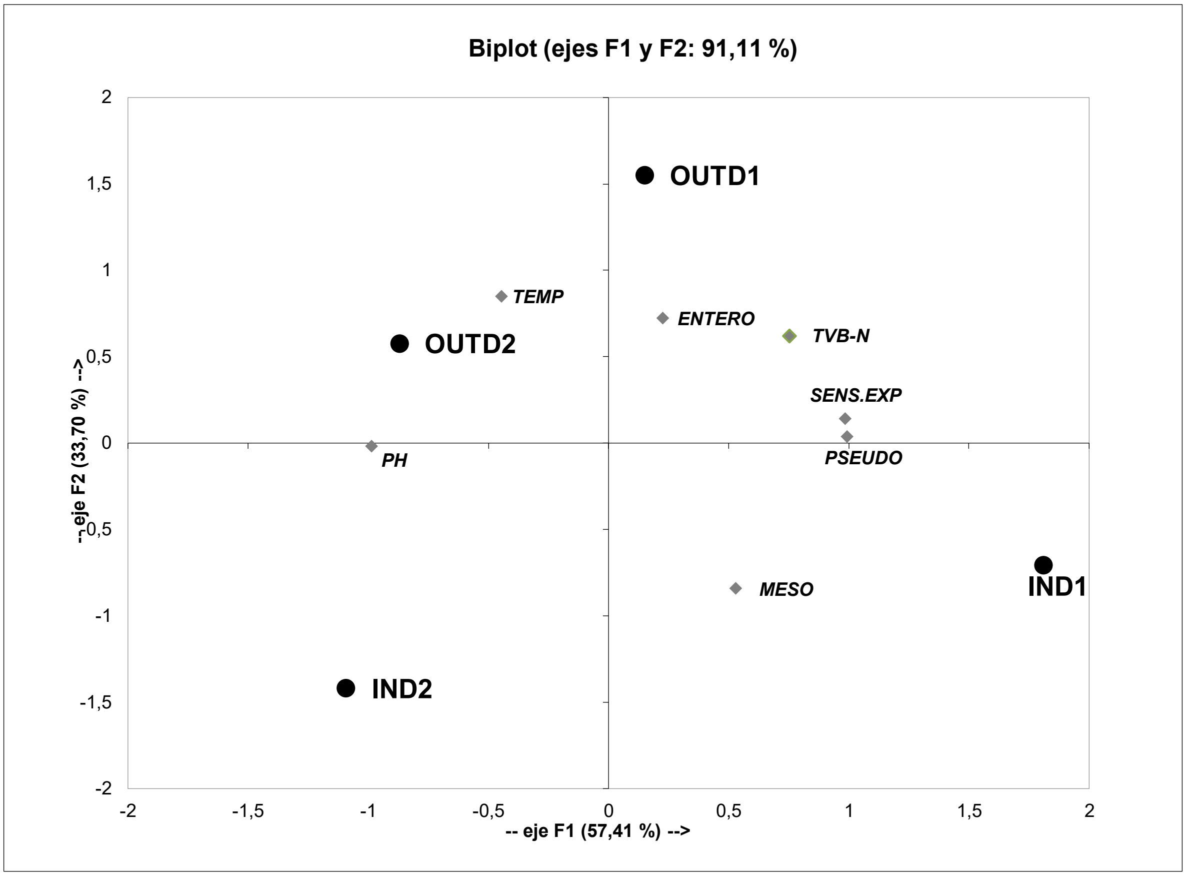

Figure 4

Graphical representation (plotter) of variables for the sardine (Sardina pilchardus) showed by PCA.

NBVT = N2 non protein, TEMP= internal temperature, MESO = mesophiles, PSEUDO = pseudomonas sp., ENTERO = *enterobacteriaceae*, PH = pH and SENS. EXP = Sensorial analysis

Table 1

TVB-N values (mg/100 g food) for salmon and sardines during the study.

	SALMON (*Salmo salar*)							
	IN1		IN2		OUT1		OUT2	
n	10		10		10		10	
	m	***s***	***m***	***s***	***m***	***s***	***m***	***s***
	19.01	2.66	20.93	5.65	18.94	3.46	29.88	4.17
	a		a		a		b	
	***		***		***		***	

	SARDINE (*Sardina pilchardus*)							
	IN1		IN2		OUT1		OUT2	
n	10		10		10		10	
	m	***s***	***m***	***s***	***m***	***s***	***m***	***s***
	14.87	3.51	29.60	5.30	24.87	3.94	28.58	4.92
	a		c		b		bc	
	***		***		***		***	

IN indicates the raw material while OUT makes for Finished Goods, the numbers 1 and 2 indicate different days and sample plots

ns: no significant difference, * = p <0.05, ** = p <0.01, *** = p <0.001.

Different letters denote differences with the significance level indicated by the asterisk

Table 2

PH values obtained by different methods for raw materials and finished products from salmon (Salmo salar) and sardine (Sardina pilchardus).

	SALMON			
METHODS	**PUNCTURE**		**HOMOGENIZATION**	
TREATMENT	**IN1 P**	**IN2 P**	**IN1**	**IN2**
n	10	10	10	10
m	6.62	6.69	6.99	6.85
s	0.15	0.09	0.22	0.15
Intratreatment	***ns***		***ns***	
n	20		20	
m	6.65		6.92	
s	0.13		0.20	
Intertreatment	*** a		*** b	
SALMON				
METHOD	**HOMOGENIZATION**			
TREATMENT	**IN1**	**IN2**	**OUT 1**	**OUT2**
n	10	10	10	10
m	6.99	6.85	6.38	6.44
s	0.22	0.15	0.12	0.14
Intratreatment	***ns***		***ns***	
n	20		20	
m	6.92		6.41	
s	0.21		0.13	
Intertreatment	*** b		*** c	
SARDINE				
METHOD	**HOMOGENIZATION**			
TREATMENT	**IN1**	**IN2**	**OUT1**	**OUT2**
n	10	10	10	10
m	5.97	6.11	6.06	6.08
s	0.05	0.10	0.02	0.11
Intertreatment	** a	** b	** b	** b

IN indicates the raw material while OUT makes for Finished Goods, INP denotes results obtained by puncture, and the numbers 1 and 2 indicate different days and sample plots

ns: no significant difference, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Different letters denote differences with the significance level indicated by the asterisk

Table 3

Temperature values (°F) obtained by different methods for raw materials and finished products from salmon (*Salmo salar*) and sardine (*Sardina pilchardus*)

	SALMON			
TEMPERATURE	**INTERNAL**		**SURFACE**	
TREATMENT	**IN1 I**	**IN2 I**	**IN1**	**IN1**
n	10	10	10	10
m	31.64	32.74	31.07	33.92
s	0.21	0.55	0.57	0.92
	***	***	***	***
	a	b	c	d
***m* (°C)**	*-0.20*	*0.41*	*-0.52*	*1.07*
	SALMON			
TEMPERATURE	**SURFACE**			
TREATMENT	**IN1 I**	**IN2 I**	**OUT1**	**OUT2**
n	10	10	10	10
m	31.07	33.92	35.95	42.12
s	0.57	0.92	1.43	1.34
	***	***	***	***
	c	d	e	f
***m* (°C)**	*-0.52*	*1.07*	*2.19*	*5.62*
	SARDINE			
TEMPERATURE	**SURFACE**			
TREATMENT	**IN1 I**	**IN2 I**	**OUT1**	**OUT2**
n	10	10	10	10
m	31.51	32.50	38.27	39.77
s	1.01	0.30	0.67	0.67
	***	***	***	***
	a	b	c	d
***m* (°C)**	*-0.27*	*0.28*	*3.49*	*4.32*

IN indicates the raw material while OUT makes for Finished Goods, IN I denotes results of internal temperature, numbers 1 and 2 indicate different days and lots of sampling.

*ns: no significant difference, * = p <0.05, ** = p <0.01, *** = p <0.001.*

Different letters denote differences with the significance level indicated by the asterisk

Table 4

Comparative table between the microbiological results obtained for salmon (Salmo salar) and sardines (Sardina pilchardus), and the parameters set out in Regulation (EC) 2073/2005.

	Aerobic Mesophillic (CFU*g^{-1})	*Enterobacteriaceae* (CFU*g^{-1})	*L. monocytogenes* (CFU*g^{-1})	*Pseudomonas spp.* (CFU*g^{-1})
REGULATION 2073/2005	**1.00E+06**	**1.00E+03**	**<100 cfu / g absence in 25g (bivalves and other marine products)**	**not specified**
Whole Salmon (*Salmo salar*)	8.70E+02	2.43E+02	<100	3.45E+02
Salmon fillets packaged	5.10E+02	5.00E+01	<100	1.30E+02
Sardine fillets (*Sardina pilchardus*) in flake ice boxes	1.13E+05	2.63E+02	<100	3.45E+02
Sardine fillets packaged	7.66E+02	6.42E+02	<100	5.97E+02

Table 5

Results obtained by sensory analysis for salmon (*Salmo salar*) and sardine (*Sardina pilchardus*) using freshness scale of the Council Regulation (EC) Nº2406/1996.

	SALMON			
	IN1	IN2	OUT1	OUT2
Skin	4.0	3.50	3.76	3.86
Mucus	4.0	3.75	3.48	3.29
Eyes	3.14	3.13	NE	NE
Gills	3.57	3.25	NE	NE
Odor	3.71	3.63	3.28	3.43
Met	3.57	3.25	5.00	3.50
m	*3.67*	*3.42*	*3.88*	*3.52*
s	*0.29*	*0.22*	*0.67*	*0.21*
% Freshness	***91.67***	***85.42***	***97.00***	***87.95***
	88.54		***92.47***	

	SARDINES			
	IN 1	***IN 2***	***OUT 1***	***OUT 2***
Skin	4.00	3.71	3.86	3.79
Mucus	4.00	3.29	3.79	3.50
Odor	3.86	3.14	3.43	3.29
Meat	3.71	3.36	3.50	3.43
m	*3.89*	*3.38*	*3.64*	*3.50*
s	*0.12*	*0.21*	*0.18*	*0.18*
% Freshness	***97.32***	***84.38***	***91.07***	***87.56***
	90.85		***89.32***	

	ítems evaluados			
	4		**6**	
CATEGORY	Weighting	%	weighting	%
optimal	*16*	100	24	100
A	*≥ 12*	75	≥ 18	75
B	*≥ 8*	50	≥ 12	50
No Accepted	*4*	25	6	25

IN indicates a Raw Material while OUT makes for Finished Goods, The numbers 1 and 2 indicate different days a sample plots, NE = No evaluate