Running title: Improved quality of ray stored in slurry ice

Title: Evaluation of a slurry ice system for the commercialization of ray (*Raja clavata*): Effects on spoilage mechanisms directly affecting quality loss and shelf-life

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Abstract

The application of slurry ice, a biphasic system formed by small spherical ice crystals surrounded by seawater at subzero temperature, was evaluated as a new storage method for ray (*Raja clavata*), the elasmobranchious fish species that exhibits highest commercial value in the European food markets. This advanced technique was performed in parallel with respect to a control batch stored 10 days in flake ice for comparison purposes. The results obtained in the sensory analysis indicated a significant extension of the overall quality (A class fish) from three days (flake ice) to six days (slurry ice). The development of ammonia external odour was the limiting parameter in both batches and was correlated with the activity of the endogenous mechanisms involved in the degradation of proteins and non-protein-nitrogen (NPN) rather than with the activity of proteolytic microorganisms. Storage of ray in slurry ice significantly ($P<0.05$) slowed down both biochemical (as estimated by the follow-up of the pH, TVB-N and K value evolution) and microbial degradation mechanisms (estimated by the development of psychrotrophes and mesophiles counts) in chilled ray muscle. According to the parameters evaluated, storage of ray in slurry ice extends the shelf-life of this elasmobranchious fish species due to a better maintenance of sensory, biochemical and microbiological quality, this facilitating its commercialization.

Keywords: Ray; Elasmobranchious fish; Chilled storage; Slurry ice; Shelf-life; Quality loss.

1. Introduction

Aquatic food products suffer a rapid quality loss *post-mortem* due to a wide number of biochemical and microbial breakdown mechanisms. The slowing down of such mechanisms requires refrigeration immediately after the capture. In this sense, aquatic food products have traditionally been cooled and stored by means of flake ice (Nunes, Batista & Morão de Campos, 1992) or refrigerated seawater (Kraus, 1992). Recently the introduction of ice slurries has provided several advantages for the preservation of aquatic food products in this type of ice-water suspensions at subzero temperature (for a review: Piñeiro, Barros-Velázquez & Aubourg, 2004). Slurry ice is a biphasic system consisting of spherical and microscopic ice crystals dispersed in refrigerated marine water that allows faster chilling rates due to its higher heat-exchange capacity, and a reduced physical damage to fish structures as compared with the aciculate crystals of flake ice. The complete coverage of the fish surface by the slurry ice mixture also allows a better protection of the fish material with respect to oxidation and dehydration events (Piñeiro, Barros-Velázquez & Aubourg, 2004).

Since Chapman (1990) reported a better maintenance of quality of finfish stored on-board in slurry ice as compared with other chilling technologies, this technique has been successfully applied to fatty fish species such as tuna (Price, Melvin & Bell, 1991) or sardine (Campos, Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005; Losada, Barros-Velázquez, Gallardo & Aubourg, 2004), and to lean fish species such as seabass (Martinsdóttir, Valdimarsdóttir, Porkelsdóttir, Olafsdóttir & Tryggvadóttir, 2002), hake (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2004; Losada, Piñeiro, Barros-Velázquez & Aubourg, 2004) or turbot (Rodríguez, Barros-Velázquez, Piñeiro, Gallardo & Aubourg, 2006). The practical advantages of slurry ice for the storage of Australian prawns (Chinivasagam, Bremner, Wood & Nottingham, 1998), or shrimp stored on-board (Huidobro, López-Caballero & Mendes, 2002) have also been reported. More recently, a slurry ice system and its combination with an antimelanotic agent have been evaluated for the storage of crustaceans (Losada, Rodríguez, Miranda, Barros-Velázquez & Aubourg, 2006; Aubourg, Losada, Prado, Miranda & Barros-Velázquez, 2007).

All such previous studies have confirmed that the rate of quality loss depends not only on the handling and storage conditions used but also on the nature of the fish species in question (Whittle, Hardy & Hobbs, 1990; Olafsdóttir, Martinsdóttir, Oehlenschläger, Dalgaard, Jensen, Undeland, Mackie, Henahan, Nielsen & Nilsen, 1997). In Spain, and indeed in other European countries, ray (*Raja clavata*) represents the most commercialized elasmobranchious fish species, mainly caught either at the European nearby coast and at the Gran Sol fishing bank (Xunta de Galicia, 2001; FAO, 2006). Thus, from the commercial point of view, ray specimens deserve considerable appreciation as a fresh consumable product because of its delicate and excellent sensory features (Pastoriza and Sampedro, 1994). However, sometimes the capture of ray in distant fishing banks usually means that the time elapsed between the catch and arrival at its destination may vary from one to eight days, underlining the need to optimise refrigeration parameters in order to provide consumers with fish of the highest quality possible. Moreover, the important mechanisms of ammonia generation occurring in ray muscle and mainly derived from the accelerated degradation of proteins and non-protein-nitrogen may limit the commercialization period of this fish species (Vyncke, 1978; Finne, 1992) as a refrigerated product. Consequently, in this study we evaluated the effects of chilling ray in slurry ice on its shelf-life determined by means of sensory,

biochemical and microbiological analyses. The results were compared with those determined in parallel in a batch stored in flake ice for 10 days.

2. Materials and methods

2.1. Slurry ice system used

The slurry ice prototype (FLO-ICE, Kinarca S.A.U., Vigo, Spain) used in this study produced a slurry ice binary mixture consisting of 40% ice and 60% water, from filtered seawater (salinity: 3.3%). The temperature of the slurry ice was -1.5°C. Flake ice was prepared with an Icematic F100 Compact device (CASTELMAC SPA, Castelfranco, Italy). The ray specimens were placed in either slurry or flake ice at a fish:ice ratio of 1:1, and stored for up to 10 days in a refrigerated room at +2°C. When required, the flake ice and the slurry ice mixture were renewed.

2.2. Fish material, processing and sampling

Specimens of ray (*Raja clavata*) were purchased the day they were caught from a local market in Vigo (Northwestern Spain) and kept under refrigeration for 1 h until arrival at our laboratory. The fish specimens were neither headed nor gutted. The length of the fish was in the 50–60 cm range and their weight was 700–900 g. The specimens were either chilled in slurry ice or in flake ice. The temperature of the ray specimens stored in slurry ice was in the range of -0.5°C to -1.0°C, while that of the counterpart specimens stored in flake ice was in the range of +0.5°C to +1.0°C. Whole specimens were taken from each batch on days 0, 3, 6, 8 and 10. Once the intact specimens had been subjected to sensory analyses, the white muscle was aseptically dissected and used for microbiological and chemical analyses. All analyses were performed by triplicate.

2.3. Sensory analyses

Sensory analysis was performed by a panel formed by five experienced judges, according to official guidelines (Table 1) concerning fresh and refrigerated elasmobranchious fish (DOCE, 1989). Four categories were ranked: highest quality (E), good quality (A), fair quality (B), and unacceptable quality (C). Sensory evaluation of the ray specimens included the following parameters: exam of skin, eyes and gills, flesh consistency and ventral side aspect, and external odour.

2.4. Microbiological analyses

Samples of 25 g of white muscle were dissected aseptically from chilled ray specimens, mixed with 225 ml of 0.1% peptone water, and homogenized in a stomacher (Seward Medical, London, UK) as previously described (Ben-Gigirey, Vieites Baptista de Sousa, Villa & Barros-Velázquez, 1998, 1999). In all cases, serial dilutions from the microbial extracts were prepared in 0.1% peptone water (Oxoid Ltd., London, UK). Total aerobes were investigated by surface inoculation in plate count agar (PCA, Oxoid) after incubation at 30°C for 72 h. Psychrotrophes were investigated in the same manner but incubation was carried out at 4°C for 7 days. Microorganisms exhibiting a proteolytic phenotype were investigated in casein-agar medium (30°C/48 h) (Phaff,

Starmer, Lachance & Ganter, 1994), as previously described (Ben-Gigirey, Vieites Baptista de Sousa, Villa and Barros-Velázquez, 2000).

2.5. Biochemical analyses

The evolution of pH values in ray muscle along storage time was determined by means of a 6-mm diameter insertion electrode (Crison, Barcelona, Spain).

Total volatile base-nitrogen (TVB-N) values were measured as previously reported (Aubourg, Sotelo & Gallardo, 1997). Briefly, fish muscle (10 g) was extracted with 6% perchloric acid and brought up to 50 ml –the TVB-N content being determined, following steam-distillation of the acid extracts rendered alkaline to pH 13 with 20% NaOH– by titration of the distillate with 10 mM HCl. The results were expressed as mg TVB-N/100 g muscle.

Nucleotide extracts were prepared according to the method of Ryder (1985) and were stored at -30°C until analysis. Nucleotide analysis was performed by HPLC using a Beckman device provided with the programmable solvent module 126 (Beckman), and the scanning detector module 167 (Beckman) connected to System Gold software, version 8.1 (Beckman). Separations were accomplished on a reverse-phase Spherisorb ODS-2 C₁₈ 250 x 4.60 mm column (Waters, Milford, MA), with an internal particle diameter of 5 µm. The composition of the mobile phase was as follows: solvent A was composed of 0.04 M KH₂PO₄ + 0.006 M K₂HPO₄, pH 7; solvent B was acetonitrile. The solvents were filtered through a 0.45 µm aqueous filter before use. Separations were carried out using a continuous gradient elution. The eluent was monitored at 254 nm and the running time was 10 min. Standard curves for adenosine 5'-triphosphate (ATP) and each compound involved in its degradation pathway [adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (Ino) and hypoxanthine, (Hx)] were constructed in the 0 to 1 mM range. All nucleotide standards were obtained from the Sigma Chemical Co. (St. Louis, MO). The widely used K value was calculated according to the following concentrations ratio: K value = 100 x (Hx + Ino)/(ATP + ADP + AMP + IMP + Ino + Hx).

2.6. Statistical analyses

Bacterial counts were transformed into log CFU/g before undergoing statistical analysis. The SPSS 11.5 for Windows software (SPSS Inc., Chicago, IL) was used to explore the statistical significance of the results obtained, this including multivariate contrasts and multiple comparisons by the Tukey test. A confidence interval at the 95% level ($P < 0.05$) was considered in all cases.

3. Results and discussion

3.1. Sensory analyses

The ray specimens stored in slurry ice exhibited overall good quality, being classified in the A category, up to day 6 (Table 1). After this sampling time, quality sharply decreased and on day 8 and 10 the ray specimens stored in slurry ice did not show acceptable quality. Remarkably, the main negative parameter linked to quality loss in the slurry ice batch corresponded to the external odour. The parameters that

deserved the best scores in the ray specimens stored in slurry ice were the aspect of skin and gills as well as the flesh consistency and ventral side aspect.

On contrary, the overall A category of sensory quality was only maintained in the ray specimens stored in flake ice only during the first three days of storage, such ray specimens being rejected on day six (Table 2). The most relevant differences with respect to the slurry ice batch were observed in the flesh consistency and ventral side aspect, while the strong external ammonia odour also revealed as the limiting factor of acceptability in this batch. A detailed comparison between batches as regards their sensory quality allows concluding that ray exhibits a very limited shelf life, although this time was doubled as a consequence of processing in slurry ice. The slowing down of mechanisms involved in the formation of ammonia odour was the most remarkable advantage of storing ray in slurry ice. Such undesirable odours are associated to the degradation of proteins and non-protein-nitrogen (NPN) compounds (Read, 1968; Finne, 1992).

According to the results shown in this work, storage of ray in slurry ice allowed a significant extension of its shelf-life, confirming the results obtained with this refrigeration system with other aquatic food products such as turbot (Rodríguez, Barros-Velázquez, Piñeiro, Gallardo & Aubourg, 2006), hake (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2004), horse mackerel (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005), sardine (Campos, Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005) and shrimp (Huidobro, López-Caballero & Mendes, 2002; Losada, Rodríguez, Miranda, Barros-Velázquez & Aubourg, 2006).

3.2. Microbiological analyses

Table 3 compiles the most remarkable results concerning microbial growth in the muscle of ray specimens stored either in either slurry ice or flake ice. Statistically significant ($P < 0.05$) differences were observed at several sampling times between both batches for aerobic mesophiles and for psychrotrophic microorganisms.

In the case of the counts of total aerobic mesophiles, the average difference between batches was 0.62 log units throughout storage. This difference was higher at advanced storage periods (day 8 and onwards), reaching a maximum value of 1.39 log units after 10 days of storage (Table 3). Although the total bacterial counts did not reach levels of 10^7 CFU/g, which are the minimum considered to be required for the spoilage of fish stored aerobically, storage of ray in slurry ice elicited a significant reduction. These results, to our knowledge the first referred to an elasmobranchious fish species, are quite in agreement with previous results obtained for lean fish species such as turbot (Rodríguez, Barros-Velázquez, Piñeiro, Gallardo & Aubourg, 2006), medium-fat fish such as horse mackerel (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005), and crustaceans such as shrimp (Losada, Rodríguez, Miranda, Barros-Velázquez & Aubourg, 2006) stored in slurry ice as compared with counterpart batches stored in flake ice.

In the case of psychrotrophes, the average difference between batches along storage was 0.49 log units. As in the case of the aerobic mesophiles, the difference between batches increased as the storage period progressed (day 6 and onwards), reaching a maximum value of 1.01 log units after 10 days of storage (Table 3). The psychrotrophes counts reached levels slightly above 10^7 CFU/g only in the flake ice batch, although this corresponded to a sampling time in which the sensory analyses had concluded the unacceptability of the batch. The counts determined for the psychrotrophes confirmed a more limited microbial growth in the slurry ice batch than in the flake ice batch,

probably derived from the sub-zero temperature of storage, characteristic of the former refrigeration system. Interestingly, the differences observed in the psychrotrophes numbers were not as high as those observed for fatty acid fish species (Campos, Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005), and were more in agreement with those determined for lean fish species such as turbot (Rodríguez, Barros-Velázquez, Piñeiro, Gallardo & Aubourg, 2006) or crustaceans (Losada, Rodríguez, Miranda, Barros-Velázquez & Aubourg, 2006).

Microbial metabolites such as peptides or amino acids, derived from protein hydrolysis, contribute significantly to undesirable sensory changes in seafood products. Thus, undesirable modifications in odour, texture and appearance lead to the spoilage of seafood products (Shewan, 1977; Makarios-Laham & Lee, 1993; Rodríguez, Barros-Velázquez, Ojea, Piñeiro & Aubourg, 2003). Moreover, ray specimens exhibit a rapid *post-mortem* quality loss mainly derived from the activity of endogenous and microbial enzymes able to degrade proteins and NPN compounds (Vyncke, 1978; Finne, 1992), an undesirable event that limits the commercialization time of this elasmobranchious fish species. Accordingly, we investigated the evolution of proteolytic bacteria in the ray batches stored in slurry or flake ice, respectively. As can be observed in Table 3, statistically significant ($P<0.05$) lower counts of proteolytic bacteria were observed in the slurry ice batch than in the flake ice batch only after 10 days of storage. However, the counts of proteolytic bacteria in ray muscle did not reach levels of 10^4 CFU/g in both batches, this indicating a very limited activity of proteolytic bacteria in ray muscle. These results do not follow the pattern observed for other fish species such as hake (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2004), sardine (Campos, Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005), horse mackerel (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005) or turbot (Rodríguez, Barros-Velázquez, Piñeiro, Gallardo & Aubourg, 2006), cases where significant contributions of proteolytic bacteria to fish muscle spoilage were observed in the flake ice batches. Thus, the results presented in this study allows concluding that the contribution of proteolytic bacteria to the degradation of ray muscle seems to be very limited, and that the formation of ammonia compounds would rather be caused by a strong activity of endogenous nitrogen-degrading enzymes present in ray muscle.

3.3. Chemical analyses

Increases in pH indicate the accumulation of alkaline compounds by means of autolytic and microbial mechanisms (Hebard, Flick & Martin, 1982). In our study on pH, statistically significant ($P<0.05$) differences were determined between both batches at all sampling times (Table 4). In this sense, the formation of urea, ammonia compounds and other alkaline metabolites provoked an important pH increase in the ray batch stored in flake ice, this leading to pH values as high as 9.0 after 10 days of storage (Table 4). On the other hand, the ray batch stored in slurry ice exhibited significantly lower pH values.

Interestingly, the pH value increases determined in muscle of ray specimens stored in slurry ice were far above those determined in other species such as turbot (Rodríguez, Barros-Velázquez, Piñeiro, Gallardo & Aubourg, 2006), horse mackerel (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005), sardine (Campos, Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005), hake (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2004) or crustaceans (Losada, Rodríguez, Miranda, Barros-Velázquez & Aubourg, 2006), or with studies that had reported steady increases in pH for other fish

species stored in conventional flake ice (Nunes, Batista & Morão de Campos, 1992; Ruíz-Capillas & Moral, 2001).

The comparative evolution of TVB-N formation in ray muscle indicated statistically significant ($P<0.05$) differences between batches at day 6 of storage and onwards (Table 4). Thus, and according to the results of this study, storage in slurry ice slowed down the formation of ammonia compounds in ray muscle as compared with flake ice. This reduction implied that after six days of storage the formation of TVB-N was reduced in a proportion of 45% (81.8 *vs* 149.5 mg/100 g, respectively) as a consequence of processing in slurry ice (Table 4). Such results exhibited a completely different profile as compared with other fish species such as hake (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2004). As in the case of the pH, the final TVB-N concentrations determined in ray muscle were far above the current levels usually determined in other fish species, such as hake, horse mackerel, sardine or turbot, a result that confirms the strong rate of spoilage mechanisms involved in the degradation of nitrogen compounds in this elasmobranchious fish species.

It should also be highlighted that TVB-N concentrations exhibited sharp increases along storage in both batches –although less important in the slurry ice batch–, while the microbial counts of proteolytic bacteria (Table 3) remained at low levels. This result points to a very limited contribution of microbial mechanisms to the formation of TVB-N and to the development of ammonia odour in ray specimens, events that revealed as the limiting factors of shelf-life for this fish species. These results are not in agreement with the patterns determined for other fish species in which significant reductions in the numbers of proteolytic microorganisms correlated directly with significant reductions in the TVB-N concentrations and with a better pH control (Rodríguez, Losada, Aubourg & Barros-Velázquez, 2004; Rodríguez, Losada, Aubourg & Barros-Velázquez, 2005).
(Si crees que vale la pena, incluye algún comentario o cita del trabajo de G. Sol sobre TVB-N).

K value

Finally, the investigation of the autolytic degradation mechanisms, estimated by means of the rate of the nucleotide catabolism pathway (Figure)also revealed significant ($P<0.05$) differences between batches in the formation of inosine and hypoxanthine, with average values of 43.3% and 54.0% being determined after six days of storage in the slurry ice and flake ice batches, respectively. In all cases, mean K values obtained for fish stored under flake ice were higher than in their counterparts kept under slurry ice conditions.

TMA: Creo que es mejor no incluirla. Los datos no son buenos ni a nivel de lo que cabía esperar (visión tradicional) ni lo que salió en el de G. Sol, donde había un clara descenso con tiempo en ambos procesos.

4. Conclusions

The storage of ray specimens in slurry ice at subzero temperature allowed a better maintenance of their high quality, from three to six days, than in a counterpart batch stored in flake ice. The formation of ammonia compounds, with the subsequent negative effect at the sensory level, revealed as the limiting factor of acceptability of the ray batches. The contribution of the proteolytic microorganisms to the formation of urea and other ammonia compounds resulted to be rather limited, these compounds mainly

being derived from the endogenous nitrogen-degrading enzymatic complexes present in ray muscle. The enhanced maintenance of quality derived from processing in slurry ice may allow the commercialization of refrigerated ray specimens with better guarantees of quality and safety.

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Table 1

Scale employed for evaluating the freshness of ray specimens along storage

Attribute	Highest quality (E)	Good quality (A)	Fair quality (B)	Unacceptable (C)
Skin	Shiny and very intense pigmentation; transparent mucus	Intense pigmentation transparent mucus	Shineless and decoloured pigmentation; opaque mucus	Decoloured skin; abundant and opaque mucus
Eyes	Convex and shiny; small pupil	Convex and slightly sunken; oval pupil	Flat; opaque pupil	Concave and yellowish
Gills	Brightly red; without odour; lamina perfectly separated	Rose coloured; without odour; lamina adhered in groups	Slightly pale; incipient fishy odour; lamina adhered in groups	Grey-yellowish colour; intense ammonia odour; lamina totally adhered
Flesh consistency and ventral side aspect	Firm and elastic flesh; presence or partial disappearance of rigor mortis symptoms; curvy flaps; white and shiny ventral side	Firm flesh; pressure signs disappear immediately and completely; rigid flaps; white and shiny ventral side with red stains	Soft flesh; presence of mechanical signs; elasticity notably reduced; soft flaps; shineless ventral side with red and yellowish stains	Flaccid flesh; internal organs blurred; flaccid flaps; yellowish to greenish ventral side with red stains
External odour	Sharply seaweedy smell	Weakly seaweedy non-ammonia odour	Incipiently sour and ammonia odour	Intense ammonia odour

Table 2

Comparative sensory acceptability of ray batches^a

	Initial	Flake ice batch					Slurry ice batch			
		(days of storage)					(days of storage)			
		0	3	6	8	10	3	6	8	10
Skin aspect	E	A	B	B	B	C	A	A	B	B
Eyes	E	A	B	B	B	C	A	A	B	C
Gills	E	A	B	B	B	C	A	A	B	B
Flesh/ventral side	E	B	B	B	C	C	A	A	B	C
External odour	E	B	C	C	C	C	A	B	C	C

^a Freshness categories are as expressed in Table 1.

Table 3

Comparative microbial growth of different microbial groups in the muscle of ray specimens stored in flake ice (FI) or in slurry ice (SI)

Storage time (days)	Aerobic mesophiles		Psychrotrophes		Proteolytic bacteria	
	FI	SI	FI	SI	FI	SI
3	4.70 a (4.65-5.16)	4.66 a (4.51-4.76)	5.18 a (5.02-5.38)	5.16 a (5.01-5.38)	3.23 a (3.05-3.38)	3.29 a (3.03-3.49)
6	5.04 a (4.83-5.16)	4.94 a (4.78-5.05)	5.64 b (5.46-5.89)	5.29 a (5.19-5.42)	3.34 a (3.15-3.52)	3.28 a (3.04-3.56)
8	6.25 b (6.14-6.56)	5.31 a (5.18-5.51)	6.07 b (5.97-6.23)	5.48 a (5.23-5.78)	3.01 a (2.76-3.45)	2.95 a (2.67-3.05)
10	6.62 b (6.41-7.01)	5.23 a (4.88-5.46)	7.02 b (6.92-7.17)	6.01 a (5.87-6.32)	3.35 b (3.13-3.53)	2.88 a (2.68-3.07)

Average counts followed by different letters are statistically different ($P<0.05$).

Ranges are indicated between brackets.

Table 4

Comparative evolution of pH and TVB-N in the muscle of ray specimens stored in flake ice (FI) or in slurry ice (SI)

Storage time (days)	pH		TVB-N (mg/100 g)	
	FI	SI	FI	SI
3	6.92 b (0.03)	6.70 a (0.05)	55.9 a (4.72)	52.5 a (8.22)
6	8.68 b (0.18)	7.46 a (0.22)	149.5 b (3.67)	81.8 a (11.3)
8	8.96 b (0.06)	8.67 a (0.17)	208.8 b (3.88)	131.5 a (2.13)
10	9.00 b (0.02)	8.49 a (0.24)	206.9 b (3.91)	125.9 a (2.08)

Average counts followed by different letters are statistically different ($P<0.05$).

Standard deviation values are indicated between brackets.

Figura 1

Figura 2

Figura 3