ANNUAL REPORT OF THE CONCERTED ACTION "EVALUATION OF FISH FRESHNESS" AIR3 CT94 2283 DECEMBER 1995- DECEMBER 1996

Gudrún Ólafsdóttir and Emilía Martinsdóttir

Icelandic Fisheries Laboratories

January 1997

Figure 1
K-values of Red Mullet during storage on ice

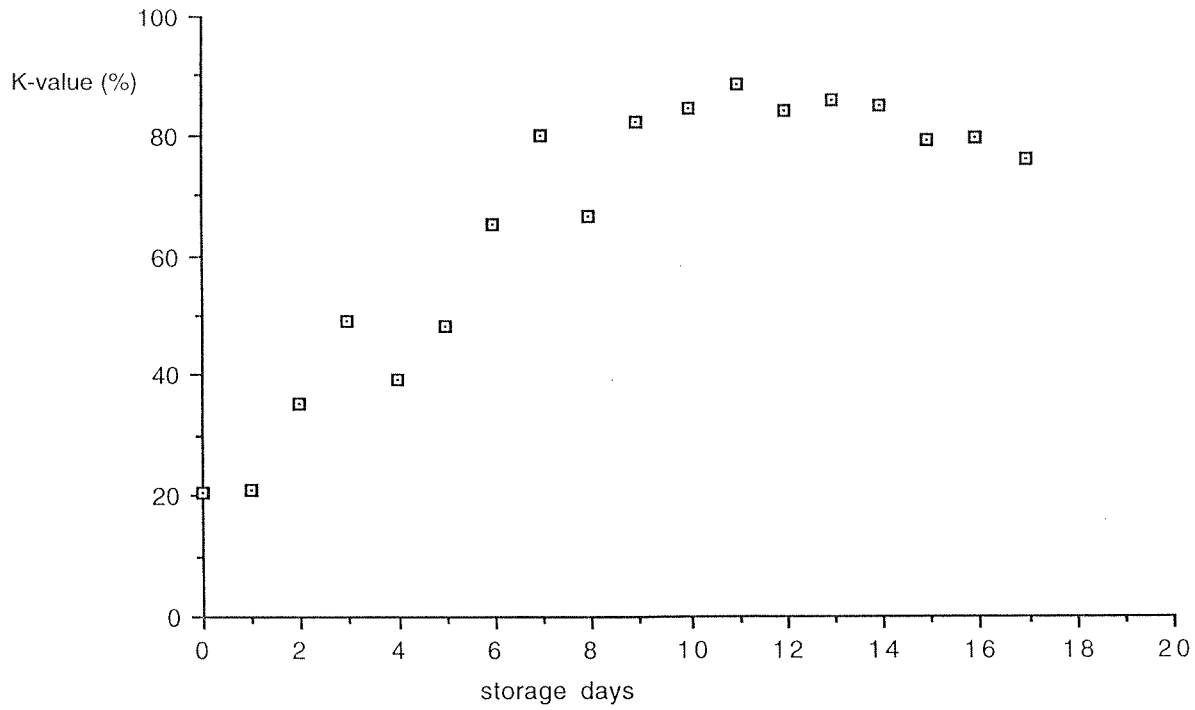

Figure 2
Changes of ATP in Red Mullet during storage on ice

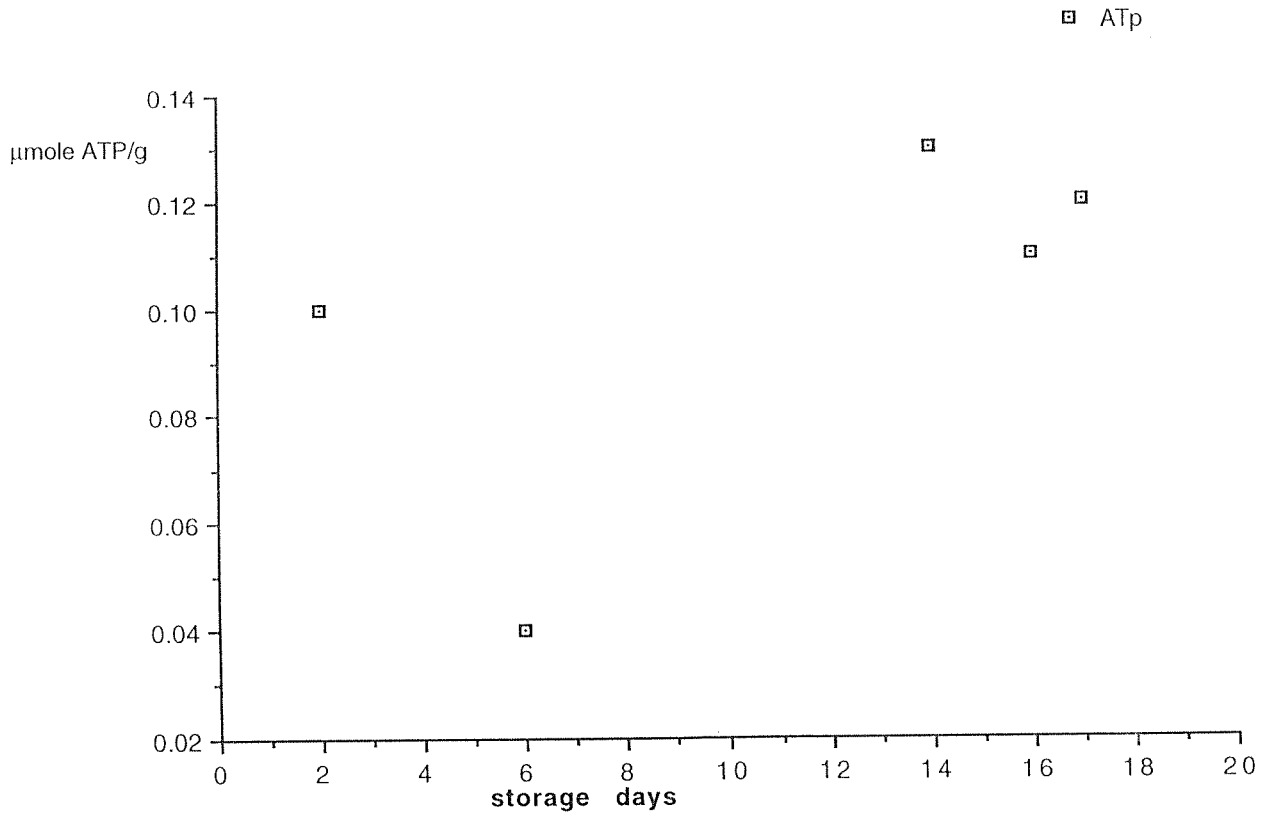

Figure 3
Changes of ADP in Red Mullet during storage on ice

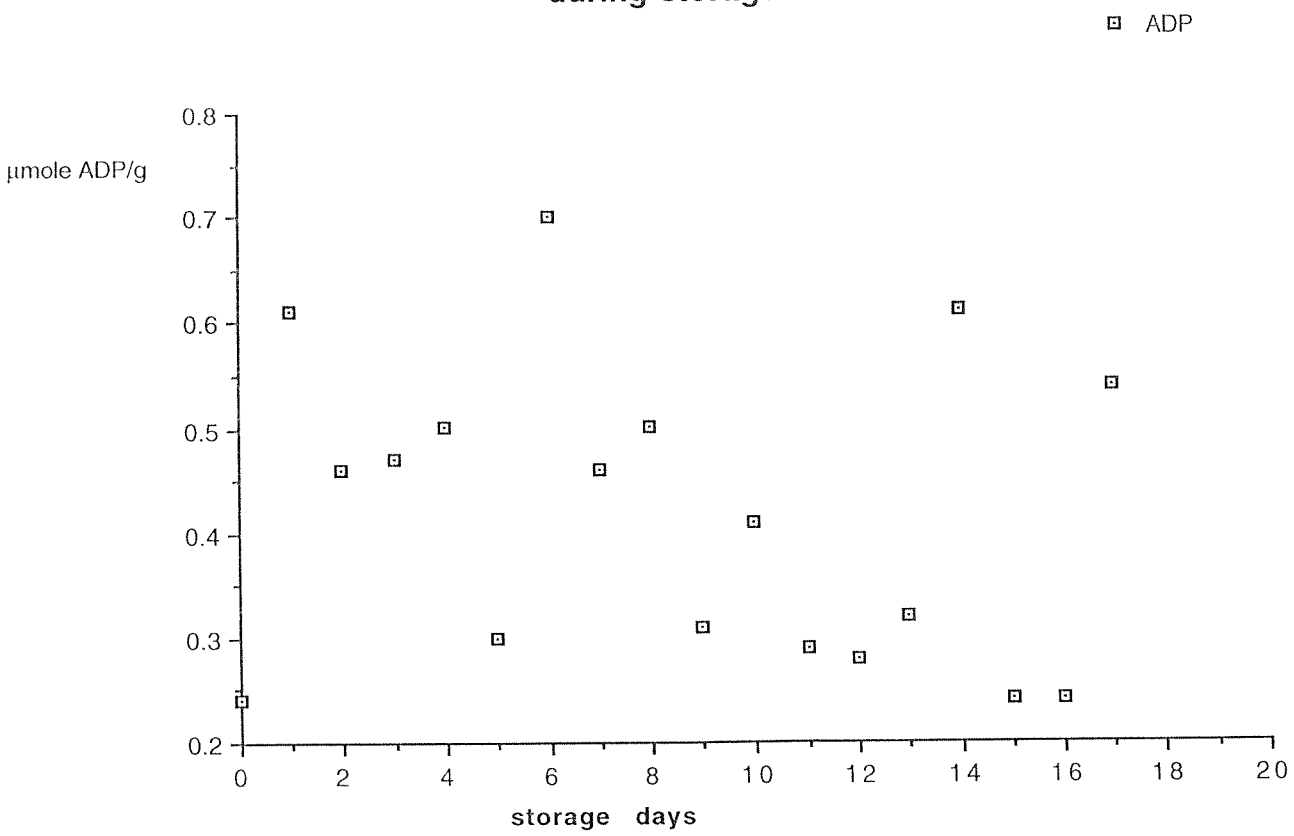

Figure 4
Changes of AMP in Red Mullet
during storage on ice

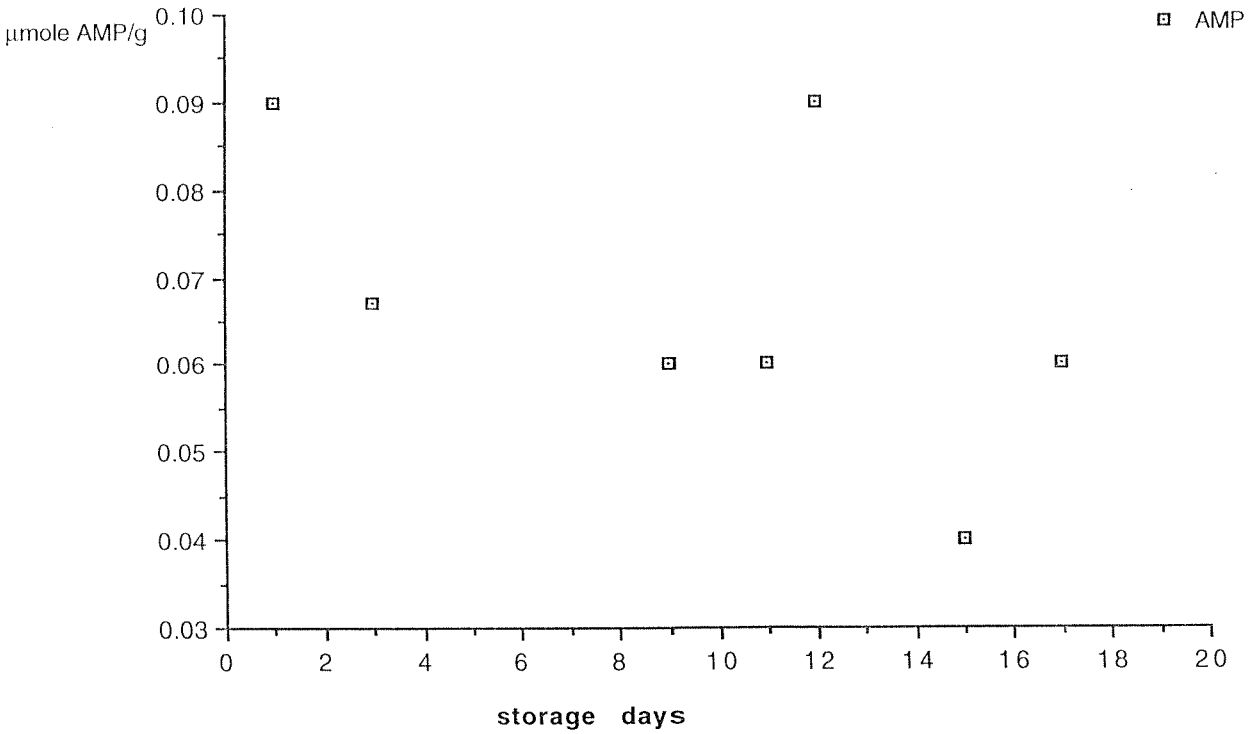

Figure 5
Changes of IMP in Red Mullet during storage on ice

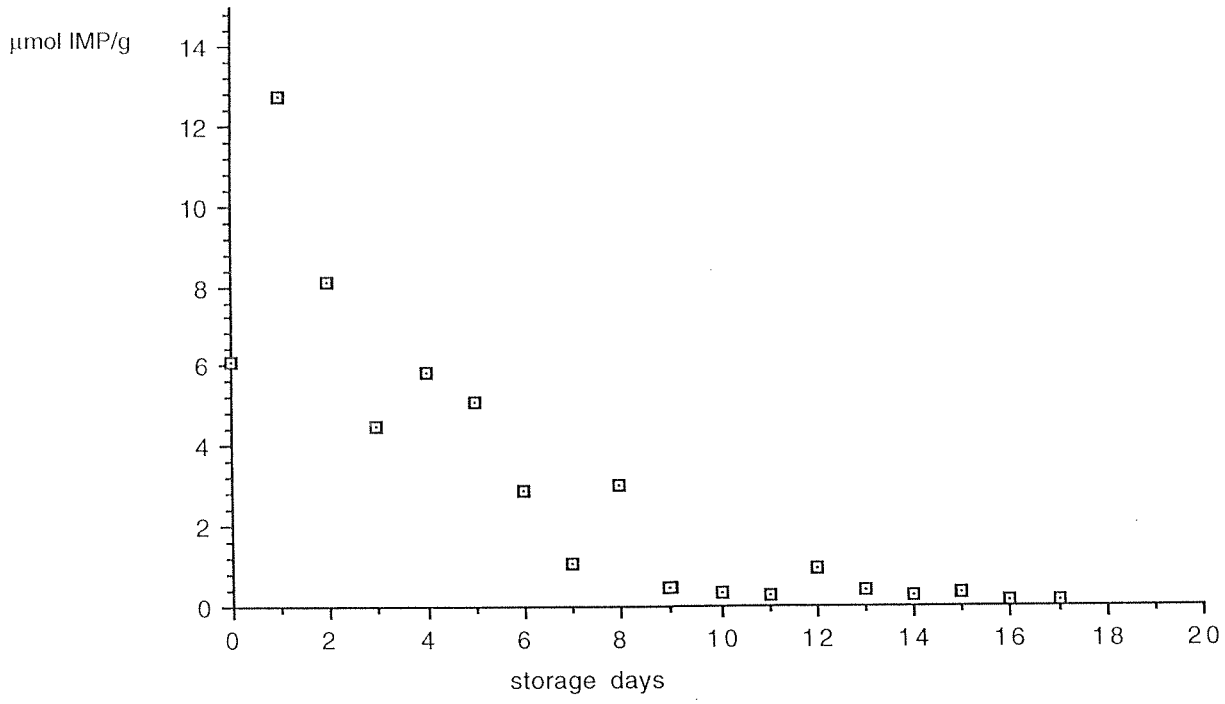

Figure 6
Changes of HxR in Red Mullet during storage on ice

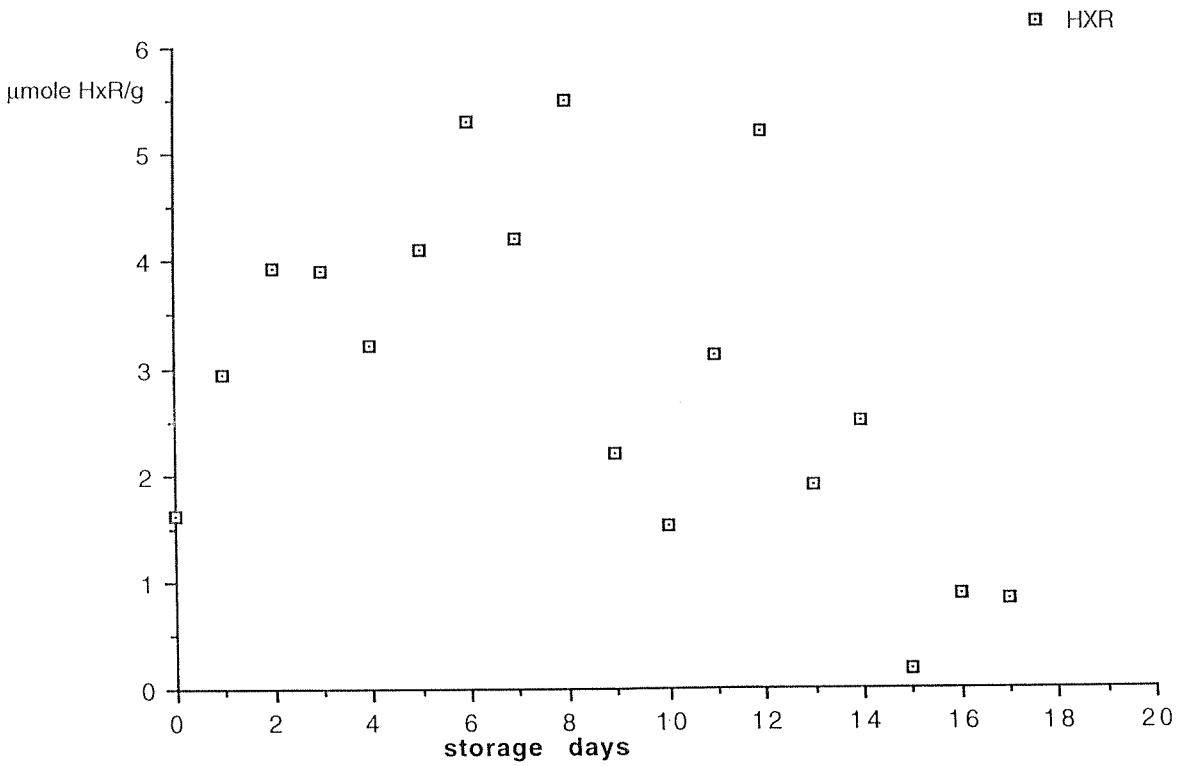

Figure 7
Changes of Hx in Red Mullet during storage on ice

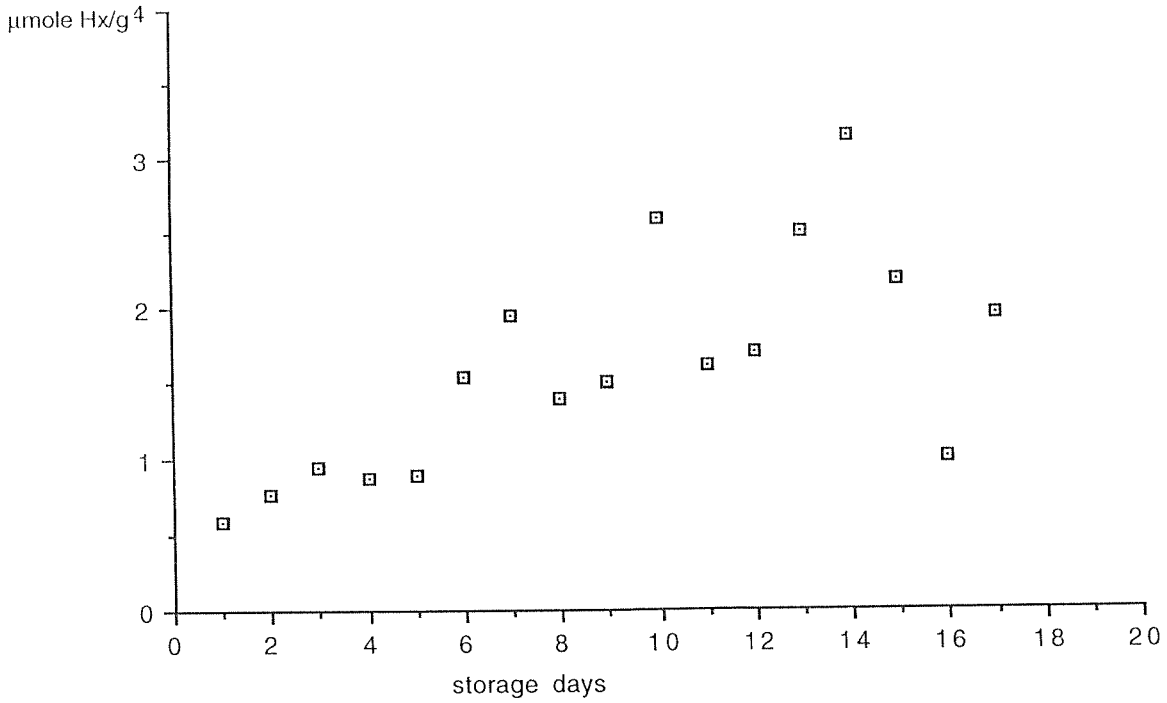

Table 1

storage days	K-value (%)	µmol Hx/g	µmol IMP/g	µmol HxR/g	µmol AMP/g	µmol ADP/g	µmol ATP/g
0	20.4		6.09	1.62		0.24	
1	20.9	0.59	12.73	2.95	0.09	0.61	
2	35.2	0.76	8.08	3.94		0.46	0.1
3	49.1	0.95	4.5	3.9	0.067	0.47	
4	39.3	0.88	5.8	3.2		0.5	
5	48.0	0.89	5.1	4.1		0.3	
6	65.2	1.53	2.9	5.3		0.7	0.04
7	79.8	1.95	1.1	4.2		0.46	
8	66.3	1.4	3	5.5		0.5	
9	82.0	1.5	0.44	2.2	0.06	0.31	
10	84.2	2.59	0.36	1.52		0.41	
11	88.3	1.6	0.27	3.1	0.06	0.29	
12	83.9	1.7	0.95	5.2	0.09	0.28	
13	85.8	2.5	0.41	1.9		0.32	
14	84.6	3.14	0.29	2.5		0.61	0.13
15	79.2	2.18	0.34	0.18	0.04	0.24	
16	79.5	1	0.13	0.86		0.24	0.11
17	76.0	1.94	0.15	0.82	0.06	0.54	0.12

Tone Jakobsen

Analysis of the post-mortem changes in Pandalus borealis muscles by isoelectric focusing and SDS-polyacrylamide gel electrophoresis.

Application for exchange visits:
"Evaluation of Fish Freshness" AIR3 CT94 2283

Name: Tone Jakobsen Profession: Engineer
Laboratory: Norwegian Institute of Fisheries and Aquaculture
Address: PO Box 2511; 9002-Tromsø; Norway

Host Laboratory: CLS Food Science Laboratory, TORRY
Address: PO Box 31; 135 Abbey Road; Aberdeen AB9 8DG, UK

Responsible scientist in the host laboratory:
Name: Dr Ian M. Mackie Profession: Head of the Chemistry Department

Duration of the visit: 14 days
Purpose of the exchange visit:

The reason for the exchange is to learn the techniques of IEF and SDS-PAGE according to Torry's standardized procedure -that, a difference to our standard procedure, permits easy interlaboratory comparisons- in order to identify the changes in myofibrillar proteins that occur in shrimps muscles during the period from 0 to 53 hours after death. This a study included in the "Evaluation of fish freshness" concerted action. The results of the collaboration will be submitted for publication.

Estimated cost
Ticket: ca. 650 ECU
Allowance: 2,520 ECU Bench fee: 100 ECU

GUIDELINES ON EXCHANGE VISITS:

Responsible scientist from each institute sends applications to the coordinators (Gudrun and Emilia in Iceland). The applications will be evaluated by the project manager team. The host laboratory has to agree and a written statement on the commitment to accept a scientist has to accompany the application. The purpose of the visitor has to be clearly related to the objectives of the concerted action regarding fish freshness and evaluation. Every laboratory is entitled to 2 exchange visits (airfares 700 ECU/visit x 2 visits; allowance 180 ECU/day x 14 days/visit x 2 visits, bench fee 100 ECU). Reimbursement will be made after a written report has been delivered to the coordinators. The report will be used for documents to Brussel.

Concerted action "Evaluation of fish freshness" AIR3 CT94 2283

Report of the exchange visit of Ms. Tone Jakobsen (Norwegian Institute of Fisheries and Aquaculture, Tromsø) to Torry (Aberdeen)

Analysis of the post-mortem changes in *Pandalus borealis* muscles by isoelectric focusing and SDS-polacrylamide gel electrophoresis

Tone Jakobsen
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Summary

We are currently studying the post-mortem changes that occur in the skeletal muscle of the Northern shrimp *Pandalus borealis* by electrophoretic techniques. It is therefore most desirable to be able to compare our results with those obtained by other laboratories, specially to achieve the identification of myofibrillar proteins. Accordingly, the aim of the present exchange was to learn Torrys' standardized procedure for the techniques of isoelectric focusing (IEF) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The analyses were performed on water-, low-salt and high-salt soluble protein extracts from shrimp (*P. borealis*) muscle. The shrimps had been stored in ice or seawater (4°C) for 0, 4, 28 and 53h after death. All extracts had been stored at -20°C in 50% glycerol for 5 months prior to analysis. IEF analysis of the water-soluble extracts showed that these proteins tend to focus in pI-range 4.5-6 with most proteins concentrated in the lower region. More acidic protein bands seemed to appear in the extracts as storage time increased. Compared to iced stored shrimps, storage in seawater accelerated the changes. SDS-PAGE analysis showed a major degradation of myosin heavy chain in high salt extracts for all storage times. In water- and low salt extracts there was a gradual breakdown of some larger proteins during storage, and the appearance of bands of smaller size is probably due to this degradation. Both extracts from iced shrimps and shrimps stored in seawater showed extensive myofibrillar degradation during the period under study and shrimps stored in seawater displayed the fastest degradation rate.

Materials and methods

Animal samples

Pandalus borealis (carapace length 15mm, 2-3 developmental stage), had been caught off Tromsø in October and kept alive in tanks with seawater until the time beginning of the experiment. At zero time, the abdominal carapace of six individuals were removed and a piece of the abdominal muscle cut and extracted. Twenty individuals were put into a glass bottle containing seawater, sealed and kept in a cold room at 4°C. Other twenty were stored in ice on a net (to allow draining of water from the melting ice) in a cold room at about 10°C. In addition, one individual was left in seawater at 10°C for 12h and extracted as indicated below.

Extraction of muscle proteins

At zero time and after 4h, 12h, 28h and 53h of storage, the muscles of three individuals from each group were extracted as follows. The whole procedure was carried out on ice. The shell was removed and about 100mg of the dorsal muscle were excised and finely minced in an Eppendorf tube with 500µl of double distilled H_2O. The contents were stirred for 10min, and after a centrifugation for 5min at 14,000rpm the water extracts were transferred to new tubes, an equal volume of ice-cold 100% glycerol was added and the samples were stored at -20°C. The pH was measured in the three first sampling times by spotting a drop of the water extract, prior to the addition of glycerol, onto pH indicator paper. For the extractions of the two last storage times, the indicator paper was cut in small pieces and one piece was completely immersed into the extract.

The low- and high-salt soluble extracts were obtained following the protocol described by d'Albis *et al.* [1]. To the pellets left in the tubes after the water extraction, 500µl of a low salt solution (20mM NaCl, 5mM sodium phosphate, 1mM EGTA, 15mM $MgCl_2$, 5mM dithiothreitol and 200µM phenylmethylsulfonyl fluoride, pH 6.5) were added. The contents were stirred for 10min, centrifuged for 5min at 14,000rpm, the extracts transferred to news tube and an equal volume of ice-cold 100% glycerol was added. To these new pellets, 500µl of a high-salt solution (100mM sodium pyrophosphate, 5mM EGTA, 15mM $MgCl_2$, 5mM dithiothreitol and 200µM phenylmethylsulfonyl fluoride, pH 8.5) were added. The contents were again stirred for 10min, centrifuged for 5min at 14,000rpm, the extracts transferred to news tube and an equal volume of ice-cold 100% glycerol was added. All the extracts were stored at -20°C until analyzed.

The protein content in the extracts was estimated by their optical density (OD_{280}=1 => 1mg/ml protein). The samples were diluted to 200µg/ml protein in SDS-PAGE sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 62.5mM Tris-HCl, pH 6.8) [2] and frozen stored.

To the insoluble pellets, 500µl of SDS-PAGE sample buffer were added, the contents were stirred for 10mins, centrifuged, and the supernatants stored at -20°C for electrophoretic analysis.

SDS-polyacrylamide gel electrophoresis

For the SDS-PAGE analyses a 12% polyacrylamide separating gel was prepared from the following reagents (enough for 2 gels, 14cm x 14cm, 1.5mm thick): 27 ml of distilled water, 20ml of 1.5M Tris-HCl pH 8.8 and 32ml of an acrylamide:BIS-acrylamide stock solution (37.5:1). The solutions were combined, degassed and 0.8ml of 10% (w/v) SDS was added. The solution was then poured into a beaker containing 0.2ml of a freshly prepared 10% ammonium persulfate solution. Then 20µl of TEMED was added and the solutions were mixed by gently swirling the beaker. The gel was immediately poured into the cassette using a syringe, leaving 4cm from the top of cassette empty. Gels were left to polymerize overnight covered by water-saturated butanol. The next day the butanol was poured off, the top of gel rinsed with distilled water and the top surface of gels blotted with folded filter paper. Stacking gels (2 gels) were prepared by mixing 12.2ml of distilled water, 5ml of 0.5M Tris-HCl pH 6.8, 2.6ml of the stock acrylamide:BIS-acrylamide (37.5:1), 0.2ml of 10% (w/v) SDS, 0.15ml of 10% ammonium persulfate and 25µl of TEMED. The preparation was made as described above for the separating gels, the gels were poured to 1cm from top of cassette and the combs inserted. After 30-45min of polymerization, the combs were removed and each well was filled with running buffer made up from 15g of Trizma base, 72g of glycine and 5g of SDS in 5l distilled water [2]. The

electrophoresis tank was filled with running buffer just below the top of the cooler, magnetic stirring was started and cold tap water was passed trough cooling rods. Sample volumes of 10 or 5µl were loaded into the wells. The reservoir was placed on top of cassette assembly and secured. Then it was placed inside the lower buffer chamber and filled with electrophoresis buffer. The running conditions were 600V, 100W and 30mA/gel for about 4h. After the electrophoresis was concluded, the gels were removed from the glass plates and placed in fixation solution containing 40% methanol and 10% acetic acid in distilled water for at least 1h.

The gels were silver stained. The following solutions were needed: reduction solution made up from 200mg of sodium thiosulphate in 1l of deionized water, silver nitrate solution made up from 2g of silver nitrate and 0.2ml of 37% formaldehyde in 1l of deionized water, developer solution containing 30g of sodium carbonate, 0.5ml of 37% formaldehyde and 5mg of sodium thiosulphate in 1l of deionized water, stop-solution made up from 5g of glycine in 1l of deionized water. The silver nitrate and developer solutions must be made up fresh each day.

Silver staining protocol

Wash	30% ethanol	3 x 20 min
Reduction	thiosulphate sol.	1 min
Wash	deionized H_2O	3 x 20 sec
Incubation	silver nitrate	20 min
Wash	deionized H_2O	3 x 20 sec
Development	developer sol.	3-5 min
Wash	deionized H_2O	2 x 30 sec
Stop	stop sol.	5 min
Wash	deionized H_2O	2 x 30 min

The staining was carried out with gentle agitation.

Isoelectric focusing

Two different gel types were used for IEF analyses of the water extracts. One was Pharmacia's wide (pH 3.5-9.5) range ready to use gel pH range, and the other was home made gels with a narrower range (pH 4.0-7.0).

The narrow range gels were prepared by mixing 3.76ml of a 40% acrylamide:BIS-acrylamide stock (29:1), 23.4ml of distilled water, 1.2ml ampholines pH 4-6, 0.6ml ampholines pH 5-7, 1ml of 2% ammonium persulfate and 30µl TEMED. The preparation of these gels was as described above. After polymerization for 30-40min at room temperature, the gel was separated from the glass plates and placed on the ceramic cooling plate of the Bio-Phoresis Cell which was thermostatically controlled at 10°C. A small amount of n-decane was applied between the gel and the plate to ensure good contact of the two. The electrode strips were evenly soaked in the appropriate electrolyte solution, and applied to each end of the gel. For the narrow range gel prefocusing was carried out at 1500V, 15W, and 15mA for 30min with anode strips soaked in 0.5M acetic acid and cathode strips soaked in 0.5M sodium hydroxide. Wide range anode strips were soaked with 1M phosphoric acid and cathode strips with 1M sodium hydroxide.

The dry sample application papers were placed on the gel 2cm in front of the cathode. A maximum of 25µl sample was loaded on every gel using double sets of application papers.

Limiting conditions for isoelectric focusing of wide range gel/narrow range gel were 1500V/2000V, 25W, 50mA/25mA for 90min. Sample application papers were removed from gel after 45/30min respectively. The gels were fixed for 30min with a solution made up from 34.5g sulphosalycilic acid and 115g trichloroacetic acid dissolved in 1l of distilled water. After 5min of destaining in a solution containing 250ml ethanol and 80ml glacial acetic acid per litre distilled water, a warm Coomassie Blue solution (60°C) was poured over the gel and allowed to stand in a water bath for 30min. The stain solution was prepared from 0.46g Coomassie Blue R250 in 400ml destain solution. Background staining was removed with several washes with destain solution until the background was perfectly clear. Then the gel was immersed in preserving solution for 1h and allowed to air-dry over night.

Results

isoelectric focusing

IEF-results show that the proteins in the water extracts tend to focus in pI-range 4.5-6 with most proteins concentrated in the acidic region (Fig. 1). In the zero time, seawater samples, three quite distinct bands are observed of pI slightly lower than pI 4.55. With increasing storage time a fourth band appears gradually (arrow). This fourth band becomes quite distinct after 53h storage. At the same time a relatively large band at pI about 5 seemed to gradually disappear (arrowhead). Iced shrimp muscle samples showed the same tendencies, but the progression in changes during storage was more slowly.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analyses of the water- and low salt-extracts were indistinguishable (Fig 2). The analyses of the proteins extracted with high salt solution showed extensive degradation of myosin heavy chain in both ice- and seawater stored shrimps (Fig. 2). A characteristic change during storage in all extracts can be seen in a band of molecular size slightly higher than 45 kDa (arrow in Fig. 2). This band was clearly noticeable in all iced extracts although its intensity decreased with storage time. In extracts from shrimps stored in seawater however, his band was almost undetectable in the 28h-stored samples (Fig 2b).

Water- and low salt extracts from both storage conditions have another strong band between 60 and 106 kDa (arrowhead in Fig 2). The band is strongly represented in iced samples, and fades gradually during storage time. For seawater samples the band fades very rapidly in the water extracts, whilst in low salt extracts it fades more gradually. At the same time other bands appear in the low molecular regions of the gels. Slightly above 18 kDa a band appears and grows stronger during storage. The band is noticeable in iced samples after 53h of storage and clearly noticeable and strong after 28 and 53 h respectively, of storage in seawater (asterisk, Fig. 2).

Water extracts stored for 5 months in freezer and then prepared for analysis by SDS-PAGE show that the supernatant and pellet fraction contain the same proteins, only less concentrated in the latter fraction (not shown).

Discussion

EIF results show that more acidic protein fragments are produced during storage, and these changes are most visible in seawater samples.

Myosin heavy chain degradation was observed from time zero and increased with storage time. The band pattern showed more small fragments all the way down the lane, and have a

relatively large amount of low molecular weight proteins present. This conclusion is stressed by the fact that low molecular weight proteins in the same positions appear in 28 and 53h water- and low salt extracts, indicating that bands in this area are a result of degradation of larger proteins. Immunoblotting of these extracts with antibodies anti-myosin heavy chain (I. Martinez, submitted) confirmed that shrimp myosin heavy chain is degraded into smaller subunits with increasing storage time.

Extracts from iced shrimps and shrimps stored in seawater showed different degradation rates, seawater-stored samples degrading faster. This may be explained in part by the higher temperature of the seawater-stored samples (4°C) *versus* ice-stored (0°C) and in part by the increased bacterial load and bacterial growth in the seawater-stored shrimps.

Conclusion

As a result of the visit to Torry, we have been able to learn the horizontal isoelectric focusing technique, that we are now standardizing at Fiskeriforskning. The results of the SDS-PAGE confirmed the results obtained with our standard technique and will make comparisons between our laboratories in the future easier.

The results of the IEF-analyses showed that the proteins in the water extracts tend to focus in pI-range 4.5-6.0 with most proteins concentrated in the acidic region. More acidic subunits seem to appear in the extracts as storage time increases. The progression in changes during storage is slower for iced samples.

SDS-PAGE showed a major degradation of myosin heavy chain in high salt extracts for all storage times. In water- and low salt extracts there is a gradual breakdown of some larger proteins during storage, and the appearance of smaller subunits is probably due to this degradation. Extracts from iced shrimps and shrimps stored in seawater show different degradation rates, seawater-stored samples degrading faster.

Acknowledgments

The author wishes to acknowledge the European Commission for the financial support to carry out the interlaboratory exchange visit through the Concerted Action AIR3 CT94 2283.

References

1 d'Albis, A., Janmot, C. and Béchet, J.J. (1986) Comparison of myosins from the masseter muscle of adult rat, mouse and guinea pig. Persistence of neonatal-type isoforms in the murine muscle. *Eur. J. Biochem.*, **156**: 291-296.

2 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

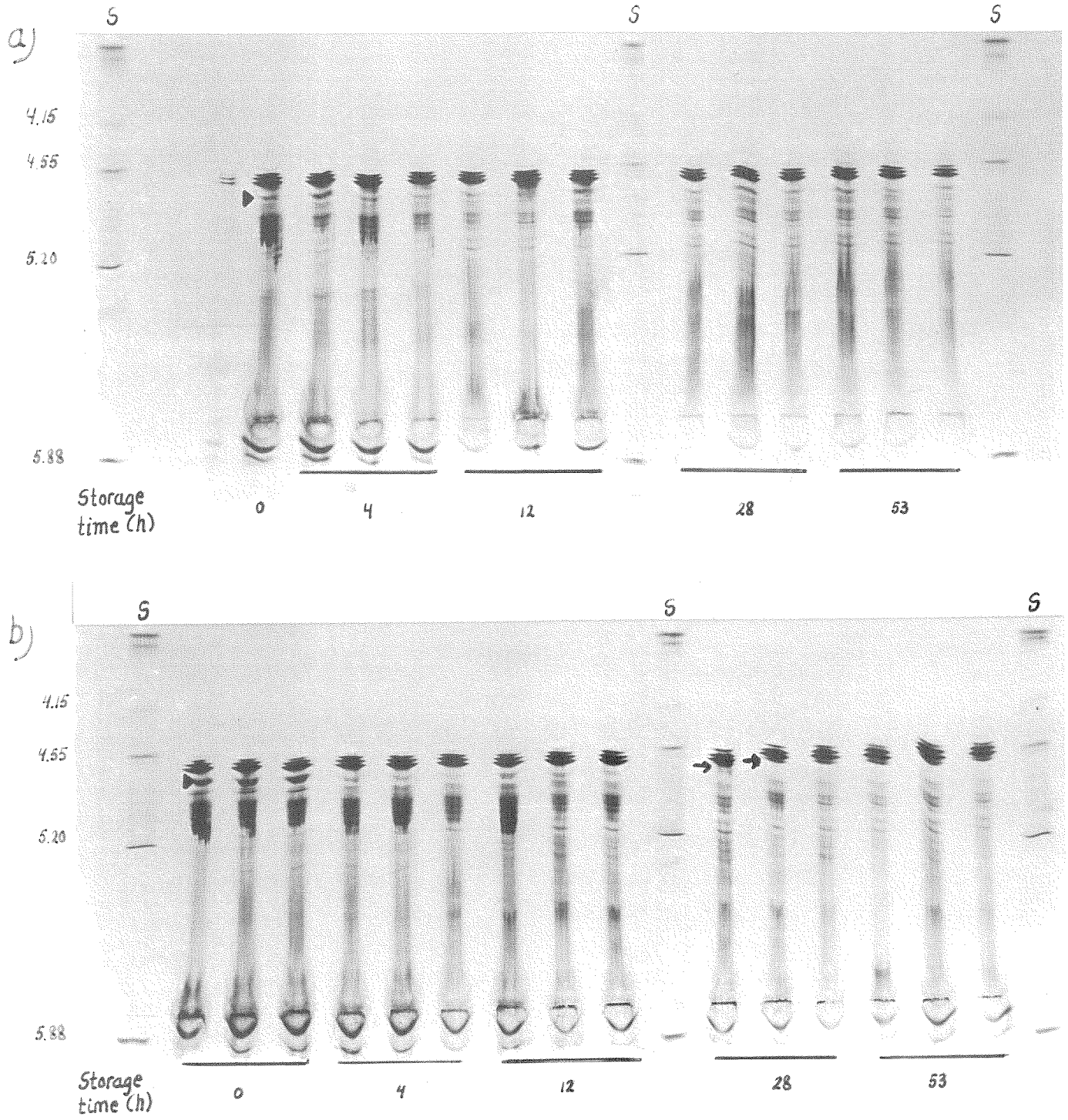

Fig. 1. Isoelectric focusing (pH 4.0-7.0) of individual shrimp muscle extracts. a) ice-stored shrimps; b) seawater-stored shrimps. S, Pharmacia's pI standards. The samples are indicated in the figure. Arrow and arrowhead indicate bands referred to in the text

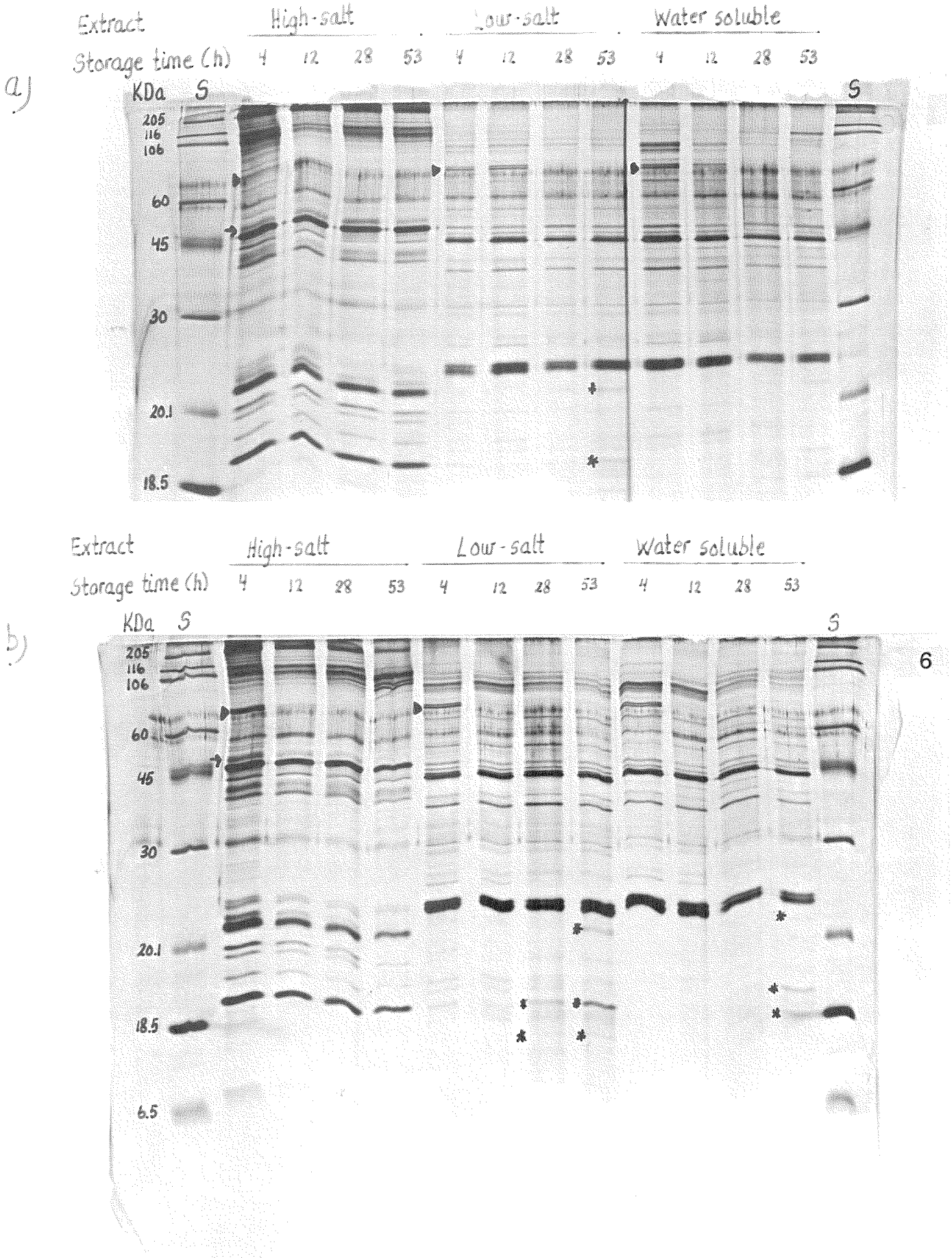

Fig. 2. SDS-12% polyacrylamide gel electrophoresis of pools (n=3) of shrimp muscle extracts. a) ice-stored shrimps; b) seawater-stored shrimps. S, Sigma's molecular size standards. The samples are indicated in the figure. Arrow and arrowhead indicate bands referred to in the text

Mercedes Careche

Changes of Northern Shrimp salt soluble proteins during ice storage measured by two dimensional and native electrophoreses.

Application form for exchange visits:
"Evaluation of Fish Freshness" AIR3 CT94 2283

Name : Mercedes CARECHE Profession: Scientist

Laboratory: INSTITUTO DEL FRIO

Address: Ciudad Universitaria s/n 28040 MADRID, SPAIN

Host Laboratory: FISKERIFORSKNING

Address: P.O. Box 2511, N-9002 Tromsö, NORWAY

Responsible scientist in the host laboratory: EDEL O. ELVEVOLL

Name: ICIAR MARTINEZ Profession: SCIENTIST

Duration of visit: 14 DAYS

Purpose of the exchange visit:

- To learn two dimensional electrophoresis of fish proteins
- To study by this technique the changes in the electrophoretic pattern of salmon water-soluble and salt soluble proteins as a function of freshness (pre-rigor (0 and 6 hours post-mortem), rigor and post-rigor), frozen storage (well and badly stored), and smoking.
- A paper would be expected from this study. Hopefully this will lead to better define ideas in order to prepare an EC proposal on changes of proteins associated with fish freshness.

Estimated cost

Ticket: ca. 867 ECU

Allowance: 2520 ECU (180ECU/day) Bench fee: 100 ECU

GUIDELINES ON EXCHANGE VISITS:

Responsible scientist from each institute sends applications to the coordinators (Gudrun and Emilía in Iceland). The applications will be evaluated by the project management team. The host laboratory has to agree and a written statement on the comittment to accept a scientist has to accompany the application. The purpose of the vistit has to be clearly related to the objectives of the concerted action regarding fish freshness evaluation. Every laboratory is entitled to 2 exchange visits (airfares 700 ECU/visit x 2 visits; allowance 180 ECU/day x 14 days / visit x 2 visits, bench fee 100 ECU). Reimbursement will be made after a written report has been delivered to the coordinators. The report will be used for documents to Brussel.

Concerted Action "Evaluation of Fish Freshness"
AIR3 CT94 2283

Report of the exchange visit of Dr Mercedes Careche (Instituto del Frío, CSIC, Madrid) to the Norwegian Institute of Fisheries and Aquaculture (NIFA), Tromso.

CHANGES OF NOTHERN SHRIMP SALT SOLUBLE PROTEINS DURING ICE STORAGE MEASURED BY TWO DIMENSIONAL AND NATIVE ELECTROPHORESIS

M. Careche
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INTRODUCTION

Muscle proteins are susceptible to proteolytic changes during storage in ice and these changes could be potentially used as markers for evaluating fish and marine species freshness degree.

In order to separate proteins and analyze their changes, electrophoretic techniques can be used in which proteins may be resolved in one dimension by one of their physico-chemical properties. However, the discrete bands which are detected after electrophoresis do not necessarily represent homogeneous proteins. The combination of two different one-dimensional electrophoretic techniques into a two-dimensional (2D) procedure has the potential to separate very complex samples containing several thousands of proteins and to resolve proteins sharing similar physico-chemical properties. The most popular two-dimensional procedure (O'Farrell, 1975) uses a combination of isoelectric focusing in the first dimension and sodium dodecyl sulphate polyacrylamide electrophoresis in the second dimension.

In the Norwegian Institute of Fisheries Research (NIFA), Dr Martinez has a vast experience in the application of 2D PAGE techniques for fish proteins which have been extensively used mainly for the study of the myofibrillar fraction of muscle, and the myosin isoforms of different fishes under several physiological conditions (Martinez, 1992; Martinez et al., 1989, 1990a,b,c, 1991, 1992).

Northern shrimps (*Pandalus borealis*) are an important fishery resource for Norway and at the NIFA, the early changes occurring in muscles of this species that can affect their quality and price at landing are currently being subject of study.

The objectives of the Concerted Action "Evaluation of fish freshness" include, among others, a) the exchange of technicians and scientists from different laboratories within existing research projects, b) the stimulation for iniciating new collaborative research projects for fish freshness and c) the comparison and validation of different techniques used for measuring fish freshness.

With all these points in mind, the aim of this exchange visit to was to learn two-dimensional electrophoretic techniques in order to implement them at the Instituto del Frío and to study by these

techniques the changes occurring in muscle proteins from shrimps stored in ice with the aim to find possible markers for the evaluation of seafood freshness.

MATERIALS AND METHODS

Preparation of extracts

Shrimps (*Pandalus borealis*) were caught off Tromso. Salt soluble proteins of the muscles of each individual were extracted after zero time and 5, 12, 24, 72 and 120 hours of storage in ice according to D'Albis et al., (1986) and Martinez (1996). The shell was removed and about 100mg of the dorsal muscle were excised and finely minced in an Eppendorf tube with 500μL of ddH_20. After stirring 10 min. and centrifuging (5 min.x14,000 rpm), the pellets were washed with a 500μL of a solution containing 20mM NaCl, 5mM Na_3PO_4, 1mM ethylene glycol-bis N,N,N',N'-tetraacetic acid (EGTA), 15mM $MgCl_2$, 5mM dithiothreitol (DTT) and 200μM phenyl methyl sulfonyl fluoride (PMSF), pH 6.5. The contents were stirred (10 min.) and centrifuged for 5 min. at 14,000rpm and the resulting pellets were extracted with a high ionic strength salt solution (100mM Sodium Pyrophosphate, 5mM EGTA, 15mM $MgCl_2$, 5mM DTT and 200μM PMSF pH 8.5). The mixture was stirred for 10 min, centrifuged for 5 minutes at 14,000rpm, and the extracts were transferred to Eppendorf tubes and mixed with an equal volume of ice-cold 100% glycerol, and stored until needed.

The protein content of the extracts was estimated by optical density at 280nm (OD280=1 ⇒ 1mg/mL protein).

For the subsequent electrophoretic analyses extracts from four individuals were pooled for each time of storage in ice.

Two-dimensional electrophoresis

Two-Dimensional (2D) gel electrophoresis was carried out basically as described by O'Farrell (1975) and Hoschstrasser et al. (1988). Two sets of 2D electrophoresis were performed with the first dimension being isoelectric focusing (IEF) at a pH range of either 3-10 iso-dalt or 3-7. The second dimension was sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

First dimension

For the IEF, the salt-soluble extracts were adjusted to 1mg/mL with sample buffer containing 9.5M urea, 2% Nonidet P-40, 2% Ampholines (range 3-10 or 3-7, depending on the run) and 5% ß-mercapto ethanol (ME).

The 3-10 iso-dalt IEF was performed in rods (1.4mm id, 10cm length) in a Hoefer Scientific Instruments rod gel apparatus. The gels performed according to Hochstrasser et al. (1988) contained 3% acrylamide, 0.08% piperazine diacrylamide (PDA). After a pre-

run (200V, 10min; 300V, 15min.; 400V, 15min) the samples were loaded (5 and 10μg) and the IEF was run at 400V, 16 hours following 800V, 1 hour. After the run the rods were transferred to eppendorf tubes, equilibrated for 15 min. at room temperature (62.5mM tris, 2.3% sodium dodecyl sulphate (SDS), metilen blue and 30% glycerol) and kept frozen (-20°C) for subsequent analyses.

The second set of 2D electrophoresis was performed at a pH range of 3-7 with the BioRad mini rod gels (1mm id, 5cm length). The pre-run conditions were 200V, 10min; 300V, 15min; 400V, 15min and the running conditions were 500V, 10min and 750V, 3hours. The rest of the conditions were the same as in the first set.

Second dimension

For both sets, electrophoresis was performed on 15% acrylamide and 0.087% PDA (Anderson et al., 1973; Hoschstrasser et al., 1988). The rods were loaded on the slab gels. SDS-PAGE was performed according to Laemmli (1970) at a constant intensity of 20mM/slab gel for about 3 hours.

In the first set, 0, 5, 12, 24, 72 and 120h sample extracts were analyzed as well as rat diaphragm myosin, 0+120h and 0h+rat. The gels were silver stained (Ansorge, 1983), except the ones used for immunoblotting.

Immunoblotting

A specific area of the 2D electrophoresis gels (7x8cm) was cut and transferred onto nitrocellulose according to Towbin et al., (1979) using the BioRad minitransfer unit at 120mA/gel for 1 hour. The nitrocellulose sheets were blocked for 1 hour with phosphate buffer saline (PBS) containing 0.5% powdered skimmed milk. The primary antibody (5μg/mL) used was anti-Artic Charr fast skeletal myosin heavy chain (Martinez and Pettersen, 1992), obtained by I. Martinez. Secondary antibody was peroxidase labelled goat anti-rabbit IgG (Sigma) and 3, 3'-diaminobenzidine was used as substrate with Co^{2+} enhancement.

Native electrophoresis

Native electrophoresis was performed according to d'Albis et al., (1979) and Hoh et al., (1976) using 4% acrylamide, 0.1% PDA in rods (6cm length, 0.5 id). After a pre-running (80mA, 30 min), 10μg of each extract (0, 5, 12, 24, 72, 120h and rat myosin diaphragm) were loaded. Electrophoresis was carried out at a constant intensity of 80 mA for 9 hours at -2°C. The samples were stained with Coomassie Brilliant Blue R-250 for 10 minutes and destained with water.

Gel slices containing the bands of interest were equilibrated for 15 min. with 62.5 mM tris, 2.3% SDS, metilen blue, 5% ME, 30% glycerol and frozen stored until needed. After thawing, the protein bands were cut in cubes from which thin gel slices were taken and loaded onto 15% polyacrylamide, 0.087% PDA gels. The

electrophoresis was performed according to Laemmli (1970) with the BioRad mini slab gels (7x8cm, 0.5mm thick) at constant 150V for about 1 hour. The gels were silver stained according to Ansorge (1983).

RESULTS

Figure 1 shows the 2D electrophoretic pattern of salt-soluble extracts from shrimps. In this gel, some spots were tentatively assigned as actin (A) and trompomyosin (TM). The spots in the low molecular weight region of the acidic side of the gel, were in the area of the myosin light chains.

A series of protein bands of molecular sizes around 100kDa (*) were present at zero time, and its relative intensities increased with the storage time in ice (figure 2), so that, at 120 hours, they grouped in two spots. A western blotting of this region of the gel showed that they gave a positive reaction with anti-myosin antiserum (figure 3) (☆).

At 0 time there was a band of about 67kDa (a) (figure 2) which was gradually disappearing until 120 hours when it was no longer visible. Also, there were four spots with a molecular size of about 50 kDa (b). The relative intensities of these bands increased with time of storage in ice. At 12h post-mortem these bands started to fuse and at 120h appeared in the 2D gels as one single spot. These 2D electrophoresis results were confirmed in the 3-7 gels (results not shown).

Electrophoresis under non-dissociating conditions of the salt-soluble extracts showed a protein band that migrated slightly faster than the myosin isoforms from rat diaphragm myosin (figure 4). With 12 hours of storage in ice it appeared a protein band (a) migrating faster than myosin which, at 24 hours became more clearly visible. At this time of storage, another faster migrating protein band (b) appeared and was more clearly seen after 74 and 120 hours post-mortem in ice. SDS-PAGE of slices of these bands (figure 5) showed that they lacked myosin heavy chain; in "a", a band of molecular size of about 94 kDa was present, whereas in "b" there were two bands in this region. These bands could be a consequence of the proteolytic breakdown of the myosin heavy chain.

DISCUSSION

The protein bands appearing at about 100kDa have also been found in 1D SDS-PAGE by Martinez (1996) for salt-soluble extracts from the same species stored in similar conditions. She attributed these bands to either proteolytic fragments of the myosin heavy chain or paramyosin, since the existence of a positive reaction with the antiserum against myosin heavy chain does not rule out the posibility of these bands belonging to paramyosin. This protein, which forms the central cores of the thick filament in most invertebrate muscles may vary in its molecular size (105-

130kDa) depending on the isoform (Martinez 1996) and it has been reported to have homologous regions of the myosin heavy chain (Hoppe and Waterson, 1996). On the other hand, myosin is susceptible to partial proteolytic breakdown in certain specific sites of the molecule which could give, depending on the conditions, fragments known as heavy meromyosin (HMM) and light meromyosin (LMM) of 130kDa and 75kDa respectively and/or subfragment S1 and rod, of molecular sizes of about 90-95kDa under SDS-PAGE (Weeds and Pope, 1977) which could account for some of these bands. Non-dissociating electrophoresis under conditions for native myosin, together with SDS-PAGE of these bands of interest also showed the presence of bands of faster mobility than myosin, with molecular sizes of about 95 kDa that were not visible in the early storage times. This suggests that these are mainly degradation fragments of myosin heavy chain as suggested previously (Martinez, 1996).

Protein bands progressively dissapearing at 67kDa with the time of storage as well as the appearance of new bands at about 50kDa confirm the results obtained by Martinez (1996) for the same species in SDS-PAGE. These bands are showing early changes during ice storage and could be used as protein markers. However, a further confirmation of these results as well as a characterization of these bands and their origin is needed, together with establishing a quantitative relationship which can be unequivocaly related to the freshness degree of this shrimps stored in ice.

CONCLUSIONS

2D electrophoresis as well as native electrophoresis have been learnt as a consequence of the exchange visit.

2D electrophoresis as well as native electrophoresis of salt-soluble extracts of shrimps has shown the existence of protein changes during ice storage which could be used as markers to help establishing the freshness degree.

ACKNOWLEDGEMENTS

Thanks are due to Ms Tone Jakobsen for preparing the extracts and to Dr Iciar Martinez for teaching me the electrophoretic techniques. The exchange visit has been financed by the European Commission under the Concerted Action AIR3 CT94 2283, Evaluation of Fish Freshness.

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LEGENDS TO FIGURES

Figure 1.- Two-dimensional electrophoretic pattern of salt-soluble proteins extracted from shrimps (*Pandalus borealis*) inmediately after death. A=actin, TM=tropomyosin, LC= light chain region.

Figure 2.- Changes in the two-dimensional electrophoretic pattern of salt-soluble proteins from shrimps (*Pandalus borealis*) after 0, 5, 12, 24, 72 and 120 hours of storage in ice. Only the region of interest is shown. IEF, 3-10 iso-dalt, SDS-PAGE, 15%A, 0.087%PDA. *=spots of molecular size of about 100kDa; a=spot of about 67kDa; b=spots of about 50kDa.

Figure 3.- Immunoblotting of salt soluble fraction proteins separated by 2D electrophoresis with antiserum anti-myosin heavy chain of Arctic charr at 0 and 5 hours frozen storage.

Figure 4.- Esquematic diagram of the native electrophoresis of salt-soluble extracts during ice storage. M=myosin.

Figure 5.- SDS-PAGE (15%A, 0.087%PDA) of the myosin (M) and proteolytic bands (a, b) appearing after 120h of ice storage as shown in figure 5.

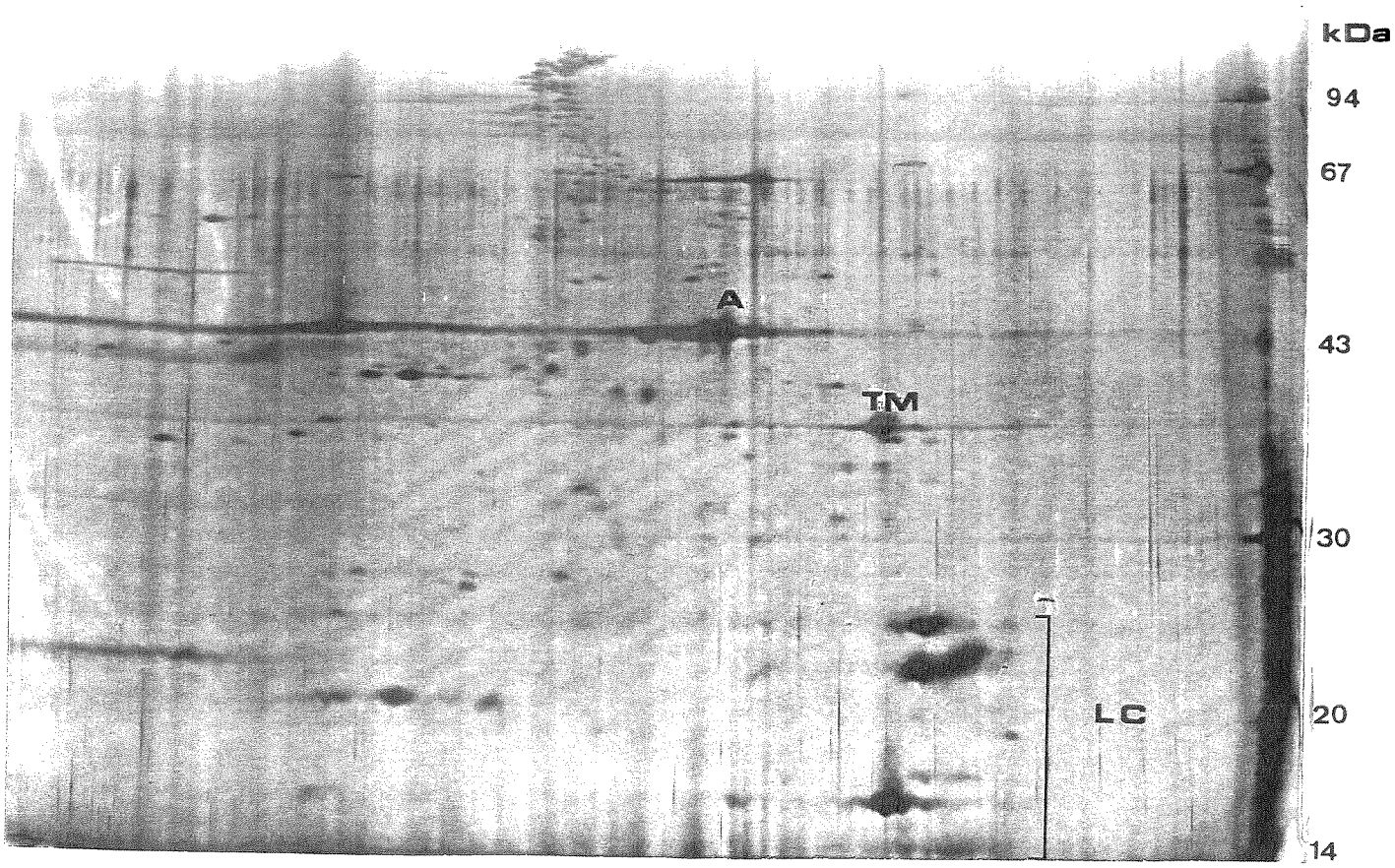
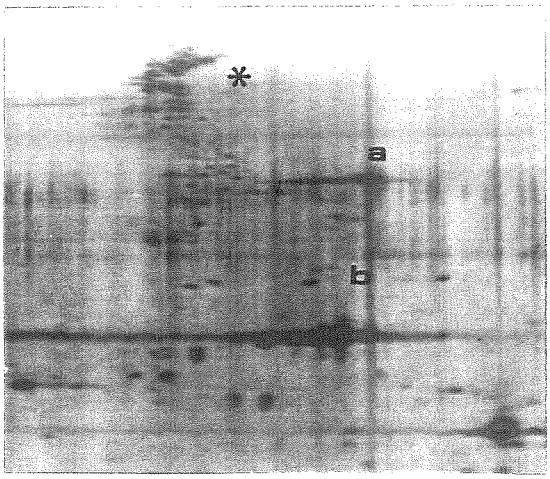
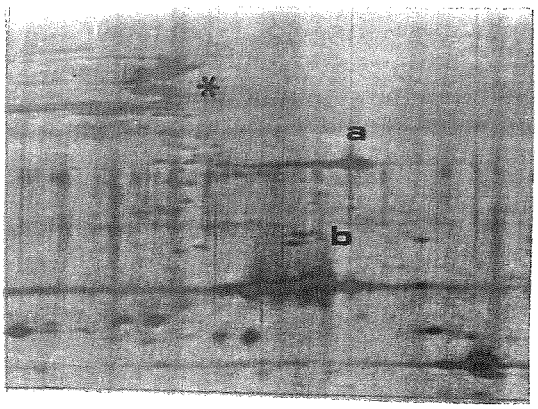


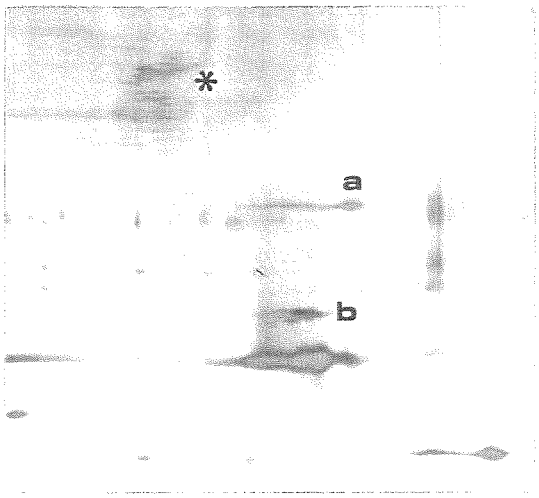
Figure 1



0 h



5 h



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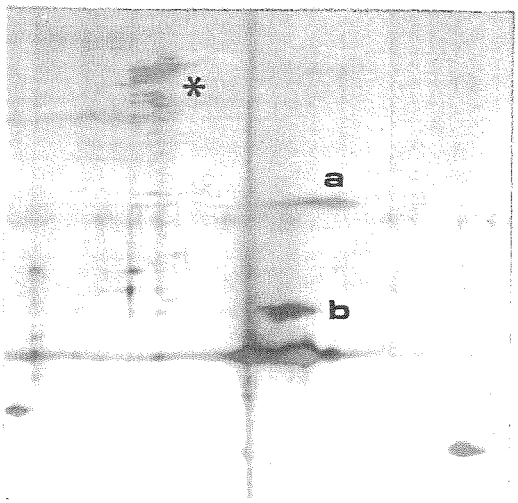

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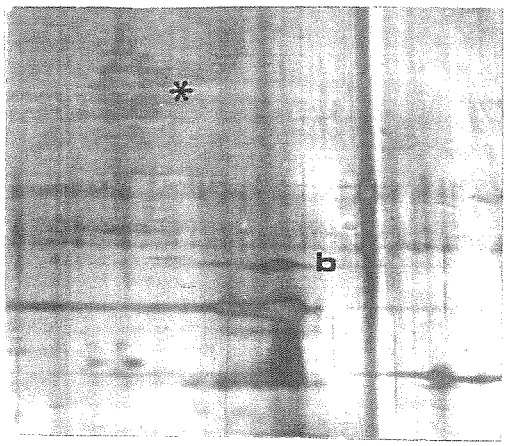
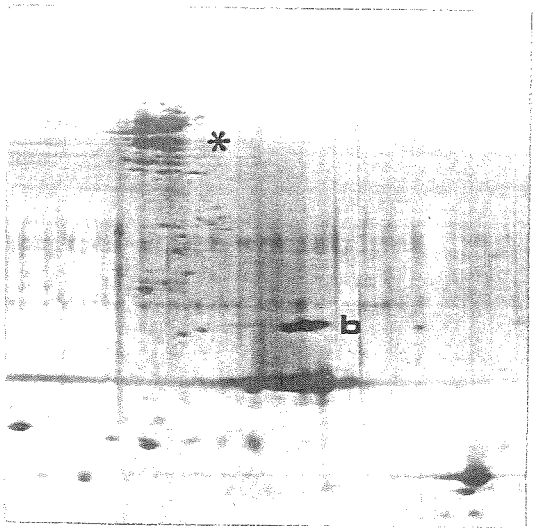


72 h



120 h

Figure 2

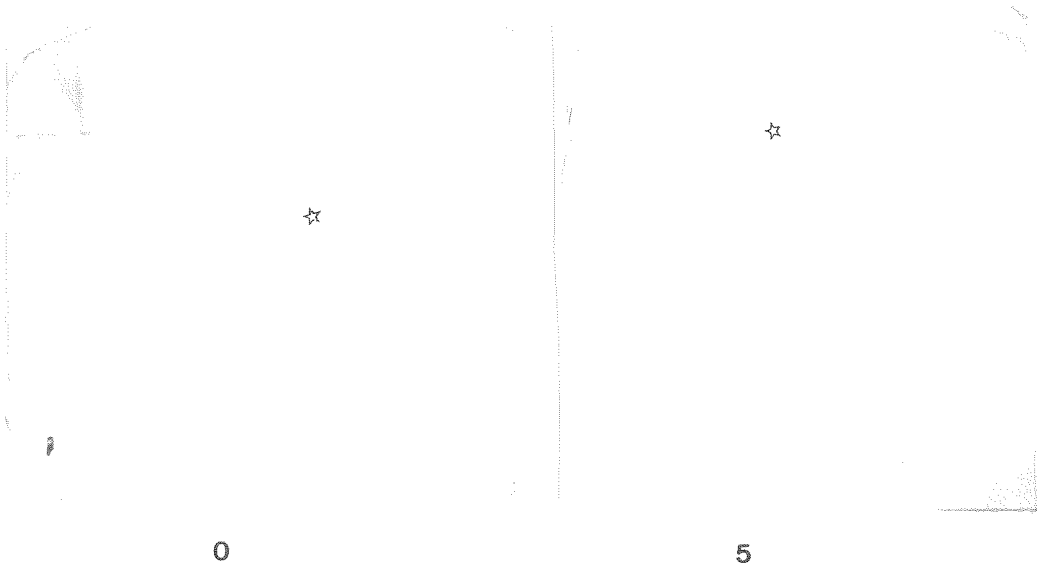


Figure 3

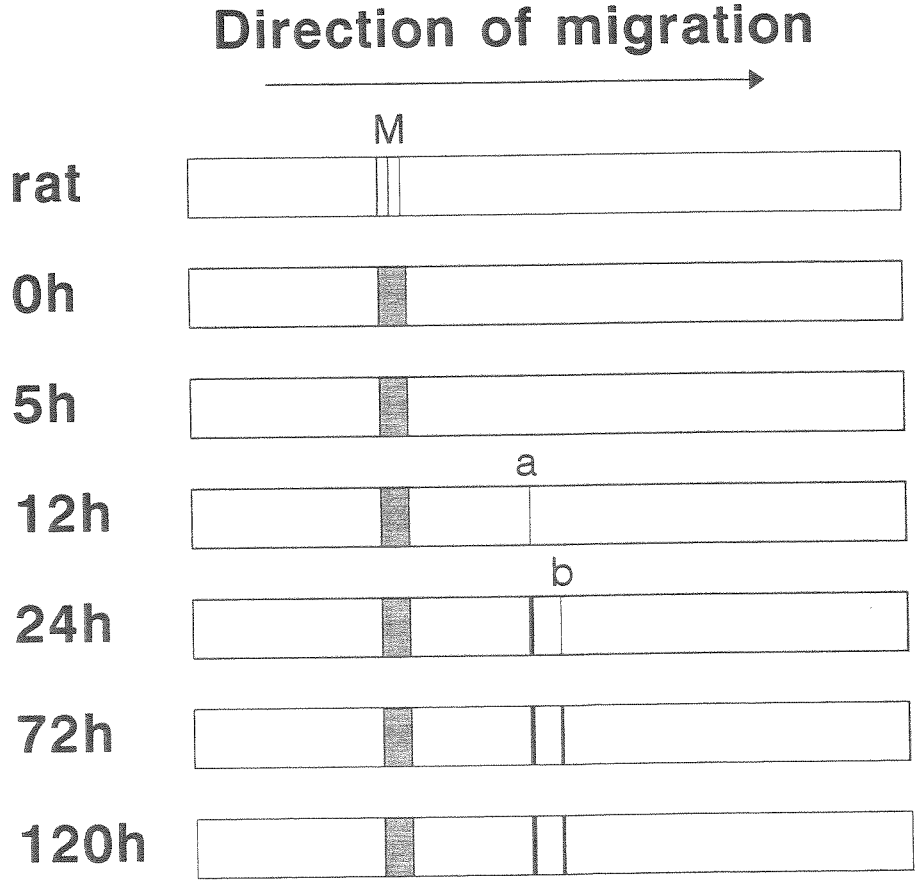


Figure 4

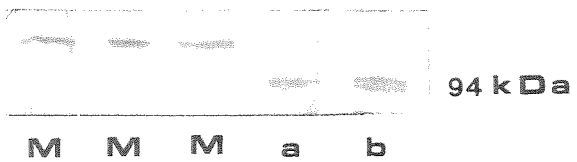


Figure 5

Narcisa Bandarra

Quality indices to evaluate lipid oxidation of sardine *Sardina pilchardus* during ice storage.

Aplication form for exchange visits:
"Evaluation of Fish Freshness" AIR3 CT94 2283

Name: Narcisa Bandarra Profession: PhD student
Laboratory:IPIMAR
Adress: Av. Brasília 1400 Lisboa, Portugal

Host Laboratory: SIK (The Swedish Institute for Food and Biotechnology)
Adress: SIK, Box 5401, 402 29 Gothenburg

Responsible scientist in the host laboratory: Gunnar Hall
Name: Ingrid Undeland Profession: scientist

Duration of visit: 14 days
Purpose of the exchange visit:

-To learn some analytical techniques related with lipid oxidation in fresh fish.

-To study the evolution of oxidation indices during ice storage of sardine by using samples of oil previously extracted.

-To elaborate a paper with the results obtained. This could also permit to elaborate an EC proposal to study the lipid degradation associated with fresh fish.

Estimate cost
Ticket: *ca* 450 ECU
Allowance: 2520 ECU (180 ECU/day) Bench fee: 100 ECU.

GUIDELINES ON EXCHANGE VISITS:

Responsible scientist from each institute sends applications to the coordinators (Gudrun and Emilia in Iceland). The applications will be evaluated by the project management team. The host laboratory has to agree and a written statement on the comittment to accept a scientist has to accompany the application. The purpose of the visit has to be clearly related to the objectives of the concerted action regarding fish freshness evaluation. Every laboratory is entitled to 2 exchanges visits (airfares 700 ECU/visit x 2 visits; allowance 180 ECU/day x 14 days/visit x 2 visits, bench fee 100 ECU). Reimbursement will be made after a written report has been delivered to the coordinators. The report will be used for documents to Brussels.

Quality indices to evaluate lipid oxidation of sardine *Sardina pilchardus* during ice storage

Responsible scientist from SIK: Ingrid Undeland

Responsible scientist from IPIMAR: Maria Leonor Nunes

Narcisa Bandarra

September 1996

Quality indices to evaluate lipid oxidation of sardine *Sardina pilchardus* during ice storage

Abstract

Sardine is a pelagic fatty species, which represents more than 40 percent of total annual catches in Portugal. The present work deals with the influence of ice storage of sardine on lipid degradation. Sardine was kept with ice for 15 days and regularly sampled for oil anlysis. Peroxide value increased during storage ranging from 5 to 241 meq O_2/kg oil. A similar increasing trend in the level of dienes and trienes was also recorded. Fluorescence results showed a similar profile, an unusual high value was recorded in the first day of storage which can be a result of a contamination or a formation of compounds related with *rigor mortis* of fish. A preliminar characterisation of the volatile compounds of sardine oil was done which allowed to identify a few typical components as well as degradative products. Among them it has to be referred the formation of an alcohol after the 6th day which was not identified. The initial content of tocopherol of sardine was 100mg/kg oil which regularly decreased during storage being completely destroyed after 4 days. This evolution suggest the utilization of this measurement as a freshness index although it is necessary to confirm the repeatibility of those results in different periods of the year.

Introduction

Small pelagic species contribute with more than 25 percent to the world's catch of fish and shellfish. Sardine, a pelagic species, is the main halieutical resource of Portugal. The lipids of this fatty species present a high proportion of polyunsaturated fatty acids (Bandarra *et al*, 1991). These compounds are claimed for health benefits reducing the risk of cardiovascular disorders, on the other hand the high number of unsaturations place some technological problems. Lipid degradation gives rise to unpleasant quality in fish leading to consumer rejection.

Several different methods for determining the rancidity in foods are currently used. Peroxide value, is a useful indicator of the extent of lipid oxidation. It measure the

hydroperoxides present in lipids, which are the primarily lipid oxidation compounds. Numerous analytical procedures for the measurement of peroxide value are described in literature. The iodometric method are widely used and are based on the measurement of the iodine produced from potassium iodide by the hydroperoxides present in oil (Rossel, 1994). The colorimetric method is based on the oxidation of Fe^{2+} to Fe^{3+} by the hydroperoxides and the reaction of the later with ammonium thiocyanate producing a red complex, ferric thiocyanate (Shantha and Decker, 1994).

The decomposition of lipid hydroperoxides, which are very unstable compounds lead to a variety of volatile compounds (Nawar, 1985) more stable with short chain like aldehydes, ketones, alcohols and hydrocarbonated compounds. Taking the advantage of high volatility of off-flavor compounds the headspace analysis has been widely used (Park, 1993).

Conjugated dienes and trienes are formed, when unsaturated fatty acids are oxidised. The conjugated acids so formed absorbed ultraviolet light, the conjugated dienes that may result from its decomposition show an absorption at 234 nm, conjugated trienes show a triple absorption band of which the principal peak is in the neighbourhood of 268 nm.

Fluorescence methods have been used in the detection of lipid oxidation damages in biological tissues (Dillard and Tappel, 1973; Trombly and Tapell, 1975). Water soluble fluorescent products derived from reaction of malonaldehyde with aminoacids and the system R-N=C-C=C-N-R is responsible for the fluorescence. Lipid soluble fluorescent chromophores from animal tissues have been described as probably being derived from the reaction of malonaldehyde with the aminogroups of phospholipids.

The unsaturated lipids in living tissues are relatively stable, due to the presence of natural antioxidants and enzymes to prevent effectively lipid oxidation (Grosch, 1987). Tocopherols (vitamin E) are considered to be the major antioxidant found in animal tissues. Vitamin E is a radical scavenger in the membrane and acts as a terminator of lipid oxidation by donating a hydrogen atom to lipid radicals, which blocks the

propagation of the process. Erickson (1993) found very good correlations between levels of α-tocopherol and oxidative products formed in minced frozen Channel catfish under temperature fluctuations. According to this author tocopherol determination can detect oxidative changes at early stages because it is not necessary to wait for oxidation products formation.

The aim of this work was to study the influence of ice storage of sardine on lipid degradation and try select the most adequate quality indices for the evaluation of sardine freshness.

Materials & Methods

Raw material

Sardine used in this work was caught in July 96 on the Portuguese coast (Peniche) by purse-seiner. Some sardines were immediately frozen on board in liquid nitrogen containers and the others kept on ice in isothermic boxes. After the arrival at the laboratory the fish frozen in liquid nitrogen (0 days) and a small part of that kept on ice (0.5 days) were prepared for the remotion of edible flesh. The other sardines were stored on ice at 5°C for two weeks and used in triplicate for lipid analysis after 1, 2, 3, 4, 6, 8, 11, 13 and 15 days.

Analytical methods

The edible flesh of sardine was minced in triplicate and used for lipid extraction by the method of Bligh and Dyer (1959) in a cold room at 0°C.

Total lipid content of sardine was done according to AOAC (1984).

Peroxide value determination was done with a system Titralab from Radiometer Copenhagen using a titrimetric method with potassium iodide and ammonium thiocianate (AOCS Cd-8-53), the amount of sample used ranged between 0.07 and 0.35 g of sardine fat depending on the degree of oxidation.

Conjugated dienes and trienes were measured at 234 and 268nm, respectively, the sample was prepared dissolving the sardine fat with isopropanol:heptane (1:1) to a concentration of 6 mg/ml for the analysis of conjugated dienes and 112,5 mg/ml for the analysis of conjugated trienes. The absorptions at these two wavelengths were then measured using Flow Injection Analysis (FIA). Thus the sample were injected into a HPLC system, methanol was used as mobile phase at a flow rate of 0.3ml/min. The detector used was a diode array HP 1090M from Hewlett Packard recording signals at 234 nm and 268 nm, respectively. No column was installed, resulting in one single peak to come out with the void-volume. The area of this peak represents the total absorption at these wavelengths.

Lipid soluble fluorescence products were recorded simultaneously with the FIA-analysis of UV-absorbance at 234 and 268nm. This was performed by connecting a fluorescence detector (JASCO FP-920, Japan) in series with the diode array detector installed in the HPLC system. The fluorescence detector was equipped with a 150W xenon lamp mounted horizontally. Excitation and emission monochromator bandpasses were set at 18 nm and 40 nm respectively.

The preparation of samples for headspace analysis was done placing 20µl of sardine oil inside a small tube fitted with glass wool and the desorption of volatiles was carried out during 50 minutes at 50°C under helium. The analysis was done in a gas chromatograph Varian 3400 equipped with a mass spectrometer Finnigan Met Incos 50 The column used was a DB1 (0.32 mm i.d. x 30 m), the temperature initial of analysis was 30°C and it was programmed to increase 4°C/min to 200°C. Tentative identifications were based on standard MS library data.

Tocopherols were determined by normal phase HPLC following the method of Piironen *et al* (1984) with minor modifications. Sample preparation was done dissolving 9 mg of fat in 250 µl of isohexane and 20µl were injected in a Rheodyne Model 7125 injector and eluted with isohexane:2-propanol (99.8:0.2) with a flow rate of 0.5ml/min in the HPLC system Beckman model 110A pump. The column used was a Lichrosorb 5 Silica column (Phenomex, Torrance, USA) 250 x 2.10 mm i.d.. For

detection a flluorescence detector Hewlett Packard 1046 A was used with excitation wavelength 292nm slits at 2 x 2mm and emission wave length 324nm slits at 4x4mm. Tocopherols were identified and quantified by comparison with standards (Merck, Germany) from which a standard curve was prepared. Peaks were integrated using a Softron PC integration program (version 1.0) each sample (n=3) was analysed in duplicate.

Results & Discussion

Sardine is a pelagic species whose lipid content varies widely with season attaining more than 20%, in the present work the fish used presented a fat content of 10.1% in the edible flesh.

During the ice storage of sardine the PV (peroxide value) was determined and the results obtained are present in figure 1. The values are represented in triplicate because each analysis was done using a different group of sardines, in order to have more representative results. The peroxide value increased during all the period of ice storage. Sardines frozen in liquid nitrogen (0 days) on board presented similar results to that kept on ice (0.5 days). The values obtained for peroxide value at the end of storage were very high (241 meq O_2/Kg fat) only possible to attain in a very oxidised fat.

The results obtained with conjugated dienes and trienes analysis during 15 days of ice storage are present in figures 2 and 3. The formation of conjugated dienes and trienes seems to have a similar evolution, increasing during all the period. Nevertheless, the values obtained for trienes were superior of dienes, which is a result of the large number of polyunsaturated fatty acids present in sardine lipids.

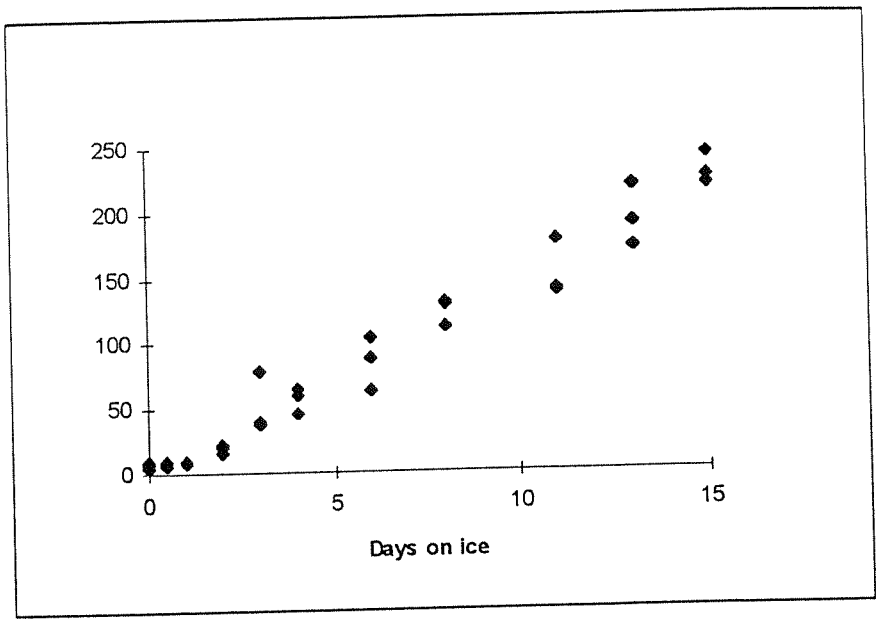


Figure 1- Evolution of peroxide value of sardine lipids during 15 days of ice storage.

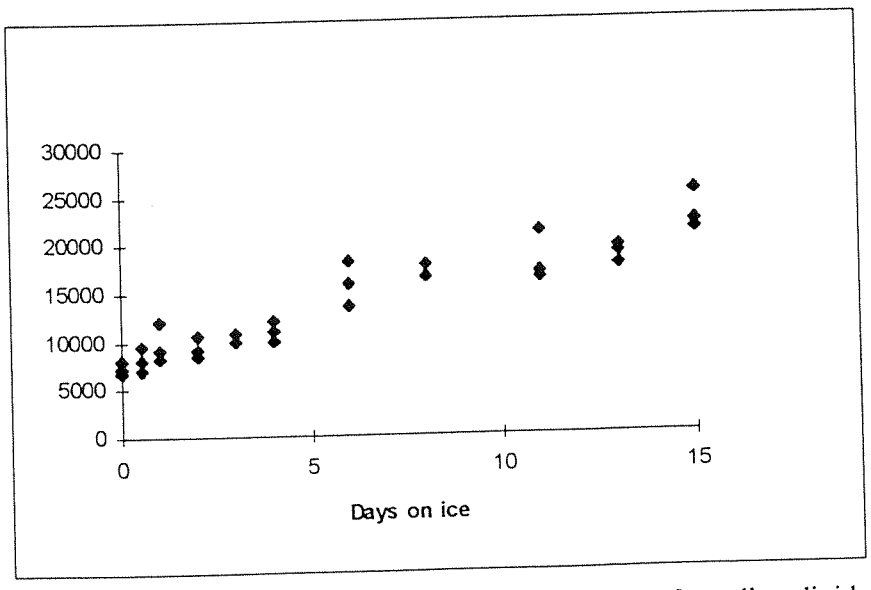


Figure 2 - Evolution of conjugated dienes of sardine lipids during 15 days of ice storage.

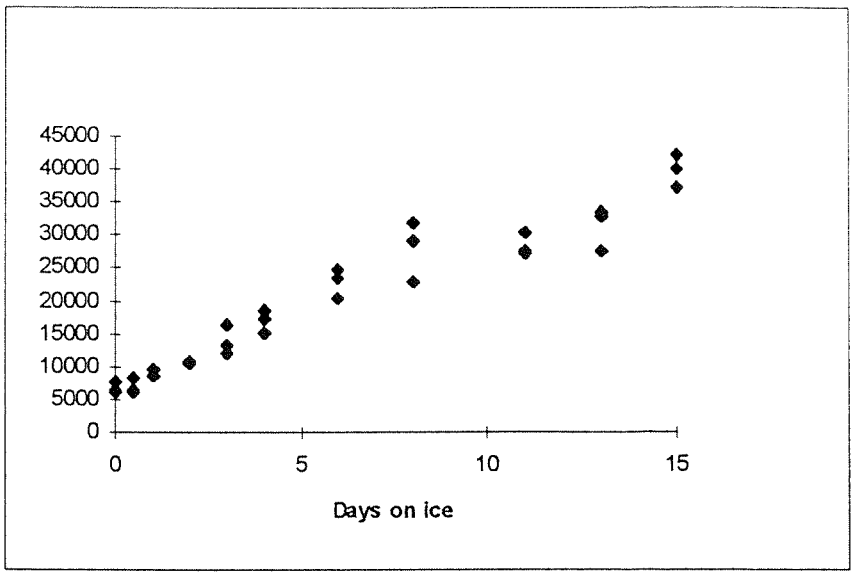


Figure 3 - Evolution of conjugated trienes of sardine lipids during 15 days of ice storage.

The results obtained in fluorescence analysis can be appreciated in figure 4, during the period of ice storage seems to have a clear tendency to increase the number of fluorescent lipidic compounds. An unusual high value was registered in the first day of storage which can be attributed to a contamination or a formation of compounds related with *rigor mortis* of fish. Further studies need to be developed.

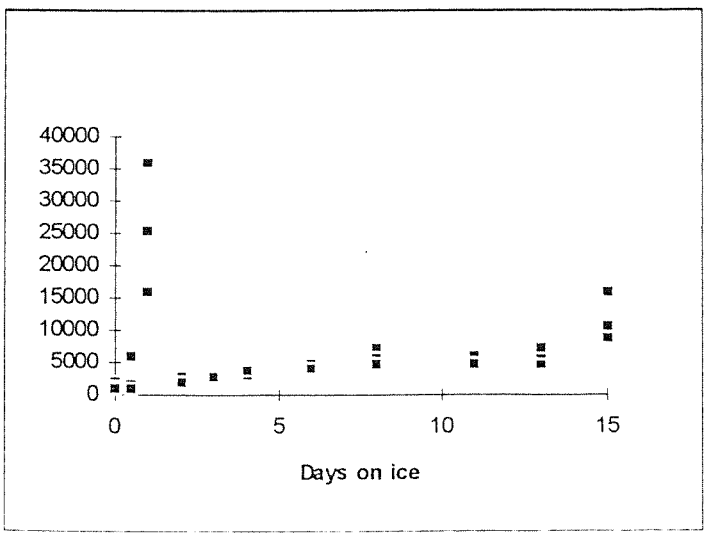


Figure 4 - Evolution of fluorescence of sardine lipids during 15 days of ice storage.

In order to appreciate the formation of volatiles in sardine lipids an headspace analysis was carried out in the beginning, after 6 and 15 days of ice storage, the results obtained are present in figures 6a, 6b and 6c. As it was expected a different profile was obtained for fresh fish, being butanoic acid butyl ester the main volatile component. After 6 days was possible to detect the presence of unsaturated aldehydes and ketones. A formation of a non identified alcohol seems to occur during the ice storage, attainning after 15 days the highest level. Additional research is needed in order to identify this compound to establish the beginning of its formation and to relate its absence with freshness state.

The most important results related with establishment of fish freshness indices were obtained with tocopherol analysis, as can be appreciated in figure 7. The level registered for α-tocopherol in the beginning of ice storage was around 100 mg/ kg of sardine fat. A destruction of α-tocopherol occurred after 3 or 4 days, and no more tocopherols were detected in lipids, as was expected seems to have an inverse relationship between the peroxide value and the tocopherol level registered in sardine lipids.

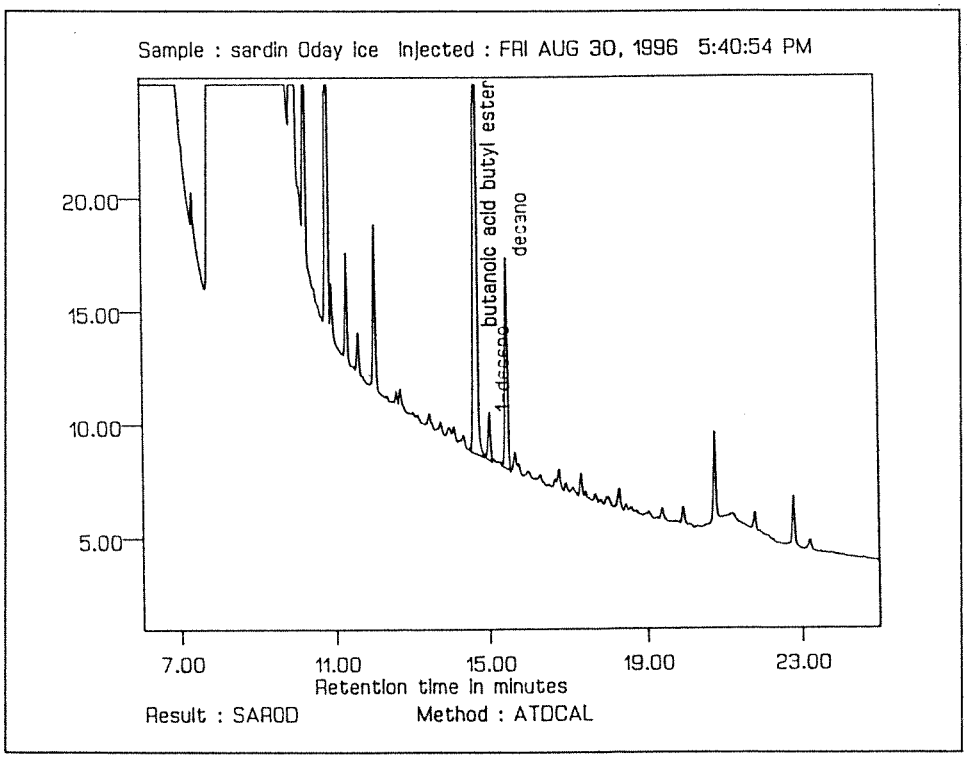


Figure 6a - Chromatogram of headspace analysis of sardine lipids in the beginning of ice storage.

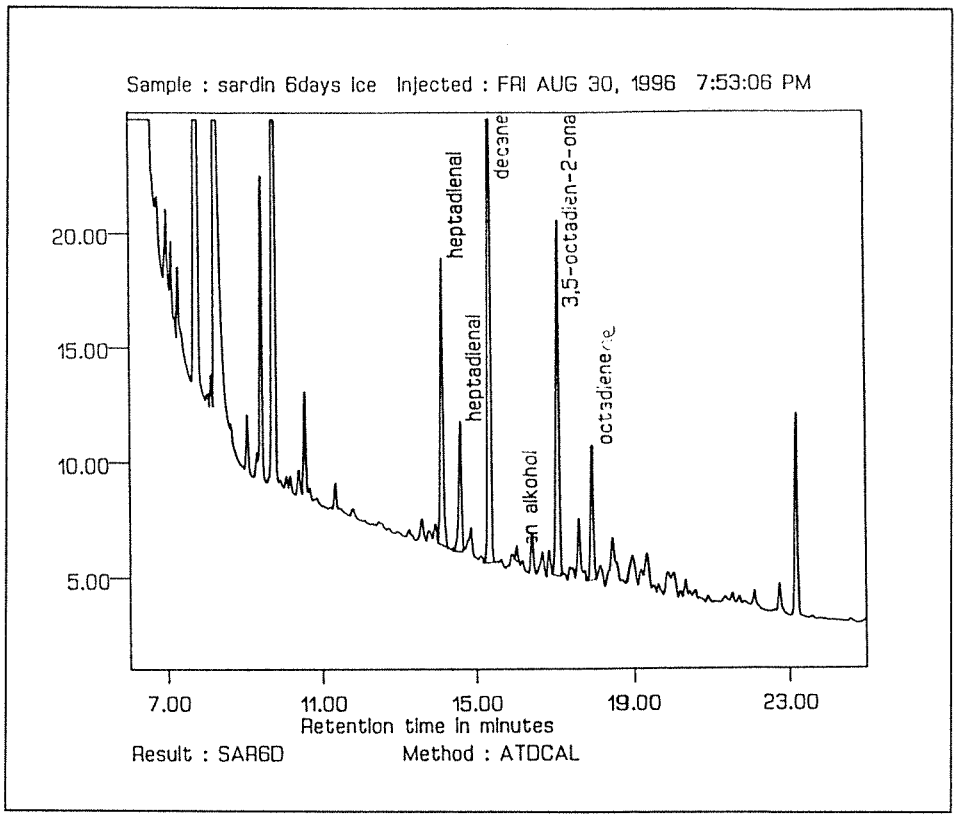


Figure 6b - Chromatogram of headspace analysis of sardine lipids after 6 days of ice storage.

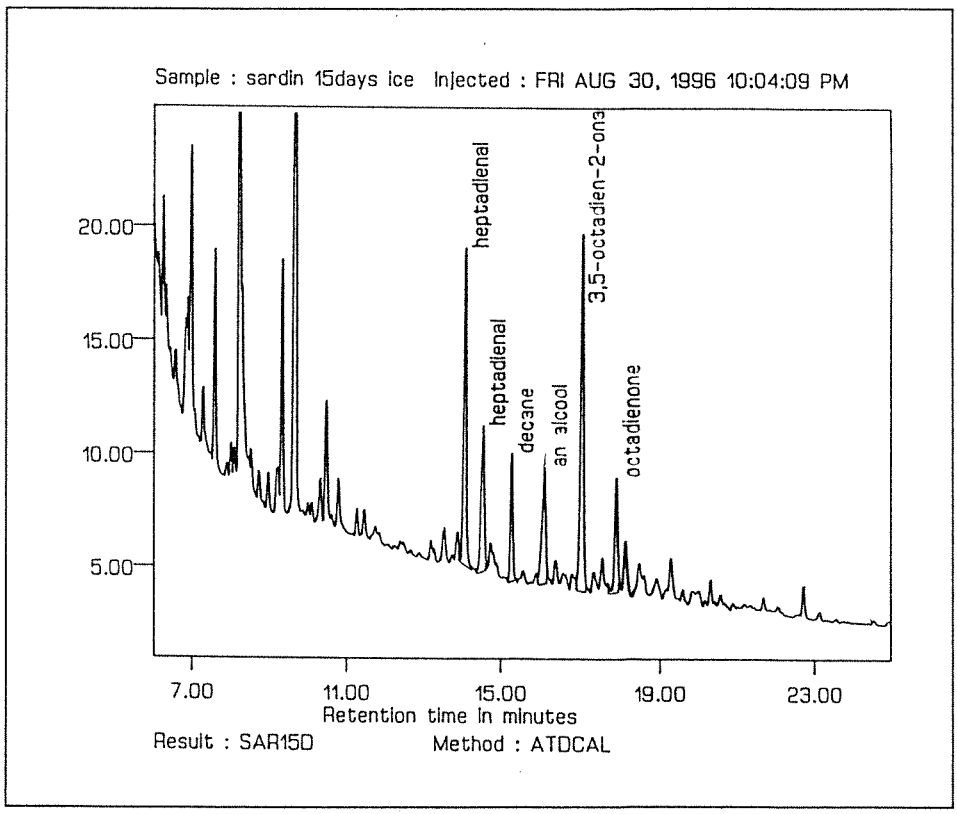


Figure 6c - Chromatogram of headspace analysis of sardine lipids after 15 days of ice storage.

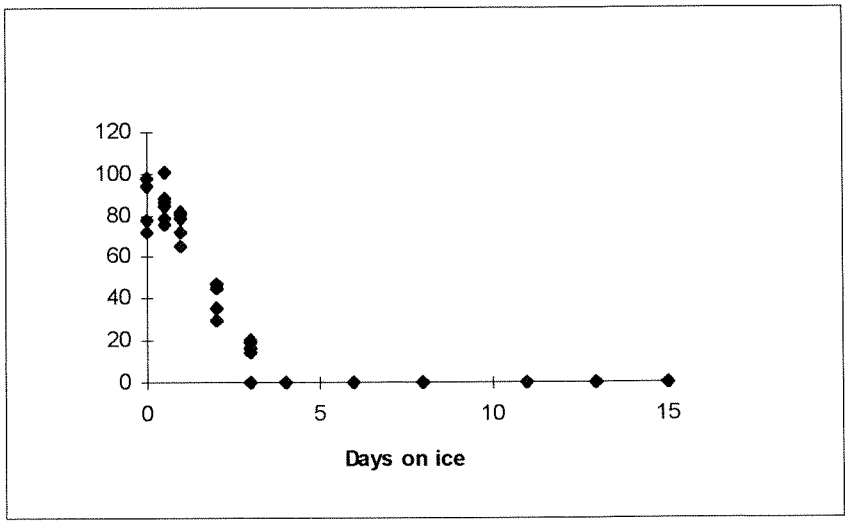


Figure 7 - Evolution of tocopherol level (mg tocopherol/Kg fat) of sardine lipids during 15 days of ice storage.

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