# ASSESSMENT OF QUALITY CHANGES IN FROZEN SARDINE (*Sardina pilchardus*) BY FLUORESCENCE DETECTION

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## ABSTRACT

The formation of fluorescent compounds was tested as a quality assessment during the frozen storage of sardine at –18°C (up to 24 months) and –10°C (up to 120 days). The fluorescence ratio between two excitation/emission maxima (393/463 nm and 327/415 nm) was studied in the aqueous ($\delta F_{aq}$) and organic ($\delta F_{or}$) extracts after Bligh and Dyer extraction of the white muscle. Fluorescence results were compared to common quality indices (total volatile base-nitrogen, TVB-N; conjugated dienes; thiobarbituric acid, TBA-i; free fatty acids, FFA). The $\delta F_{aq}$ showed good correlations with the storage time (r = 0.80 and r = 0.72, at –18 and –10°C, respectively) and the TBA-i (r = 0.92 and r = 0.81). The principal component analysis grouped the $\delta F_{aq}$ with quality indices that are sensitive for the assessment of fish damage during the frozen storage at both temperatures (TBA-i and FFA at –18°C; BVT-N, TBA-i and FFA at –10°C). According to the present results, fluorescence detection of interaction compounds in the aqueous phase can provide an accurate method to assess quality differences during the frozen storage of fish.



Key Words: Fluorescence, frozen storage, interaction compounds, lipid damages, sardine.

Running Title: Fluorescence in frozen sardine

## INTRODUCTION

Canned fish and other marine species are products of large economic importance in many countries. Many of the problems encountered with poor quality canned fish can be related with the quality of the raw material (1). Frozen storage is the most utilized method in canneries for preserving fish prior to canning. Fish species employed possess a high lipid content (2, 3), which in addition have a high proportion of polyunsaturated fatty acids (PUFA) typical of marine lipids (4). During the storage period enzymatic and non enzymatic lipid oxidation (5, 6) can become a very important factor responsible for fish damage. A close relationship has been found between the lipid damages and the quality of the raw material employed for canning, and as a result with the quality of the final canned product (7, 8).

Many methods have been used to measure lipid oxidation in foods as a means for determining the degree of damage (9, 10). However, some difficulties were found with common methods when quality has to be assessed because oxidation products are unstable and tend to react with biological amino constituents (proteins, peptides, free amino acids and phospholipids), leading to interaction compounds (11-14).

The analysis of the above interaction products by fluorescence detection has become a complementary method to the other more sophisticated measurements for assessing lipid damage (15-18). Recent studies have measured the fluorescent properties of thermally treated fish at different excitation/emission maxima (19-21). Increments of lipid oxidation, time and temperature of processing produced a fluorescence shift towards higher wavelength maxima. The fluorescence ratio between two of these maxima (393/463 nm and 327/415 nm) showed an interesting correlation with fish quality after cooking and during chilling (20-22).

The present work was design to test the fluorescent compounds detection as a quality assessment index for frozen stored sardine. Two frozen storage temperatures (-18°C and -10°C) were studied. The fluorescence shift, measured as the above mentioned ratio, was studied together with common quality estimations (total volatile base-nitrogen, conjugated dienes, thiobarbituric acid index, free fatty acids).

## MATERIALS AND METHODS

### Raw material, freezing, frozen storage and sampling

Fresh sardines (*Sardina pilchardus*) were obtained in a local market. Upon arrival in our laboratory, individual fish were frozen at -40°C and then distributed into two storage conditions: -18°C and -10°C. At each storage temperature, sardines were divided into three batches that were analyzed separately along the whole experiment. Sardines stored at -18°C were sampled at month 0.5, 2, 4, 8, 12,

18 and 24 and those stored at -10ºC were sampled at day 3, 10, 25, 60 and 120. In each batch of each storage temperature, analyses were performed on the homogenized white muscle from three individual sardines.

Water and lipid contents

Water content was determined by weight difference of the homogenized muscle (1-2g) before and after 24 hours at 105°C. Lipids were extracted by the Bligh and Dyer (23) method. Quantification was carried out according to Herbes and Allen (24).

Total volatile base-nitrogen (TVB-N) determination

TVB-N was measured by the Antonacopoulos (25) method with some modifications. Fish muscle (10g) was extracted with perchloric acid (6%) and made up to 50 mL. TVB-N content was determined by steam distillation of the acid extracts made alkaline to pH 13 with NaOH (20%), followed by titration of the distillate with 10 mM hydrochloric acid. Data were expressed as mg TVB-N/ 100g muscle.

Determination of lipid damages

Conjugated dienes (CD) formation was measured at 233 nm (10). Results are expressed according to the following formula (15): CD = B x V / w, where B is the absorbance reading at 233 nm, V denotes the volume (mL) of the sample and w is the mass (mg) of the lipid sample.

The thiobarbituric acid index (TBA-i) (mg malondialdehyde / kg sample) was determined according to Vyncke (26).

Free fatty acids (FFA) content was determined by the Lowry and Tinsley (27) method based on complex formation with cupric acetate-pyridine. Results are expressed as g FFA / 100g lipids.

Fluorescence analysis

A Perkin-Elmer LS 3B fluorescence spectrophotometer was employed. Fluorescence formation was studied at 393/463 nm and 327/415 nm according to previous experiences (21, 22). The relative fluorescences (RF) were calculated as: $RF = F / F_{st}$, where F is the sample fluorescence at each excitation/emission maximum, and $F_{st}$ is the corresponding fluorescence intensity of a quinine sulfate solution (1 μg/mL in 0.05 M $H_2SO_4$). The fluorescence shift (δF) was calculated as the ratio between both RF values: $\delta F = RF_{393/463\ nm} / RF_{327/415\ nm}$. The δF value was studied in the aqueous ($\delta F_{aq}$) and organic ($\delta F_{or}$) phases resulting from the lipid extraction (23); the ratio between both values ($\delta F_{or} / \delta F_{aq}$) was also evaluated.

Statistical analysis

The *Statistica* package (28) was employed. Data from the different damage measurements were subjected to the ANOVA one-way method ($p < 0.05$), correlation analysis and factor analysis (principal components) ($p < 0.05$ and $p < 0.01$). A varimax normalized rotation was employed for factor rotation.

## RESULTS

Water contents ranged between 68% and 71% in all samples. No significant differences ($p < 0.05$) were obtained as a result of temperatures of frozen storage (-18°C and -10°C). Lipid contents ranged between 4% and 7% (wet basis). Little differences observed between samples, could be explained as a result of lipid content variations in individual fishes, and not as a result of the frozen storage.

Quality measurements

Sardines stored at –18°C produced TVB-N during the storage period at a slower rate than those stored at –10°C. A slight increase with the storage time was observed at –10°C while no clear tendency was detected at –18°C. The amount of volatile amines nitrogen has been widely employed for the estimation of fish quality during and after several processes (29, 30). In the case of frozen stored fish, volatile amines result from the breakdown of either trimethylamine oxide (in the case of gadoid fish possessing) or amino acids, leading to the accumulation of dimethylamine and $NH_3$, respectively (31). Since non gadoid fish do not show trimethylamine oxide demethylase activity (32), it might be expected that the TVB-N formed during the frozen storage of sardines comes mainly from the deamination of amino acids.

Primary oxidation products (conjugated dienes) showed some significant differences although a definite pattern during the storage time at both frozen temperatures could not be observed (Tables 1 and 2). It is concluded that the CD content was not suitable as a quality measurement since dienes are relatively unstable and capable of interacting with other constituents (21, 22, 33).

Secondary lipid oxidation products were measured by the TBA-i. TBA-i determined of this index at –18°C (Table 1) showed a significant increase after 4 months of storage. Thereafter, progressive increases were found along the whole storage. A similar behavior was obtained at –10°C (Table 2), where a significant increase was obtained after 10 days of storage. According to previous experiences (34, 35), the TBA-i has shown to be an accurate way of assessing quality changes during the frozen storage time.

Lipid hydrolysis was measured by the FFA. A progressive increase of the FFA content was observed throughout the storage at both frozen temperatures (Tables 1 and 2). At -18°C increases were detected after 0.5, 12 and 24 months of storage. At -10°C differences were observed after 3 and 25 days

of storage. According to previous experiences (34, 36), the FFA formation as a result of the lipid hydrolysis (triglycerides and phospholipids classes) has provided a suitable means for assessment of fish damage during the frozen storage.

Fluorescence measurements

The fluorescence shift measured in the organic phase resulting from the Bligh and Dyer (23) extraction ($\delta F_{or}$) of the different frozen samples was studied. Both storage temperatures (Tables 1 and 2) showed a progressive increase of the $\delta F_{or}$ value with time, except for the end of the storage where a decrease was detected (24 months at –18°C and 120 days at –10°C). Significant increases were obtained after 0.5 and 18 months (at –18°C) and after 10 and 60 days (at –10°C).

However, measurement of fluorescence in the aqueous phase resulting from Bligh and Dyer (23) extraction ($\delta F_{aq}$ value) showed a progressive increase along the whole storage at both temperatures (Tables 1 and 2). At -18°C significant increases were detected after 12, 18 and 24 months, while at -10°C increases were observed after 60 and 120 days.

In order to study the relative formation of fluorescent compounds that are soluble in organic solvents and water, the $\delta F_{or}/\delta F_{aq}$ ratio was evaluated. A similar behavior was obtained at both frozen temperatures (Tables 1 and 2). An increase in the $\delta F_{or}/\delta F_{aq}$ ratio was observed during the first steps of the storage, that was followed by a sharp decrease after 12 months (at –18°C) and after 25 days (at –10°C).

A similar behavior was observed by studying the $\delta F_{or}/\delta F_{aq}$ ratio during sardine storage at 0°C and 10°C (22). In the above work, during the first steps of the storage, fluorescent compounds responsible for the $\delta F$ value were mostly lipid-soluble; however, as the lipid damage increased, these kinds of compounds became progressively more soluble in the aqueous phase.

Fluorescent compounds studies based on a single excitation/emission maximum have been mostly carried out on organic extracts (lipids) and have shown high correlations with sensory measurements and storage times (16, 17). However, experimental evidence has demonstrated that fluorescent substances formed from oxidized membrane lipids remain attached to the amino constituents resulting in compounds quite insoluble in organic solvents (37-39). According to our results based on fluorescence ratios, the analysis of the aqueous phase can provide a more accurate assessment of the fluorescent properties of the interaction compounds formed as a result of the fish damage.

Correlation and multivariate analyses

The different damage indices were tested for correlations with storage time and also with each other (Tables 3 and 4). Almost every index correlates well with the storage time (ST) at both temperatures, except for the CD which did not vary with storage time. Indices behave similarly at both storage temperatures. Two indices (TBA-i and FFA) correlate best with storage time at both temperatures and

according to previous experiences could be used as an indication of quality deterioration of frozen sardine (34-36).

Concerning the fluorescence measurements, both indices ($\delta F_{or}$ and $\delta F_{aq}$) reflected good correlations with the storage time at both temperatures and showed to be strongly correlated with those lipid damage indices that also show variation with storage time (FFA and TBA-i). Correlation data are better for the $\delta F_{aq}$ than for the $\delta F_{or}$ , especially in the case of –18°C storage.

Principal component analysis showed that the 80.0 % and 80.9 % of the variability observed at –18 and –10°C, respectively, could be explained by two factors. These factor loadings are graphically displayed in Figures 1 and 2. Figure 1 (-18°C) shows that ST, FFA, TBA-i and $\delta F_{aq}$ are grouped together at high loading in the Factor 1 axis, whereas their loading values for Factor 2 are low; meanwhile CD, TVB-N and $\delta F_{or}$ have clearly higher loadings in Factor 2. In the case of –10°C (Figure 2) similar results can be concluded, with the exception that the TVB-N index has a high loading in Factor 1 axis and is grouped together with the ST, FFA, TBA-i and $\delta F_{aq}$.

## DISCUSSION

In previous experiences related to chilling, cooking and canning lipid deterioration compounds (peroxides and carbonyls) have caused the formation of interaction compounds with fluorescent properties (20-22). Accelerated by the temperature, fluorescent compounds formed in the first stages of interaction led to the formation of other fluorescent compounds showing excitation/emission maxima at higher wavelengths than their precursors. The fluorescence ratio between two of these maxima (393/463 nm and 327/415 nm) provided an interesting method for quality assessment.

The aim of the present work was to study the fluorescence formation during the frozen storage and to test the fluorescence ratio as a way of assessing fish quality changes. Previous research on frozen storage (40, 41) had detected some fluorescence that could be related to the quality of product.

In the present experiment, fluorescence detection of interaction compounds was accomplished in both the aqueous and organic phases resulting from the lipid extraction. As in the case of chilling, the fluorescence analysis of the aqueous phase ($\delta F_{aq}$) provided better results than that of the organic one ($\delta F_{or}$). $\delta F_{aq}$ showed a good correlation with the storage time and grouped together with indices that reflect fish damage at –18°C (TBA-i and FFA) and at –10°C (TBA-i, FFA and TVB-N).

The satisfactory correlation of fluorescence measurements with secondary lipid oxidation (TBA-i) is in accord with the general theory about fluorescent compounds formation as a result of interaction between carbonyl compounds (electrophilic molecules) formed during lipid oxidation and amine compounds (nucleophilic molecules) present in the fish muscle (13, 14, 42).

A good correlation between lipid hydrolysis (FFA) and lipid oxidation (TBA-i) has also been obtained. Some previous experiences have proved the influence of the lipid hydrolysis on the lipid oxidation (43, 44). According to the correlations obtained, a positive influence of the FFA content on the fluorescent compounds formation can be inferred.

Results obtained in this work reinforce the role of fluorescence detection of interaction compounds as a tool for quality assessment, especially when processing food with a high content of unsaturated lipids and amino compounds. This is the case of marine species, that are known to contain a high amounts of polyunsaturated fatty acids (4) and of free amino nitrogen compounds (45).

Acknowledgments

The authors thank *Mr. Oscar Bellón* and *Mr. Marcos Trigo* for technical assistance and the Xunta de Galicia for financial support (*Project XUGA 402 01B93*).

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TABLE 1: Quality measurements* and fluorescence shifts* during frozen storage at -18°C**.

| ST (months) | TVB-N | CD | TBA-i | FFA | $\delta F_{or}$ | $\delta F_{aq}$ | $\delta F_{or}/\delta F_{aq}$ |
|---|---|---|---|---|---|---|---|
| 0 | 25.6 a (0.67) | 1.2 bc (0.12) | 0.5 a (0.05) | 1.1 a (0.14) | 0.4 a (0.04) | 0.4 a (0.02) | 1.2 b (0.10) |
| 0.5 | 24.2 a (0.26) | 1.5 cd (0.14) | 1.6 ab (0.28) | 4.2 b (0.63) | 1.7 b (0.20) | 0.3 a (0.02) | 5.1 d (0.33) |
| 2 | 29.5 b (0.52) | 0.9 ab (0.03) | 2.8 ab (0.61) | 3.0 b (0.33) | 1.9 b (0.14) | 0.3 a (0.02) | 5.7 d (0.64) |
| 4 | 27.2 ab (1.08) | 1.2 bc (0.01) | 5.0 bc (0.87) | 4.1 b (0.20) | 1.5 b (0.02) | 1.2 ab (0.10) | 1.3 b (0.13) |
| 8 | 24.1 a (0.96) | 0.8 ab (0.07) | 3.9 ab (0.11) | 4.0 b (0.16) | 2.4 b (0.40) | 1.0 ab (0.07) | 2.3 c (0.30) |
| 12 | 35.6 c (0.30) | 2.2 e (0.37) | 8.6 c (0.67) | 7.1 c (0.37) | 2.3 b (0.60) | 2.4 b (0.33) | 0.9 ab (0.15) |
| 18 | 29.6 b (2.63) | 1.9 de (0.14) | 9.6 c (2.10) | 6.4 c (0.60) | 3.7 c (0.15) | 5.9 c (0.42) | 0.6 ab (0.04) |
| 24 | 30.2 b (0.56) | 0.7 a (0.03) | 29.9 d (2.32) | 11.8 d (0.63) | 2.2 b (0.45) | 12.6 d (1.28) | 0.2 a (0.06) |

* Mean values of three determinations. Values in the same column followed by different letters are significantly different ($p < 0.05$). Standard errors of the means are indicated in brackets.

** Abbreviations: ST (storage time), TVB-N (total volatile base-nitrogen), CD (conjugated dienes), TBA-y (thiobarbituric acid index), FFA (free fatty acids), $\delta F_{or}$ (fluorescence shift in the organic phase), $\delta F_{aq}$ (fluorescence shift in the aqueous phase)

TABLE 2: Quality measurements* and fluorescence shifts* during frozen storage at –10°C**.

| ST (days) | TVB-N | CD | TBA-i | FFA | $\delta F_{or}$ | $\delta F_{aq}$ | $\delta F_{or}/\delta F_{aq}$ |
|---|---|---|---|---|---|---|---|
| 0 | 25.6 b (0.67) | 1.2 abc (0.12) | 0.5 a (0.05) | 1.1 a (0.14) | 0.4 a (0.04) | 0.4 a (0.02) | 1.2 a (0.10) |
| 3 | 28.5 c (1.13) | 1.4 c (0.06) | 1.9 ab (0.16) | 3.8 b (0.47) | 0.7 a (0.05) | 0.3 a (0.02) | 2.6 a (0.23) |
| 10 | 22.2 a (0.52) | 1.3 bc (0.16) | 2.9 b (0.41) | 3.1 ab (0.52) | 2.0 b (0.20) | 0.3 a (0.02) | 8.1 c (1.36) |
| 25 | 29.3 cd (0.45) | 1.1 ab (0.02) | 5.4 c (0.41) | 6.9 c (0.40) | 1.8 b (0.08) | 0.4 a (0.05) | 5.2 b (0.65) |
| 60 | 28.4 c (0.93) | 1.0 a (0.07) | 5.2 C (0.20) | 7.8 C (1.74) | 3.1 C (0.59) | 0.5 b (0.07) | 5.7 B (0.93) |
| 120 | 31.1 d (0.28) | 1.4 c (0.09) | 8.7 d (1.07) | 8.5 c (0.07) | 1.8 b (0.14) | 2.2 c (0.05) | 0.8 a (0.08) |

* Mean values of three determinations. Values in the same column followed by different letters are significantly different ($p < 0.05$). Standard errors of the means are indicated in brackets.

** Abbreviations as specified in Table 1.

Table 3: Correlation matrix for different parameters*** (storage time and quality estimations) measured during sardine storage at -18ºC.

| | TVB-N | CD | TBA-i | FFA | $\delta F_{or}$ | $\delta F_{aq}$ |
|---|---|---|---|---|---|---|
| ST | 0.52* | 0.09 | 0.79** | 0.82** | 0.65* | 0.80** |
| TVB-N | | 0.43* | 0.36 | 0.46* | 0.35 | 0.34 |
| CD | | | -0.22 | -0.10 | 0.23 | -0.15 |
| TBA-i | | | | 0.87** | 0.35 | 0.92** |
| FFA | | | | | 0.50* | 0.83** |
| $\delta F_{or}$ | | | | | | 0.36 |

* Significance: $p < 0.05$.

** Significance: $p < 0.01$.

*** Abbreviations as specified in Table 1.

Table 4: Correlation matrix for different parameters (storage time and quality estimations) measured during sardine storage at -10ºC.

| | TVB-N | CD | TBA-i | FFA | $\delta F_{or}$ | $\delta F_{aq}$ |
|---|---|---|---|---|---|---|
| ST | 0.55* | -0.07 | 0.94** | 0.88** | 0.72* | 0.72* |
| TVB-N | | 0.07 | 0.61* | 0.71* | 0.14 | 0.60* |
| CD | | | 0.14 | -0.03 | -0.24 | 0.27 |
| TBA-i | | | | 0.84** | 0.56* | 0.81** |
| FFA | | | | | 0.73* | 0.59* |
| $\delta F_{or}$ | | | | | | 0.17 |

* Significance: $p < 0.05$.

** Significance: $p < 0.01$.

*** Abbreviations as specified in Table 1.

## FIGURE LEGENDS

Figure 1: Principal component analysis for different parameters (storage time and quality indices) measured at –18°C storage (abbreviations as specified in Table 1).

Figure 2: Principal component analysis for different parameters (storage time and quality indices) measured at –10°C storage (abbreviations as specified in Table 1).