QUALITY ASSESSMENT OF SARDINES DURING STORAGE BY MEASUREMENT OF FLUORESCENT COMPOUNDS

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ABSTRACT

In the present study, sardine alterations during chilled storage (0°C) were investigated by measuring the fluorescence ratio between two excitation/emission maxima (393/463 nm 327/415 nm) and compared with common quality indices. Sardines were also maintained at 15°C to accelerate all the reactions occurring at 0°C. The behavior of quality indices (total volatile base-nitrogen, free fatty acids content, formation of conjugated dienes, and thiobarbituric acid values) were determined and the use of the fluorescence ratio (fluorescence shift, δF) was analyzed. A good correlation was found between the δF and the total volatile base-nitrogen ($r = 0.93$, at 0°C; $r = 0.92$, at 15°C).

Key Words: Quality, lipids, interaction compounds, fluorescence, sardines.

INTRODUCTION

Fish foods have been recognized as playing a positive role in human nutrition because of the highly unsaturated composition of their lipids (Kinsella, 1987; Ackman and Ratnayake, 1990). However, during processing and/or storage, fish might lose quality as a result of a number of factors. One of the most important factors concerns the oxidation of the highly unsaturated lipid composition (Pearson et al., 1977; Pigott and Tucker, 1987).

Many methods have been used to measure primary (peroxides) and secondary (carbonyl compounds) oxidation products in foods as a way of determining the degree of food damage (Melton, 1983; Kim and Labella, 1987). Further, the influence of lipid hydrolysis on the formation of oxidation products has also attracted attention (Miyashita and Takagi, 1986; Han and Liston, 1988).

However, it has been proved that primary and secondary oxidation products may react with biological amino constituents (proteins, peptides, free amino acids and phospholipids) to produce interaction compounds (Pokorny, 1977; Gardner, 1979; Leake and Karel, 1985; Montfoort et al., 1987). The analysis of these kinds of products by means of their fluorescent properties has become a rapid and useful way of assessing lipid damage. So far, these measurements have been conducted only at a single excitation/emission wavelength maximum (Lubis and Buckle, 1990; Miyagawa et al., 1991) and are considered now as complementary of other more developed measurements.

Previous studies carried out have measured the fluorescence properties of processed fish samples at different excitation/emission maxima (Aubourg et al., 1992a, 1992b, 1995). From a qualitative point of view, a fluorescence shift towards higher

1 wavelength maxima was observed as a result of increasing the presence of lipid
2 oxidation products and the time and temperature of processing. Indeed, the fluorescence
3 ratio between two of these maxima (F3/F1, where F3 is 393/463 nm and F1 is 327/415
4 nm) showed an interesting correlation with fish quality (Aubourg et al., 1992b, 1995).

5 In the present study, sardine alterations during chilled storage (0°C) were
6 investigated. As a complementary experiment, sardines were also maintained at 15°C in
7 order to accelerate all the reactions occurring at 0°C. The fluorescence shift measured
8 as the F3/F1 ratio was studied together with other quality estimations (total volatile
9 base-nitrogen and free fatty acids contents, formation of conjugated dienes,
10 thiobarbituric acid index) (El Marrakchi et al., 1990; Lubis and Buckle, 1990; Hwang
11 and Regenstein, 1993). The behavior of all the quality indexes was tested and the
12 usefulness of the fluorescence ratio was analyzed.

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14

15 **MATERIALS AND METHODS**

16

17 **Raw material, processing and sampling**

18 Fresh sardines (*Sardina pilchardus*) were obtained in a local market. Upon
19 arrival in our laboratory, individual fish were stored in isothermal rooms at 0°C (on ice)
20 and 15 °C. At each temperature, sardines were divided into three batches. Samples were
21 taken for analyses at day 1, 3, 9 and 16 in the case of storage at 0 °C, and at day 1, 2, 4
22 and 8 at 15 °C. In each batch of both storage temperatures, analyses were carried out on
23 the homogenized white muscle from four individual sardines.

24

25

Basic analyses and total volatile base-nitrogen (TVB-N) determination

Water content was determined by weight difference between the fresh homogenized muscle (1-2 g) and after 24 hr at 105 °C (Aursand et al., 1994). Results are expressed as g water/100g muscle. Lipids were extracted by the Bligh and Dyer (1959) method. Quantification was carried out according to Herbes and Allen (1983). Results are expressed as g lipids/100g wet muscle.

TVB-N were measured by the Antonacopoulos (1960) method with some modifications. Ten grams fish muscle was extracted with perchloric acid (6 %) and made up to 50 mL. TVB-N content was obtained 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 are expressed as mg TVB-N/100g muscle.

Lipid damage measurements

Free fatty acids (FFA) content was determined by the Lowry and Tinsley (1976) method based on complex formation with cupric acetate-pyridine. Results are expressed as g FFA/100g lipids.

Conjugated diene (CD) formation was measured at 233 nm (Kim and Labella, 1987). The result is expressed according to the following formula (Smith et al., 1990):

$$CD = \frac{B \times V}{w}$$

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 extract measured. The thiobarbituric acid (TBA) index (mg malondialdehyde/kg sample) was determined according to Vyncke (1970).

Fluorescence analyses

A Perkin-Elmer LS 3B fluorescence spectrophotometer was employed. The fluorescence shift (δF) was calculated as follows:

$$\delta F = \frac{F3 / F3_{st}}{F1 / F1_{st}}$$

where F3 and F1 are the fluorescence intensities of the sample at 393/463 nm and 327/415 nm, respectively. $F3_{st}$ and $F1_{st}$ are the fluorescence intensities of a quinine sulfate solution (1 μg/mL in 0.05 M H_2SO_4) at the corresponding wavelength. This fluorescence shift was calculated on the aqueous (δF_{aq}) and organic (δF_{or}) phases resulting from the lipid extraction (Bligh and Dyer, 1959).

A mixture of 5 g sardine muscle, 5 mL chloroform and 2 mg propyl gallate was prepared. Methanol and water were added to obtain a 1/2/0.8 chloroform-methanol-water ratio. The mixture was homogenized and then chloroform and water were added so that a 2/2/1.8 chloroform-methanol-water ratio was attained. The mixture was centrifuged at 3000xg for 10 min. The resulting aqueous phase was directly employed for the fluorescence determination (δF_{aq}). The remaining organic phase (lipids) was washed with 0.5 % sodium chloride (5 mL), then with water (5 mL) and finally dried

over anhydrous sodium sulfate. The lipids extract was made up to 15 mL with chloroform and directly employed for fluorescence detection (δF_{or}).

Samples were previously exposed to U.V. light (350 nm) for 30 s, to destroy any retinol present. Sensitive setting was 5 (samples) and 0.5 (quinine standard solution). Fluorescence was measured at room temperature (16-18°C) with a 10 mm path-length quartz cuvette.

Statistical analyses

Data from the different measurements of quality were subjected to the ANOVA one-way method according to Sokal and Rohlf (1981). Correlation analysis and factor analysis (principal components) were carried out with all the variables studied. A varimax normalized rotation was employed for factor rotation.

RESULTS AND DISCUSSION

Water and lipid contents showed very little differences between the samples investigated (Tables 1 and 2). A slight increase in water content was observed at storage at 0°C (on ice) that can be explained as a result of contact with ice.

Quality measurements

TVB-N content did not differ significantly (Table 1) during the first nine days of storage at 0°C. At 16 days an increase was observed indicating the end of the lag phase of microorganisms. Similarly, Bennour et al. (1991) observed a large increase after ten days during chilling of mackerel.

1 At 15°C the production of volatile amines was much more rapid (Table 2). In
2 this case, no lag phase could be noticed. A gradual increase was obtained throughout the
3 storage period. El Marrakchi et al. (1990) observed a similar behavior in TVB-N
4 production during the storage of sardines at 22-24°C.

5 The FFA content at 0°C increased after the third day, and then remained fairly
6 constant till the end of the storage (Table 1). Hwang and Regenstein (1993) stored
7 minced white muscle of mackerel at 2-3°C. During the first seven days of storage,
8 similar behavior was obtained when compared to the present results. However, at the
9 end of their storage (day 15) an increase in FFA content was obtained which is more
10 like our values at 15°C. However, in the present experiment at 15°C (Table 2) values
11 also increased after day 4.

12 At 0°C the CD content increased the first day and then remained quite constant
13 (Table 1). At 15°C no increase in CD until day 4 was observed, and then it remained
14 constant till the end of the storage (day 8) (Table 2). It can be concluded that the CD
15 content did not fit as a quality measurement since dienes are relatively unstable
16 molecules capable of interacting with other food constituents (Shimasaki et al., 1977;
17 Cho et al., 1989; Aubourg et al., 1995). However, in the case of starting damages, the
18 CD measurement has been used satisfactorily as a quality determination (Sergent et al.,
19 1993; Vossen et al., 1993).

20 The TBA index (TBA-i) increased on day 3 at 0°C (Table 1) but decreased on
21 day 16. At 15°C (Table 2) an increase was observed after the first day. Then, no
22 differences were obtained throughout the storage. As in the case of CD, the TBA-i did
23 not fit as a quality measurement since carbonyl compounds that could be measured by
24 this method are relatively unstable molecules capable of interacting with food
25 constituents (Igene et al., 1979; Gardner, 1979; Kim and Labella, 1987).

1

2 **Fluorescence measurements**

3 At 0°C (Table 1) both fluorescence shifts in the aqueous (δF_{aq}) and organic
4 (δF_{or}) phases showed an increase after day 9 and at the end of the storage. During
5 storage at 15°C (Table 2) an increase in both fluorescence shifts was also obtained after
6 days 4 and 8 of storage in the aqueous phase; in the case of the organic phase,
7 differences were only detected after day 8.

8 With the aim of studying the relative formation of fluorescenct compounds that
9 are organic and water soluble, the δF_{or} / δF_{aq} ratios were evaluated at both temperatures
10 of storage. At 0°C (Table 1) an increase in the proportion of the fluorescence shift of the
11 organic phase was observed after days 3 and 9; then a decrease was obtained after the
12 end of the storage. According to the behavior of chilled sardine (El Marrakchi et al.,
13 1990) and the present results on TVB-N, it seemed as if the δF_{or} / δF_{aq} increased during
14 the first stages of fish damaging (days 3 and 9); then, after day 9, when fish damage
15 became very important, a relative decrease of the fluorescence shift of the organic phase
16 was observed. At 15°C no changes in the δF_{or} / δF_{aq} ratio were observed through day 4
17 but an increase was obtained after day 8, compared to 0 days.

18 Fluorescent compounds studies have been mostly carried out on organic extracts
19 (lipids) and have led to good correlations with sensory measurements and storage times
20 (Bouzas et al., 1985; Maruf et al., 1990; Lubis and Buckle, 1990). However,
21 experimental evidence has been reported demonstrating that fluorescent substances
22 formed from oxidized membrane lipids possibly with amino compounds remain
23 attached to the amino constituent resulting in interaction compounds quite insoluble in
24 organic solvents (Shimasaki et al., 1984; Iio and Yoden, 1988; Hasegawa et al., 1992,

1 1993). According to the present results, the use of both kinds of determinations may be
2 necessary to provide a more accurate study of interaction compounds formation.
3
4 **Correlation and multivariate analyses**

5 The different quality measurements were tested for correlation with storage time
6 and also among themselves. Table 3 (0°C on ice) and Table 4 (complementary
7 experiment at 15°C) present the correlation matrices for the different indicators of
8 quality loss at both temperatures.

9 According to the results previously shown in Table 1, at 0°C only three indexes
10 (TVB-N, δF_{aq} and δF_{or}) showed significant values with respect to the time of storage.
11 The remaining quality indexes (TBA-i, FFA and CD) did not show satisfactory
12 correlations with the storage time. The best correlation between quality measurements
13 was obtained for the TVB-N and δF_{aq} ($r = 0.93$) (Table 3). These two measurements are
14 compared in Fig. 1.

15 Very different results were obtained in the case of storage at 15°C (Table 4).
16 This can be explained by the fact that conditions of storage accelerated all damaging
17 events, so that most of the indexes increased easily with time (Table 2). All the quality
18 measurements showed significant relationships with storage time. The highest
19 correlations were observed for the TVB-N ($r = 0.99$), δF_{aq} ($r = 0.93$) and FFA ($r = 0.93$)
20 (Table 4). Again, the δF_{aq} ratio showed good correlations with other quality
21 measurements such as TVB-N ($r = 0.92$) and FFA ($r = 0.92$) (Table 4). Comparison of
22 TVB-N and δF_{aq} measurements can be observed in Fig. 1.
23
24 In order to seggregate the different quality methods into different factors
25 principal component analysis (PCA) was carried out with the results obtained during

chilling storage. Using this method, 87.29 % of the variability of the seven variables under study could be explained with only two factors. Following a Varimax rotation (Table 5), it was found that Factor 1 alone accounted for 60.52 % of the variability. Factor 1 had a very high loading (> 0.90) on the following variables: time, TVB-N, δF_{aq} and δF_{or}. Factor 2 accounted for about 26.77 % of the variability and showed high loadings (> 0.82) on TBA-i, CD and FFA.

Figure 2 presents the loadings of both factors. Variables can be seen divided into different groups. The first one includes time of storage together with quality methods that show better correlations with storage time (TVB-N and both fluorescence shifts). Another group accounts for the TBA-i and FFA. Finally, the CD content can be shown isolated.

Conclusions

During storage at 0°C on ice (chilling) both fluorescence shifts (δF_{aq} and δF_{or}) were useful in assessing fish damage. The remaining lipid damage measurements did not permit quality assessment throughout the whole storage period, because compounds measured in such indexes were unstable and capable of reacting with other food constituents. Concerning the complementary experiment at 15°C a different situation was observed. All the methods tested (traditional quality methods and fluorescence measurements) showed good correlation with storage time. The fluorescence shift measured in the aqueous phase was found more suitable than the one in the organic phase, although the use of both kinds of extracts could provide a more accurate study of fluorescence formation. Lipid damage compounds (peroxides, carbonyls) have led to the formation of interaction compounds, whose fluorescent properties have provided a valuable method for the assessment of quality.

ACKNOWLEDGEMENTS

The authors thank Mr. Oscar Bellón for technical assitance and Xunta de Galicia for financial support (Project XUGA 402 01B93).

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1 **TABLE 1** Chemical analysis, quality measurements and fluorescence shifts during
2 sardine chilling (0°C)*
3
4

5 Measurement**	Storage time (days)				
7	0	1	3	9	16
9-10 H_2O	68.2 a (2.19)	69.7 ab (1.76)	71.8 bc (0.22)	72.4 bc (1.85)	74.1 c (0.29)
12-13 LC	7.11 a (1.80)	7.90 a (1.06)	5.71 a (0.94)	7.25 a (1.26)	6.30 a (0.86)
15-16 TVB-N	25.55 a (0.94)	26.74 a (1.13)	25.71 a (0.82)	26.67 a (2.59)	64.48 b (1.29)
18-19 FFA	1.11 a (0.19)	2.02 ab (0.47)	4.41 c (1.13)	3.47 bc (1.56)	3.96 bc (0.35)
21-22 CD	1.19 a (0.17)	2.31 b (0.26)	2.29 b (0.30)	2.03 b (0.42)	1.82 b (0.19)
24-25 TBA-i	0.48 a (0.08)	2.04 a (0.41)	6.43 bc (0.81)	8.03 c (1.66)	4.70 b (0.42)
27-28 δF_{aq}	0.37 a (0.03)	0.32 a (0.04)	0.36 a (0.04)	0.89 b (0.19)	2.17 c (0.33)
30-31 δF_{or}	0.43 a (0.05)	0.63 a (0.12)	1.12 a (0.14)	4.93 b (0.96)	6.71 c (1.05)
33-34 $\delta F_{or}/\delta F_{aq}$	1.15 a (0.14)	2.01 a (0.27)	3.15 b (0.64)	5.55 c (0.58)	3.09 b (0.04)

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36
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38
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40 * Mean values of three determinations. Values in the same row followed by different
41 letters are significantly different ($p<0.05$). Standard deviations are indicated in brackets.
42
43 **Abbreviations and units employed: **LC** (lipid content; g lipids/100g muscle), **TVB-N**
44 (total volatile base-nitrogen; mg TVB-N/100g sample), **FFA** (free fatty acids; g
45 FFA/100g lipids), **CD** (conjugated dienes; calculated as expressed in the Materials and
46 Methods section), **TBA-i** (thiobarbituric acid index; mg malondialdehyde/Kg sample),
47 $\boldsymbol{\delta F_{aq}}$ (fluorescence shift in the aqueous phase) and $\boldsymbol{\delta F_{or}}$ (fluorescence shift in the
48 organic phase) (both fluorescence determinations calculated as expressed in the
49 Materials and Methods section).

TABLE 2 Chemical analysis, quality measurements and fluorescence shifts during sardine storage at 15°C*

Measurement**	Storage Time (days)				
	0	1	2	4	8
H_2O	68.2 a (2.19)	68.2 a (1.42)	70.7 a (1.14)	68.4 a (1.59)	70.7 a (0.49)
LC	7.11 a (1.80)	7.37 a (0.93)	5.55 a (1.09)	5.66 a (0.38)	6.70 a (1.04)
TVB-N	25.55 a (0.94)	39.78 b (2.83)	86.41 c (6.49)	183.22 d (4.09)	400.11 e (10.31)
FFA	1.11 a (0.19)	4.44 ab (0.30)	4.36 ab (0.24)	7.53 b (0.72)	15.90 c (3.76)
CD	1.19 a (0.17)	1.29 a (0.32)	1.34 a (0.17)	2.46 b (0.06)	2.60 b (0.36)
TBA-i	0.48 a (0.08)	3.11 b (0.74)	3.80 b (0.69)	4.23 b (0.44)	3.92 b (0.87)
δF_{aq}	0.37 a (0.03)	0.34 a (0.01)	1.00 a (0.34)	1.83 b (0.28)	2.75 c (0.57)
δF_{or}	0.43 a (0.05)	0.69 a (0.09)	1.13 a (0.10)	2.93 ab (0.30)	7.28 b (4.53)
$\delta F_{or}/\delta F_{aq}$	1.15 a (0.14)	2.01 ab (0.24)	1.26 ab (0.40)	1.64 ab (0.28)	2.50 b (1.21)

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

Abbreviations and units employed: **LC (lipid content; g lipids/100g muscle), **TVB-N** (total volatile base-nitrogen; mg TVB-N/100g sample), **FFA** (free fatty acids; g FFA/100g lipids), **CD** (conjugated dienes; calculated as expressed in the Materials and Methods section), **TBA-i** (thiobarbituric acid index; mg malondialdehyde/Kg sample), δF_{aq} (fluorescence shift in the aqueous phase) and δF_{or} (fluorescence shift in the organic phase) (both fluorescence determinations calculated as expressed in the Materials and Methods section).

1 **TABLE 3**

Correlation matrix[*] for different parameters (storage time and quality analyses) measured during sardine chilling (0°C) [**]

	STT	TBA-i	CD	TVB-N	FFA	δF_{aq}	δF_{or}
STT		0.50	0.04	**0.86**	0.50	**0.94**	**0.95**
TBA-i			0.48	0.06	**0.78**	0.28	**0.57**
CD				-0.11	**0.57**	-0.03	0.10
TVB-N					0.31	**0.93**	0.75
FFA						0.42	**0.51**
δF_{aq}							**0.92**

[*] Significant values ($p<0.05$) are expressed in bold print.

[**] Abbreviations and units employed: **STT** (storage time; days), **LC** (lipid content; g lipids/100g muscle), **TVB-N** (total volatile base-nitrogen; mg TVB-N/100g sample), **FFA** (free fatty acids; g FFA/100g lipids), **CD** (conjugated dienes; calculated as expressed in the Materials and Methods section), **TBA-i** (thiobarbituric acid index; mg malondialdehyde/Kg sample), $\boldsymbol{\delta F_{aq}}$ (fluorescence shift in the aqueous phase) and $\boldsymbol{\delta F_{or}}$ (fluorescence shift in the organic phase) (both fluorescence determinations calculated as expressed in the Materials and Methods section).

1 **TABLE 4**
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7 Correlation matrix* for different parameters (storage time and quality analyses)
8 measured during sardine storage at 15°C**
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	STT	TBA-i	CD	TVB-N	FFA	δF_{aq}	δF_{or}
STT		**0.58**	**0.85**	**0.99**	**0.93**	**0.93**	**0.77**
TBA-i			**0.61**	0.51	**0.61**	**0.59**	0.48
CD				**0.84**	**0.84**	**0.90**	**0.76**
TVB-N					**0.93**	**0.92**	**0.77**
FFA						**0.92**	**0.91**
δF_{aq}							**0.83**

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25 * Significant values ($p<0.05$) are expressed in bold print.
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27 ** Abbreviations and units employed: **STT** (storage time; days), **LC** (lipid content; g
28 lipids/100g muscle), **TVB-N** (total volatile base-nitrogen; mg TVB-N/100g sample),
29 **FFA** (free fatty acids; g FFA/100g lipids), **CD** (conjugated dienes; calculated as
30 expressed in the Materials and methods section), **TBA-i** (thiobarbituric acid index; mg
31 malondialdehyde/Kg sample), $\boldsymbol{\delta F_{aq}}$ (fluorescence shift in the aqueous phase) and $\boldsymbol{\delta F_{or}}$
32 (fluorescence shift in the organic phase) (both fluorescence determinations calculated as
33 expressed in the Materials and Methods section).

TABLE 5

Factor loadings resulting from the principal component analysis of different parameters (storage time and quality analyses) measured during sardine chilling (0°C) *

	Factor 1	Factor 2
STT	0.952	0.251
TBA-i	0.253	0.865
CD	-0.159	0.824
TVB-N	0.941	-0.082
FFA	0.349	0.845
δF_{aq}	0.982	0.093
δF_{or}	0.905	0.324

* Abbreviations and units employed: **STT** (storage time; days), **LC** (lipid content; g lipids/100g muscle), **TVB-N** (total volatile base-nitrogen; mg TVB-N/100g sample), **FFA** (free fatty acids; g FFA/100g lipids), **CD** (conjugated dienes; calculated as expressed in the Materials and Methods section), **TBA-i** (thiobarbituric acid index; mg malondialdehyde/Kg sample), **δF_{aq}** (fluorescence shift in the aqueous phase) and **δF_{or}** (fluorescence shift in the organic phase) (both fluorescence determinations calculated as expressed in the Materials and Methods section).

Comentario [JG1]:

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6 Plots of the δF_{aq} (fluorescence shift in the aqueous phase) against the TVB-N
7 (total volatile base-nitrogen content; mg TVB-N/ 100g muscle) measured during storage
8 at 0°C and 15°C.

9
10
11 Figure 2

12 Principal component analysis for the different parameters (storage time and
13 quality indexes) measured during chilling storage (STT, storage time; TVB-N, total
14 volatile base-nitrogen; FFA, free fatty acids; CD, conjugated dienes; TBA-i,
15 thiobarbituric acid index; δF_{aq}, fluorescence shift in the aqueous phase; and δF_{or},
16 fluorescence shift in the organic phase).

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