INFLUENCE OF FORMALDEHYDE IN THE FLUORESCENCE FORMATION
RELATED TO FISH DETERIORATION

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ABSTRACT

In previous studies fluorescence detection was accomplished at different excitation/emission maxima during common fish processing. A bathochromic shift towards higher wavelength maxima was observed and measured as the ratio between absorption at two of the maxima tested. This fluorescence ratio (δF) value correlated with increased fish damage. In the present work, the influence of formaldehyde (FA) in the formation of the same δF value was studied. For it, FA reacted in model systems at 30°C during 25 days with propylamine and fish muscle. It was observed that FA showed a lower ability in producing fluorescent compounds than the common fish oxidation products (propanal and hexanal) that were also tested. However, in the presence of both lipid oxidation aldehydes, the FA containing mixtures produced a higher δF value. Model systems consisting of FA and fatty fish (sardine) muscle produced a higher fluorescence development than in the case of FA and lean fish (cod), as a result of a greater presence in the first case of lipid oxidation compounds formed during the reaction conditions. From the muscle systems results, it is concluded that the presence of FA in a reacting medium enhances the fluorescence formation, so that the δF value could be an accurate tool in order to assess fish damage. This determination could be profitable in processes such as gadoid fish freezing where both the FA and lipid oxidation are produced.

Running title: Formaldehyde and fluorescence formation.

Key Words: Formaldehyde, amines, fish, fluorescence, lipid oxidation.

INTRODUCTION

Rancidity can become a very important factor of fish damage during processing (1, 2) because of the high proportion of polyunsaturated fatty acids (PUFA) found in marine lipids (3). It has been proved that primary and secondary lipid oxidation products may react with biological amino constituents (proteins, peptides, free amino acids and phospholipids) to produce interaction compounds (4-7). As a result browning (8), flavour changes (9, 10) and loss of essential nutrients (11, 12) have been observed.

The analysis of these interaction products by fluorescence detection has become a complementary way of other more developed measurements when assessing lipid damage (13, 14). Recent studies have measured the fluorescent properties of processed fatty fish samples at different excitation/emission maxima. A fluorescence shift towards higher wavelength maxima was observed as a result of increasing the time and temperature of processing. The fluorescence ratio between two of the maxima tested (393/463 nm and 327/415 nm) showed an interesting correlation with fish quality (15-17).

Lipid damages have been studied during lean fish freezing (18, 19). However, the biggest attention, specially in gadoid fish species has been given to the FA formation. During such species freezing, FA is produced along with dimethylamine by enzymatic reduction of trimethylamine oxide and has been recognised as a highly reactive molecule leading to inter- and intramolecular linkages between protein chains. As a result, protein denaturation and quality loss in frozen fish have been associated to formaldehyde formation (20-23).

Many efforts have been carried out in order to clarify the possible mechanism of FA-mediated protein denaturation (23, 24). Interaction of FA with amino compounds has been monitored (25-27), the relative influence of FA and lipid damage products in texture changes has been evaluated (28-30) and some fluorescence development related to quality during freezing has been recognised (31, 32).

The present work was envisaged in order to study the influence of FA in the fluorescence development during interaction compounds formation in processed fish. Model systems consisting of FA and a primary amine (propylamine) and fish (cod and sardine) muscle were studied. The fluorescence ratio detected during fatty fish processing was measured (15-17). A 30°C heating temperature was chosen so that interaction compounds formation could be accelerated. Resulting fluorescence values in FA systems were compared with the ones obtained by common lipid oxidation products (propanal and hexanal). The presence of both aldehydes in the FA containing mixtures was also investigated.

MATERIALS AND METHODS

Chemicals employed along the present work were reagent grade (E. Merck, Darmstadt, Germany).

Propylamine model systems

A 0.1 M solution of propylamine and a 0.5 M solution of each aldehyde (FA, propanal and hexanal) were prepared in aq. 86% ethanol.

In a first type of reacting mixture, 10 ml of each aldehyde solution, 10 ml of propylamine solution and 10 ml of aq. 86% ethanol solution were mixed and placed in 30 ml stoppered tubes.

In a second type of reacting mixture, 10 ml of propylamine solution, 10 ml of FA solution and 10 ml of propanal or hexanal solution were mixed and placed in 30 ml stoppered tubes.

Both kinds of reacting mixtures were incubated at 30°C in dark without stirring and sampled at 1, 3, 7, 14 and 25 days of storage for fluorescence analysis. Triplicated reaction mixtures were carried out throughout the whole experiment. Blanks consisting of the starting compounds (propylamine and aldehydes) were also examined at the same reaction conditions.

Fish muscle model systems

Fresh cod (*Gadus morhua*; six individuals) was divided into three batches. In each batch, the cod white muscle was separated. Minced cod portions (4g) of each batch were mixed with 7 ml of aq. 86% ethanol solution and 3 ml of each 0.5 M aldehyde (formaldehyde, propanal and hexanal) solution and placed in 30 ml stoppered tubes.

Fresh sardine (*Sardina pilchardus*; three dozens) was divided into three batches. In each batch, the sardine white muscle was separated. Minced sardine portions (4 g) of each batch were mixed with 7 ml of aq. 86% ethanol solution and 3 ml of 0.5 M FA solution and placed in 30 ml stoppered tubes.

In both kinds of fish muscle systems, the reaction mixtures were held at 30°C in dark without stirring and at definite time intervals (1, 7 and 25 days) subjected to fluorescence analysis. Blanks consisting of the starting reagents (minced cod and

sardine muscles, FA, propanal and hexanal) were also examined throughout the same reaction conditions.

Fluorescence analysis

Reaction mixtures containing fish muscle were centrifuged (3000g for 10 min) and filtered. The liquid part was exposed to UV light (350 nm) for 30s to destroy any retinol present in the extract and then analysed by fluorometry. In the case of model systems containing propylamine, the reaction mixtures were directly analysed.

Fluorescence measurements (Perkin-Elmer LS 3B) were made at 327/415 nm and 393/463 nm excitation/emission maxima. 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 fluorescence intensity of a quinine sulphate solution (1 µg/ml in 0.05 M H₂SO₄) at the corresponding wavelength. The fluorescence ratio (δF) was calculated as: $\delta F = RF_{393/463nm} / RF_{327/415nm}$ (16-17).

Statistical analysis

Data corresponding to δF were subjected to the ANOVA one-way method. Mean results were compared by the LSD test using the Statistica package (33). Significance was declared at $p < 0.05$.

RESULTS

As a first step, the role of formaldehyde (FA) in the fluorescent compounds formation was studied in a model system with a primary amine (propylamine). The FA containing medium reflected a little increase in the δF value after 25 days of reaction (Table 1). A comparison with two lipid oxidation compounds was carried out. For it, propanal and hexanal were chosen because of their wide formation during ω 3-PUFA and ω 6-PUFA (respectively) oxidation (34, 35). Both aldehydes systems led to a progressive increase in the δF value along the reaction time reaching higher values at the end of the storage than the FA medium. All the aldehyde (FA, propanal and hexanal) blank solutions provided no significant changes throughout the experiment. The same result was observed in the case of propylamine (Table 1).

Propanal showed a higher δF formation than hexanal, that could be explained on the basis of being a smaller molecule and having a greater electrophilic reactivity. The positive inductive effect of the penthyl radical is known to be bigger (36) than the one of the ethyl radical, so that the carbonilic carbon would be more reactive (a stronger electrophile) in the case of propanal.

A different conclusion about the effect of the aldehydes chain length was obtained by Montfoort et al. (37), where a comparative study of fluorescence formation was carried out by exposing phosphatidyl ethanolamine (PE) containing liposomes to a variety of aldehydes. The fluorescence formation increased with chain lengths in saturated aldehydes. However fluorescence was investigated at a single excitation/emission, so that a bathochromic shift could not be evaluated. Another difference with the present experiment is that a more lipophilic amine (PE) was employed, so that a longer chain length in the aldehyde could facilitate the interaction with such amine.

The next experiment was to study the fluorescence formation in mixtures containing both FA and the same primary amine in the presence of lipid oxidation compounds. Again, propanal and hexanal were employed. Results are indicated in Table 2. It can be concluded that such mixtures produced a larger δF formation than model systems with only FA and amine (Table 1). However, hexanal and propanal systems without the FA presence had produced higher δF values. This result could be explained on the basis of the higher reactivity of FA (smaller molecule having a more electrophilic carbonilic carbon) that would be able to interact faster with the amine, which is five times less concentrated in the reacting medium than any of the aldehydes.

The following step was to study a more real system consisting of fish muscle. The effect of the addition of FA, propanal and hexanal to the white muscle of a gadoid fish (cod) (Table 3) was studied. The blank muscle medium did not provide a significant increase in the δF value along the storage. At the same time, the FA addition to cod muscle produced a significant increase after 25 days of reaction. Accordingly, FA has exerted a positive effect in the fluorescence formation. However, this value was clearly lower than the ones obtained in the reacting mixtures concerning both lipid oxidation aldehydes, according to the above results. Again, the propanal medium showed a greater fluorescence development than the hexanal one.

Finally, the role of FA in the interaction compounds formation with fluorescent properties was studied in a reaction medium consisting of sardine muscle. Results are also indicated in Table 3.

The addition of FA to a fatty fish muscle led to a higher δF increase than in the case of cod. A significant increase was already obtained after seven days of reaction. This higher fluorescence development can be explained on the basis that fatty fish undergoes a greater lipid oxidation than lean one (1, 9). At the same time, the blank

sardine muscle provided a significant increase at the end of the storage that can be explained because of the interaction between amino compounds from the muscle and oxidation molecules formed from muscle lipids during the reaction conditions (15-17). This increase was clearly lower than the one obtained in the presence of FA, indicating that FA has played a significant role in the fluorescence formation as in the case of the cod system.

DISCUSSION

In previous experiences related to fatty fish processing (15-17), lipid deterioration compounds have caused the formation of interaction compounds, whose fluorescent properties provided a valuable method for quality assessment. The present experiment was envisaged in order to study such fluorescent compounds formation in fish samples where FA and lipid oxidation compounds are present as a result of processing damage.

Different kinds of reacting model systems were tested. Although with different degrees, all kinds of FA experiments showed a bathochromic shift (δF increase), according to the general theory consisting of a progressive formation of addition compounds where amino and aldehyde compounds are involved (5, 6, 8).

In spite of its higher reactivity, FA has showed a lower ability than the lipid oxidation compounds in producing the fluorescence development. However, results obtained in both kinds of fish model systems indicated that FA produced a positive effect in the δF value, so that an interaction could be pointed out by fluorescence spectroscopy. On the basis of these results it is postulated that fluorescence detection of interaction compounds could be employed as a complementary tool of quality assessment in fish species processing where FA formation (freezing of gadoid species)

and lipid oxidation (enzymatic and non enzymatic) take place. In this sense, further research is needed in order to compare such fluorescence detection with common damage indexes during freezing of gadoid species.

The implication of FA in toxicological and nutritional issues has attracted a great attention too (23). The participation of FA in the fluorescent compounds formation could bridge gaps in our knowledge in the relationship observed between the interaction compounds formation during storage/processing of foods and the pigmented and fluorescent granules found in human and animal tissues (lipofuscin) (7, 38, 39).

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TABLE 1: Fluorescence ratio (δF) values* obtained from the aldehyde-propylamine reacting systems**

Reaction Time (days)	Reacting Mixture			
	PAM	FA + PAM	Pal + PAM	Hal + PAM
1	1.50 b (0.20)	0.77 a (0.03)	8.34 d (0.07)	2.01 c (0.07)
3	1.58 b (0.20)	0.93 a (0.05)	17.84 d (0.17)	6.09 c (0.27)
7	1.50 a (0.20)	1.04 a (0.02)	33.05 c (0.71)	15.95 b (0.16)
14	1.56 a (0.20)	1.10 a (0.04)	49.92 c (0.88)	29.93 b (0.18)
25	1.66 a (0.20)	1.12 a (0.10)	75.13 c (1.92)	49.52 b (0.53)

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

** Abbreviations: PAM (propylamine), FA (formaldehyde), Pal (propanal) and Hal (hexanal).

TABLE 2: Fluorescence ratio (δF) values* obtained by reaction of FA with PAM in the presence of other aldehydes**

Reaction Time (days)	Reacting Mixture		
	FA + PAM	FA + PAM + Pal	FA + PAM + Hal
1	0.84 c (0.01)	0.77 b (0.04)	0.58 a (0.03)
3	0.90 b (0.04)	0.96 b (0.01)	0.78 a (0.03)
7	1.07 a (0.06)	1.23 b (0.04)	1.28 b (0.06)
14	1.13 a (0.01)	1.57 b (0.05)	2.28 c (0.07)
25	1.17 a (0.02)	1.58 b (0.04)	3.11 c (0.05)

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

** Abbreviations as specified in Table 1.

TABLE 3: Fluorescence ratio (δF) values* obtained from the aldehyde-fish muscle reaction systems**

Reacting Mixture	Reaction Time (days)		
	1	7	25
MCM	0.88 ab (0.10)	0.97 a (0.16)	1.18 a (0.31)
FA + MCM	1.18 b (0.01)	1.18 a (0.04)	1.53 a (0.10)
Pal + MCM	6.03 d (0.79)	22.63 d (2.61)	37.08 d (1.57)
Hal + MCM	3.23 c (0.23)	18.17 c (1.48)	26.81 c (2.18)
MSM	0.53 a (0.07)	0.65 a (0.11)	1.21 a (0.09)
FA + MSM	1.48 b (0.11)	3.46 b (0.09)	6.73 b (0.30)

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

** Abbreviations: MCM (minced cod muscle), MSM (minced sardine muscle). Aldehydes as expressed in Table 1.

TABLE 1: Fluorescence measurements (327/415 and 393/463 nm)* in aldehyde-MSM model systems**

Time of reaction	MSM control		FA + MSM		AcH + MSM		Pr + MSM		Hex + MSM		Hx + MSM		Bz + MSM													
Starting MSM	0.38	a	0.39	a																						
Initial aldehyde			0.10	a	0.12	a	0.10	b	0.12	a	0.10	a	0.15	a	0.02	0.16	a	nd	0.08	a						
1	0.67	b	0.35	a	0.25	b	0.37	a	0.23	c	1.05	b	0.20	b	0.78	b	0.28	c	0.72	b	nd	0.83	b	nd	0.29	b
7	0.89	c	0.57	b	0.44	c	1.53	b	0.02	a	5.30	c	0.14	a	1.47	c	0.22	b	1.61	c	nd	1.45	d	nd	0.42	c
25	1.77	d	2.14	c	0.64	d	4.35	c	0.01	a	4.86	c	0.24	b	2.99	d	0.26	c	3.66	d	nd	1.07	c	nd	1.13	d

* Abbreviations: MSM (minced sardine muscle), FA (formaldehyde), AcH (acetaldehyde), Pr (propanal), Hex (hexanal), Hx (2-hexenal) and Bz (benzaldehyde).

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

*** nd: not detected (lower than 0.01).

TABLE 3: Fluorescence measurements (327/415 and 393/463 nm)* in aldehyde-propylamine model systems**

Time of reaction	Pam		FA + Pam		AcH + Pam		Pr + Pam		Hex + Pam		Hx + Pam		Bz + Pam													
Starting Pam	0.09	a	0.19	c																						
Initial aldehyde			0.10	a	0.12	a	0.10	b	0.12	a	0.10	a	0.16	a	0.02	0.16	b	nd	0.08	a						
1	0.09	a	0.14	ab	0.17	c	0.14	b	0.16	c	6.50	c	0.65	b	5.39	b	0.49	b	0.99	b	nd	2.42	d	nd	0.12	b
7	0.09	a	0.14	a	0.14	b	0.15	b	0.02	a	1.53	b	0.96	c	31.81	c	0.93	d	14.80	c	nd	0.37	c	nd	0.30	c
25	0.11	a	0.18	bc	0.14	b	0.14	b	nd		nd		0.63	b	47.22	d	0.76	c	37.81	d	nd	0.02	a	nd	0.63	d

* Abbreviations: Pam (propylamine). Remaining reagents as indicated in Table 1.

** Mean values of three determinations. For each reagent (aldehydes or propylamine) values in the same column followed by different letters are significantly different ($p < 0.05$).

*** nd: not detected (lower than 0.01).

TABLE 2

Mean fluorescence ratio (δF) values ($\pm$ SD, n=3) obtained by reaction at 30°C in the aldehyde-MSM model systems**

Reacting mixture	Starting MSM/ aldehyde	1	7	25
MSM	1.03 $\pm$ 0.09 a	0.53 $\pm$ 0.06 a	0.65 $\pm$ 0.09 a	1.21 $\pm$ 0.07 a
FA + MSM	1.20 $\pm$ 0.02 ab	1.48 $\pm$ 0.09 b	3.46 $\pm$ 0.07 b	6.73 $\pm$ 0.24 b
AcH + MSM	1.27 $\pm$ 0.02 ab	4.62 $\pm$ 0.31 d	267.33 $\pm$ 10.78e	523.79 $\pm$ 28.23d
Pr + MSM	1.27 $\pm$ 0.02 b	4.03 $\pm$ 0.55 d	10.23 $\pm$ 0.86 d	12.92 $\pm$ 0.75 c
Hex + MSM	1.58 $\pm$ 0.21 c	2.61 $\pm$ 0.08 c	7.38 $\pm$ 0.52 c	14.17 $\pm$ 0.42 c

* Values in the same column followed by different letters are significantly different (p<0.05).

** Abbreviations as specified in Table 1.

TABLE 4

Mean fluorescence ratio (δF) values ($\pm$ SD, n=3) obtained by reaction at 30°C in the aldehyde-propylamine model systems**

Reacting mixture	Starting Pam/ aldehyde	1	7	25
Pam	2.06 $\pm$ 0.01 c	1.52 $\pm$ 0.31 ab	1.54 $\pm$ 0.32 a	1.65 $\pm$ 0.03 a
FA + Pam	1.20 $\pm$ 0.02 a	0.77 $\pm$ 0.03 a	1.04 $\pm$ 0.01 a	1.02 $\pm$ 0.08 a
AcH + Pam	1.27 $\pm$ 0.02 a	39.60 $\pm$ 1.38 d	90.03 $\pm$ 7.66 d	—
Pr + Pam	1.27 $\pm$ 0.02 a	8.34 $\pm$ 0.06 c	33.05 $\pm$ 0.58 c	75.13 $\pm$ 1.57 c
Hex + Pam	1.58 $\pm$ 0.21 b	2.01 $\pm$ 0.05 b	15.95 $\pm$ 0.13 b	49.52 $\pm$ 0.43 b

* Values in the same column followed by different letters are significantly different (p<0.05).

** Abbreviations as specified in Table 2.

TABLE 1: Fluorescence measurements (327/415, 393/463 and 479/516 nm)* in aldehyde-MSM model systems**

Reacting Mixture	Reaction Time (days)	327/415 nm	393/463 nm	479/516 nm
MSM	0	0.38 a	0.39 a	4.94 a
MSM	1	0.67 b	0.35 a	7.26 a
MSM	7	0.89 c	0.57 b	5.26 a
MSM	25	1.77 d	2.14 c	10.87 b
FA	0	0.10 a	0.12 a	0.35 a
MSM + FA	1	0.25 b	0.37 a	5.15 b
MSM + FA	7	0.44 c	1.53 b	7.15 c
MSM + FA	25	0.64 d	4.35 c	13.41 d
AcH	0	0.10 b	0.12 a	0.33 a
MSM + AcH	1	0.23 c	1.05 b	7.00 b
MSM + AcH	7	0.02 a	5.30 c	28.93 c
MSM + AcH	25	0.09 a	4.86 c	36.83 d
Pr	0	0.10 a	0.12 a	0.34 a
MSM + Pr	1	0.20 b	0.78 b	5.59 b
MSM + Pr	7	0.14 a	1.47 c	7.55 c
MSM + Pr	25	0.24 b	2.99 d	9.24 d
Hex	0	0.10 a	0.15 a	0.33 a
MSM + Hex	1	0.28 c	0.72 b	5.80 b
MSM + Hex	7	0.22 b	1.61 c	7.13 b
MSM + Hex	25	0.26 c	3.66 d	11.48 c
Hx	0	0.00 a	0.16 a	0.39 a
MSM + Hx	1	0.00 a	0.83 b	37.57 b
MSM + Hx	7	0.00 a	1.45 d	85.86 c
MSM + Hx	25	0.00 a	1.07 c	136.08 d

* Abbreviations: MSM (minced sardine muscle), FA (formaldehyde), AcH (acetaldehyde), Pr (propanal), Hex (hexanal) and Hx (2-hexenal).

** Mean values of three determinations. For each reagent (aldehyde or minced sardine muscle) values in the same column followed by different letters are significantly different ($p < 0.05$).

TABLE 2: Fluorescence measurements (327/415, 393/463 and 479/516 nm)* in aldehyde-propylamine model systems**

Reacting Mixture	Reaction Time (days)	327/415 nm	393/463 nm	479/516 nm
Pam	0	0.09 a	0.19 c	0.34 ab
Pam	1	0.09 a	0.14 ab	0.40 b
Pam	7	0.09 a	0.14 a	0.32 a
Pam	25	0.11 a	0.18 bc	0.50 c
FA	0	0.10 a	0.12 a	0.36 a
FA + Pam	1	0.17 c	0.14 b	0.37 a
FA + Pam	7	0.14 b	0.15 b	0.38 a
FA + Pam	25	0.14 b	0.14 b	0.48 b
AcH	0	0.10 b	0.12 a	0.33 a
AcH + Pam	1	0.16 c	6.50 c	59.79 b
AcH + Pam	7	0.02 a	1.53 b	174.38 c
AcH + Pam	25	0.00 a	0.00 a	54.37 b
Pr	0	0.10 a	0.12 a	0.34 a
Pr + Pam	1	0.65 b	5.39 b	0.46 a
Pr + Pam	7	0.96 c	31.81 c	2.79 b
Pr + Pam	25	0.63 b	47.22 d	8.91 c
Hex	0	0.10 a	0.16 a	0.33 a
Hex + Pam	1	0.49 b	0.99 b	0.49 b
Hex + Pam	7	0.93 d	14.80 c	1.11 c
Hex + Pam	25	0.76 c	37.81 d	3.84 d
Hex	0	0.02 b	0.16 b	0.39 a
Hex + Pam	1	0.03 c	2.42 d	220.10 d
Hex + Pam	7	0.00 a	0.37 c	125.02 c
Hex + Pam	25	0.00 a	0.02 a	35.73 b

* Abbreviations: Pam (propylamine). Remaining reagents as indicated in Table 1.

** Mean values of three determinations. For each reagent (aldehydes or propylamine) values in the same column followed by different letters are significantly different ($p < 0.05$).

TABLE 3: Fluorescence detection* at 528/556 nm in acetaldehyde and 2-hexenal model systems **.

Reacting Mixture	Reaction Time (days)		
	1	7	25
AcH + Pam	151.25 a	284.00 b	373.75 b
Hx + Pam	390.00 a	1562.30 b	1712.50 b
AcH + MSM	13.23 a	80.40 b	85.67 b
Hx + MSM	113.67 a	312.73 b	481.67 c

* Abbreviations: As specified in Tables 1 and 2.

** Mean values of three determinations. Values in the same row followed by different letters are significantly different ($p < 0.05$). Initial values for Pam, MSM, AcH and Hx were: 2.68, 7.27, 2.54 and 2.82, respectively.