Effect of pH on the fluorescence formation related to fish deterioration

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ABSTRACT

Earlier studies have investigated fluorescence at different excitation/emission maxima during common fish processing. A shift towards higher wavelength maxima was observed and measured as the ratio between two of the maxima tested. This fluorescence ratio (δF) correlated with increased fish damage. The present work is focused on the effect that pH can have on the fluorescent compounds formation in fish muscle systems. Minced hake muscle was homogenised with 0.1 M phosphate buffer of different pH values (5, 6, 7 and 8) and was stored at 30°C up to 30 days. Lipid damage measured as the δF value of the aqueous reaction medium and the fish lipid extract provided little differences between pH values at the conditions employed in the present experiment. The presence of formaldehyde (FA) in the same reaction medium was also evaluated. In this case, it was observed that FA had a positive effect in the fluorescence shift formation in the aqueous reaction medium so that a higher δF value was observed for pH 7 and 8, especially for the last one. A special interest is concluded in determining changes in the δF value during fish storage and/or processing where in addition to FA and lipid oxidation formations, significant pH increases are expected to occur as a result of damaging.

Running Title: pH and fluorescence formation

Key Words: Fish muscle, fluorescence, formaldehyde, lipid oxidation, pH value

INTRODUCTION

Marine lipid composition has proved to possess a high content of polyunsaturated fatty acids (PUFA) (1, 2). During processing and/or storage, PUFA degradation can lead to the formation of significant quantities of primary and secondary lipid oxidation products, which can finally produce browning (3), fluorescent compounds (4, 5), off flavour (6) and the loss of essential nutrients (7).

Our previous studies of lipid damage during fish processing have been focused on the fluorescence detection. In them, compounds formed as a result of interaction between lipid oxidation products and biological amino constituents were studied at different excitation/emission maxima. Relatively more fluorescence was observed at higher wavelength maxima as the quality of the fish decreased. This fluorescence shift was measured as the ratio between two of the maxima tested (393/463 nm and 327/415 nm) and proved to be a more effective index of fish quality than other common quality assessment methods (8-11).

Investigation concerning the influence of different factors on the fluorescence ratio (δF) value has been carried out. Accurate model systems were prepared so that temperature and different kinds and quantities of aldehydes and amines were tested (12-14). The effect of formaldehyde (FA) in the δF value was also checked (14). FA showed less ability than the lipid oxidation compounds to produce the bathochromic shift of fluorescence; however, results indicated that FA had an increasing effect on the δF value, so that an interaction could be inferred by fluorescence spectroscopy.

Variations in the fish pH have been reported from catching till it is consumed as a food. Aspects such as species, glycogen content before catching, capture conditions, post-mortem changes and formation of degradation molecules can be considered responsible of such pH fluctuations (15-18).

The present work reports an investigation of the effect that pH can have on the fluorescence formation measured as the δF value. Different pH values commonly found in processed/stored fish species were investigated. Model systems starting from minced hake muscle were heated at 30°C up to 30 days. Lipid oxidation development was checked by conjugated diene formation. The presence of FA in the reaction mixtures was also studied, so that interactions between FA, lipid oxidation compounds and biological amino constituents could be evaluated by fluorescence detection at different pH values.

MATERIALS AND METHODS

Raw material and chemicals

Fresh hake (*Merluccius merluccius*; six individuals) purchased from a commercial source was divided into three batches. In each batch, the white muscle was separated, minced and considered for the present study.

Solutions of 0.1 M phosphate buffer at pH 5.0, 6.0, 7.0 and 8.0 were prepared. Solutions of 0.5 M FA in 0.1 M phosphate buffer at pH 5.0, 6.0, 7.0 and 8.0 were prepared.

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

Model systems preparation

Minced hake portions (5 g) of each batch were mixed with 11 ml of 0.1 M phosphate buffer at each pH and placed in 30 ml stoppered tubes.

Minced hake portions (5 g) of each batch were mixed with 8 ml of 0.1 M phosphate buffer at each pH and 3 ml of the corresponding 0.5 M FA solution and placed in 30 ml stoppered tubes.

For both kinds of fish muscle systems, the reaction mixtures were held at 30°C in the dark without stirring and, at definite time intervals (1, 2, 4, 7, 15 and 30 days) were subjected to analysis. Blanks consisting of the starting pH solutions (with and without FA) were also examined using the same reaction conditions throughout.

Sample analysis

Reaction mixtures were centrifuged (3000 g for 10 min) and filtered. The liquid part was exposed to UV light (350 nm) for 30 s to destroy any retinol present in the extract and then analysed by fluorimetry.

Lipids from the hake muscle were extracted by the Bligh and Dyer method (19). Lipid quantification was carried out according to Herbes and Allen (20). Conjugated dienes (CD) formation was measured at 233 nm (21) in a Beckman DV-64 spectrophotometer; results were determined according to the following formula (4): $CD = (B \times V) / w$, where B is the absorbance reading at 233 nm, V denotes the volume (ml) of the sample and w is the mass (mg) of lipid extract measured. Lipid extracts were exposed to UV light as explained above before being analysed by fluorimetry.

Fluorescence measurements (Perkin-Elmer LS 3B) were made at 327/415 nm and 393/463 nm excitation/emission maxima. Relative fluorescence values (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 $\mu\text{g/ml}$ in 0.05 M H_2SO_4) at the corresponding wavelength. The fluorescence ratio was calculated as $\delta F = \text{RF}_{393/463\text{nm}} / \text{RF}_{327/415\text{nm}}$ (8-10).

Statistical analysis

Data corresponding to δF values were subjected to the ANOVA one-way method (22). Mean results were compared by the LSD test using the Statistica package Statsoft, Tulsa, USA. Significance was declared at $p < 0.05$.

RESULTS

CD formation (Table 1)

Primary lipid oxidation compounds formed in the hake muscle were monitored by conjugated diene detection. As a result of the heating treatment a significant increase in the CD value was obtained at all pH studied after one day of reaction (initial mean value: 1.08) and then again after two days. The highest values were obtained after 7 days (pH 5) and 15 days (pH 6, 7 and 8). At the end (after 30 days) a significant decrease was obtained in all cases compared to the highest values.

Very little significant differences were detected between pH studied in each reaction time, so that a definite tendency concerning the pH value could not be inferred.

Fluorescent compounds detection

None of the pH blank solutions (with and without FA) produced any significant changes throughout the course of the experiment. Accordingly, results are evaluated in mixtures containing the fish muscle in the pH medium with and without FA.

a) Model systems without FA

Concerning the aqueous reaction medium, a gradual increase of the δF value with the reaction time was obtained at the four pH (Table 2). However, little significant differences were obtained by comparison of the different pH values at each reaction step. Only after 30 days of reaction a higher δF value at pH 8 than at pH 5 was obtained.

By studying the corresponding lipid extracts (Table 3), an increasing tendency for the δF value (initial mean value: 0.60) was also obtained as long as the reaction time increased, although this pattern was not so regular as in the case of the aqueous reaction medium. Comparison of the different pH values at each step of the reaction time provided some differences, although a clear tendency related to pH value could not be inferred; pH 8 showed in most cases the lowest value, although differences were not always significant.

b) Model systems with FA

The aqueous reaction medium revealed (Table 4) a progressive increase with the reaction course in the four pH studied; indeed, a sharp increase was obtained for the δF value in the cases of pH 7 and 8. Comparison of the different pH media in the different times of reaction showed some significant differences. δF values obtained for pH 7 and 8 showed to be higher than the ones of pH 5 and 6. At the end of the reaction pH 8 provided a higher value than pH 7.

The corresponding lipid extracts showed (Table 5) a significant increase at all pH (initial mean value: 0.60) after 1 day of reaction. Then, a general increase was observed after 4 days, followed by no more significant changes till the end of the experiment. Comparison of pH at the different reaction times provided different conclusions than in the case of the above mentioned aqueous reaction medium. Values obtained for the pH 7 and 8 were lower than for pH 5 and 6 along the whole experiment except for the end of it, where no significant differences could be detected.

DISCUSSION

The present investigation has provided a comparison of the effect of different pH values in terms to produce interaction compounds with fluorescent properties. Accelerated by the temperature (30°C) and time of reaction, a similar bathochromic shift than in the case of fish processing (cooking, sterilization, chilling and freezing; 8-11) was observed leading to the formation of addition compounds where amino and aldehyde molecules are involved (3, 23-26).

During the present experiment, lipid oxidation showed to occur since an increase of CD content at all pH studied was observed after one and two days of reaction (Table 1). However, the fact that this index showed a general decrease at the end of the experiment, lead to conclude that CD are relatively unstable and capable of interacting with other constituents in order to produce interaction compounds (9, 27, 28).

Interaction compounds formation measured as δF value indicated (Tables 2 and 3) that lipid oxidation was produced with the reaction time, but very little differences were produced as a result of varying the pH medium. In previous research, lipid

oxidation appeared to be accelerated at higher pH (29, 30), although polyunsaturated fatty acids model systems were tested, and not a lean fish muscle (mean initial lipid content: 0.63g/ 100g muscle) as in the present case.

The presence of FA in the reaction medium has provided notorious differences. In the case of the aqueous medium, a greater δF development than in absence of FA was observed, specially in the cases of pH 7 and 8 (comparison of Tables 2 and 4). According to previous results (14), FA has showed a positive effect in order to produce fluorescent compounds which can now be extended to different kinds of pH, but is largely greater in the case of the two highest pH studied (7 and 8).

The larger fluorescence development found in the cases of pH 7 and 8 can be explained by analysing the pKa values of the biological amine compounds involved (31). As long as pH increases, nucleophilic ability of N-terminal groups of proteins and R-terminal groups of basic amino acids (lysine, tyrosine and arginine) is enlarged so that interaction compounds formation is favoured. At the same time, condensation reactions (Cannizaro type, and so on) of FA are facilitated as long as the medium basicity increases (32).

Some differences can be observed by comparing the δF values obtained in the aqueous reaction media and the corresponding lipid extracts. Increases due to the FA presence (Tables 4 and 5) were smaller in the lipid fraction which could be explained by the difficulty of FA in reacting with non-water soluble molecules such as lipids. At the same time, δF values obtained for the lipid fraction provided a different fluorescence formation pattern than in the aqueous phase; thus, greater δF values were obtained in the cases of pH 5 and 6 than in pH 7 and 8 along the different reaction times, although at the end (30 days) no differences could be assessed.

Present results can be of great interest for fish processes where in addition to FA and lipid oxidation formations, sensible pH increases are expected to occur as a result of damaging. This is the case of freezing and frozen storage of gadoid fish species where much work has been carried out in order to clarify the possible mechanisms of FA-mediated protein denaturation and lipid damage (33-35). According to the present results, fluorescence detection as the δF value shows to provide an accurate way of assessing fish damage that should be compared to commonly used damage indices during such processes in complementary studies.

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REFERENCES

1. Pigott G, Tucker B (1987) *Food Rev Intern* 3: 105-138
2. Ackman R (1989) Fatty acids. In: Ackman R (ed) *Marine biogenic lipids, fats and oils*. CRC Press, Boca Raton, Florida (USA), Vol 1 pp 103-137
3. Pokorný J (1981) *Prog Food Nutr Sci* 5: 421-428
4. Smith G, Hole M, Hanson S (1990) *J Sci Food Agric* 51: 193-205
5. Lubis Z, Buckle K (1990) *Int J Food Sci Technol* 25: 66-77
6. Kunert-Kirchhoff J, Baltes W (1990) *Z Lebensm Unters Forsch* 190: 14-16
7. Nielsen H, Finot P, Hurrell R (1985) *Brit J Nutrit* 53: 75-86
8. Aubourg S, Medina I, Pérez-Martín R (1995) *Z Lebensm Unters Forsch* 200: 252-255
9. Aubourg S, Sotelo C, Gallardo J (1997) *J Food Sci* 62: 295-299
10. Aubourg S, Medina I (1997) *J Agric Food Chem* 45: 3617-3621
11. Aubourg S, Sotelo C, Pérez-Martín R (1998) *J Amer Oil Chem Soc* (accepted for publication)
12. Aubourg S, Pérez-Martín R, Medina I, Gallardo J (1992) *J Agric Food Chem* 40: 1805- 1808
13. Aubourg S, Gallardo J (1997) *Int J Food Sci Technol* 32: 153-158
14. Aubourg S (1998) *Z Lebensm Unters Forsch* 206: 29-32
15. Cheftel J, Cheftel H (1976) *Introducción a la Bioquímica y Tecnología de Alimentos*. Ed. Acribia, Zaragoza (Spain) pp 70-73
16. Whittle K, Hardy R, Hobbs G (1990) Chilled fish and fishery products. In: Gormley T (ed) *Chilled foods. The state of the art*. Elsevier Applied Science, London, New York pp 87-116
17. Nunes M, Batista I, Morao de Campos R (1992) *J Sci Food Agric* 59: 37-43

18. Chalmers M, Careche M, Mackie I (1992) *J Sci Food Agric* 58: 375-383
19. Bligh E, Dyer W (1959) *Can J Biochem Physiol* 37: 911-917
20. Herbes S, Allen C (1983) *Can J Fish Aquat Sci* 40: 1315-1317
21. Kim R, Labella F (1987) *J Lipid Res* 28: 1110-1117
22. Sokal R, Rohlf F (1981) *Biometry*. Freeman, San Francisco, pp 208-270
23. Gardner H (1979) *J Agric Food Chem* 27: 220-229
24. Esterbauer H (1982) Aldehydic products of lipid peroxidation. In: McBrien D and Slater T (eds) *Free radicals, lipid peroxidation and cancer*. Academic Press, London, pp 101-128
25. Kikugawa K, Beppu M (1987) *Chem Phys Lipids* 44: 277-297
26. Montfoort A, Bezstarosti K, Groh M, Koster J (1987) *FEBS Lett* 226: 101-104
27. Cho S-Y, Endo Y, Fujimoto K, Kaneda T (1989) *Nippon Suisan Gakkaishi* 55: 545-552
28. Melton S (1983) *Food Technol* 37: 105-111, 116
29. Saunders D, Coleman J, Hampson J, Wells P, Riemenschneider R (1962) *J Amer Oil Chem Soc* 39: 434-439
30. Huang S-W, Frankel E, Schwarz K, German B (1996) *J Agric Food Chem* 44: 2496-2502
31. Lehninger A (1979) *Bioquímica*. Ediciones Omega, S A, Barcelona, pp 80-82
32. Finar I (1966) *Química Orgánica*. Editorial Alhambra S A Madrid, pp 200-203
33. Rehbein H (1988) *J Sci Food Agric* 43: 261-276
34. Oehlenschläger J, Schreiber W (1988) *Fat Sci Technol* 89: 38-41
35. Rehbein H, Orlick B (1990) *Int J Refrig* 13: 336-341

TABLE 1: Conjugated diene values* obtained by heating minced muscle at 30°C in the reaction system at different pH

Reacting Time (days)	pH value			
	5	6	7	8
1	2.86 (1.095)	3.39 (0.867)	4.30 (0.924)	3.22 (0.382)
2	4.16 a (0.344)	6.61 a, b (1.506)	5.56 a, b (0.959)	7.23 b (1.783)
4	6.15 (1.202)	5.65 (0.842)	4.95 (1.528)	7.44 (1.839)
7	6.71 a, b (1.436)	5.16 a, b (1.285)	4.29 a (0.159)	6.84 b (1.819)
15	4.34 a (1.146)	7.66 b (0.846)	7.38 b (0.946)	7.99 b (2.468)
30	4.48 (0.906)	4.17 (0.035)	4.79 (1.626)	4.50 (1.662)

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

TABLE 2: Fluorescence ratio values* obtained from the aqueous medium of the reaction system without formaldehyde at different pH

Reacting Time (days)	pH value			
	5	6	7	8
1	0.65 (0.230)	0.76 (0.398)	0.89 (0.089)	1.10 (0.172)
2	0.92 (0.457)	1.24 (0.516)	1.07 (0.129)	1.19 (0.203)
4	1.45 (0.435)	1.82 (0.426)	1.43 (0.232)	1.41 (0.314)
7	2.32 (0.712)	2.40 (0.450)	1.99 (0.435)	1.77 (0.354)
15	3.30 (0.594)	3.39 (0.304)	3.00 (0.328)	3.02 (0.360)
30	4.49 a (0.211)	4.81 a, b (0.331)	4.88 a, b (0.422)	5.59 b (0.680)

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

TABLE 3: Fluorescence ratio values* obtained from the hake lipid extracts of the reaction system without formaldehyde at different pH

Reacting Time (days)	pH value			
	5	6	7	8
1	1.00 a, b (0.174)	1.05 b (0.383)	0.77 a, b (0.240)	0.51 a (0.220)
2	1.46 b (0.066)	1.26 b (0.048)	1.03 b (0.444)	0.46 a (0.222)
4	1.13 (0.381)	1.91 (0.713)	1.52 (0.998)	0.75 (0.313)
7	1.02 a (0.351)	2.16 b (0.492)	1.36 a, b (0.541)	0.73 a (0.135)
15	3.13 a (1.213)	3.40 b (0.915)	2.55 a, b (0.473)	1.74 a (0.707)
30	6.22 a, b (0.850)	5.65 a, b (0.856)	8.50 b (3.085)	4.40 a (1.872)

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

TABLE 4: Fluorescence ratio values* obtained from the aqueous medium of the reaction system with formaldehyde at different pH

Reacting Time (days)	pH value			
	5	6	7	8
1	0.80 a (0.045)	0.96 a (0.113)	1.88 c (0.109)	1.59 b (0.207)
2	1.00 a (0.234)	1.30 a (0.109)	2.68 b (0.248)	2.43 b (0.092)
4	1.36 a (0.403)	1.51 a (0.059)	7.38 c (1.159)	5.36 b (0.263)
7	1.78 a (0.235)	2.46 a (0.567)	9.08 b (1.781)	9.79 b (0.807)
15	2.94 a (0.643)	3.51 a (0.720)	16.41 b (3.295)	17.94 b (1.336)
30	4.98 a (0.899)	6.57 a (1.743)	42.49 b (9.110)	63.79 c (9.065)

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

TABLE 5: Fluorescence ratio values* obtained from the hake lipid extracts of the reaction system with formaldehyde at different pH

Reacting Time (days)	pH value			
	5	6	7	8
1	2.28 c (0.359)	2.35 c (0.041)	1.80 b (0.145)	1.12 a (0.082)
2	3.22 b (0.307)	2.77 b (0.247)	2.06 a (0.175)	2.00 a (0.325)
4	5.52 b (0.274)	6.65 b (0.409)	4.16 a (0.444)	3.89 a (1.185)
7	4.44 a, b (0.685)	5.81 b (0.870)	4.25 a (1.045)	3.56 a (0.289)
15	5.54 b (0.468)	5.29 b (0.178)	4.39 a (0.394)	4.21 a (0.478)
30	4.67 (0.344)	5.00 (0.596)	4.20 (0.421)	3.67 (1.198)

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

TABLE 5: Et + FA

	5	6	7	8
1	0.24 a (0.005)	0.24 a (0.069)	0.26 a (0.043)	0.30 a (0.044)
2	0.17 a (0.025)	0.15 a (0.013)	0.18 a (0.012)	0.27 b (0.015)
4	0.15 a (0.004)	0.15 a (0.024)	0.16 a (0.005)	0.33 b (0.014)
7	0.16 a (0.016)	0.14 a (0.001)	0.15 a (0.002)	0.44 b (0.111)
15	0.15 a (0.004)	0.14 a (0.001)	0.15 a (0.003)	0.33 b (0.043)
30	0.12 a (0.002)	0.13 a (0.002)	0.14 a (0.003)	0.29 b (0.076)

TABLE 6: Et + Ac

	5	6	7	8
1	0.74 a (0.011)	0.73 a (0.049)	0.57 b (0.007)	0.95 c (0.011)
2	0.60 a (0.039)	0.56 a (0.018)	0.60 a (0.122)	1.06 b (0.004)
4	0.55 a (0.085)	0.53 a (0.022)	0.55 a (0.008)	1.22 b (0.007)
7	0.57 ab (0.039)	0.55 a (0.026)	0.60 b (0.007)	1.36 c (0.007)
15	0.65 b (0.029)	0.57 a (0.033)	0.66 b (0.002)	1.49 c (0.017)
30	0.54 a (0.010)	0.54 a (0.011)	0.65 b (0.020)	1.47 c (0.008)

TABLE 7: Et + Ac + FA

	5	6	7	8
1	0.25 b (0.003)	0.22 a (0.010)	0.21 a (0.004)	0.27 c (0.019)
2	0.19 a (0.015)	0.17 a (0.002)	0.17 a (0.006)	0.30 b (0.046)
4	0.18 a (0.016)	0.17 a (0.002)	0.16 a (0.004)	0.31 b (0.029)
7	0.20 a (0.003)	0.18 a (0.001)	0.16 a (0.003)	0.34 b (0.049)
15	0.22 a	0.20 a	0.19 a	0.30 b

	(0.006)	(0.002)	(0.044)	(0.025)
30	0.21 b	0.18 a	0.17 a	0.24 c
	(0.003)	(0.021)	(0.003)	(0.004)