**FLUORESCENCE CHANGES IN AMINE MODEL SYSTEMS RELATED TO
FISH DETERIORATION**

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SUMMARY

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 absorption at two of the maxima tested. This fluorescence ratio (δF) value correlated with increased fish damage. In the present work, different kinds of amines were tested to compare the fluorescence produced with that measured during fish processing. Amines with different substitution degree, and primary amines with different steric hindrance and chain length were reacted with cod liver oil and glutaraldehyde at 30°C in model systems.

All the amine classes produced changes in the fluorescent properties of the systems, higher δF values being associated with lower degree of substitution and steric hindrance of the $-NH_2$ group, and also as a result of a longer chain length and higher concentration of the amine compound.

Running title: Amines producing fluorescence changes

Key Words: Cod liver oil, fish deterioration, glutaraldehyde, lipid oxidation, steric hindrance, substitution degree.

INTRODUCTION

Some products of reaction of amines and lipid oxidation products during food deterioration have been detected by increase in fluorescence, usually at a single excitation/emission wavelength maximum (Maruf *et al.*, 1990; Lubis & Buckle, 1990). This was thought to be a useful index of lipid deterioration, although most results have been complementary to other more established measurements of food deterioration (Melton, 1983; Smith *et al.*, 1990).

Our previous studies of lipid damage by means of the fluorescence properties of the compounds formed during fish processing, tested different excitation/emission maxima. Relatively more fluorescence was observed at higher wavelengths maxima as 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 (Aubourg *et al.*, 1992, 1995 & 1997).

The present work reports an investigation of this fluorescence ratio (δF) in model amine systems, comparing different kinds of amines (substitution degree, steric hindrance and chain length). For this purpose, amines have been reacted at 30°C using cod liver oil (CLO) as generator of lipid oxidation compounds and with glutaraldehyde (GL). The influence of the amine content and the time of reaction were also evaluated.

MATERIALS AND METHODS

All chemicals were reagent grade (E. Merck, Darmstadt, Germany).

CLO (Bama Geve, S. A., Barcelona, Spain) was obtained from a commercial source. It was stored under N₂ at 4° C. Before employing it, acid value (1.1 mg KOH/g), peroxide value (2.4 meq O₂/Kg) (Williams, 1950) and fatty acid composition (Medina et al., 1994) were analyzed. The following proportions (%) of the major fatty acids were obtained: 5.08 (C 14:0), 13.8 (C 16:0), 5.67 (C 16:1 ω 7), 3.47 (C 18:0), 26.9 (C 18:1 ω 9), 3.39 (C 18:1 ω 7), 8.25 (C 18:2 ω 6), 3.22 (C 18:3 ω 3), 2.06 (C 20:1 ω 9), 2.22 (C 18:4 ω 3), 2.67 (C 20:4 ω 6), 10.7 (C 20:5 ω 3), 1.96 (C 22:5 ω 3) and 5.83 (C 22:6 ω 3).

Model systems preparation

All the reacting mixtures were incubated in triplicate and sampled at 1, 7 and 25 days for fluorescence analysis. 25 ml of each reacting mixture were placed in 30 ml stoppered tubes in dark at 30°C without stirring. Blanks consisted of the individual starting compounds under the same reaction conditions.

Amine substitution degree and concentration

A 0.05 M solution of amine (n-butylamine, BA; dibutylamine, DBA; tributylamine, TBA) was prepared in aq. 86% ethanol. CLO portions (0.5 g) were mixed with 0.1, 0.4, 2 and 10 ml of each amine solution and made up to 25 ml with aq. 86% ethanol leading to 0.2, 0.8, 4 and 20 mM amine mixtures, respectively.

Glutaraldehyde (GL) solution (5 ml, 1 M in aq. ethanol 86%) was mixed with amine (BA, DBA and TBA) solution (1 ml, 1 M in aq. ethanol 86%) and made up to 25 ml with aq. 86% ethanol (40 mM amine).

Chain length and steric hindrance of primary amines

Methylamine (MA) and ethylamine (EA) solutions (0.1 M) were prepared in aqueous 86% ethanol; propylamine (PA), octylamine (OA), n-butylamine (BA), isobutylamine (i-BA), sec-butylamine (s-BA) and tert-butylamine (t-BA) solutions were prepared (0.1 M) in chloroform. CLO (0.5 g) was mixed with 5 ml of each amine solution; then, aq. 86% ethanol and chloroform were added in order to obtain a 25 ml solution in the 1:1 ratio for both solvents.

Fluorescence analysis

Fluorescence measurements (Perkin-Elmer LS 3B) were made at 327/415 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 ($1 \mu\text{g ml}^{-1}$ in 0.05 M H_2SO_4) at the corresponding wavelength. The fluorescence ratio (δF) was calculated as: $\delta F = RF_{393/463\text{nm}} / RF_{327/415\text{nm}}$.

Statistical analysis

Fluorescence ratios (δF) were subjected to one-way ANOVA (Sokal & Rohlf, 1981); $p < 0.05$.

RESULTS AND DISCUSSION

Influence of the substitution degree

As a first step to analyze the effect of the substitution degree on the ability of amine molecules to change the fluorescent properties of the reacting mixtures, a comparison between a primary (BA), a secondary (DBA) and a tertiary (TBA) amine was carried out. The fluorescence ratio (δF) obtained in the different reacting mixtures with CLO are shown in Table 1. For each amine δF increased significantly with time. The most substituted amine (TBA) showed a much lower δF value than the primary one (BA); DBA showed an intermediate value.

In order to study in a more particular way the effect of the substitution degree, the interaction between the same amines (BA, DBA and TBA) and a dialdehyde molecule (glutaraldehyde, GL) was tested. A smaller δF value was detected as a result of increasing the substitution degree in the amino compound, leading again to the decreasing sequence: BA > DBA > TBA (Table 2). Increased reaction times produced progressively greater δF for all three amines.

These results can be explained by the greater reactivity of non-substituted amines since nucleophilicity of the nitrogen atom is not hindered and it interacts easily with lipid oxidation compounds.

Influence of the steric hindrance in primary amines

As primary amines showed the highest δF value, the effect of their steric hindrance on the fluorescence formation was investigated in model systems using BA, i-BA, s-BA and t-BA. For all the amines (Table 3), a significant increase was obtained with the reaction time. From the beginning of the reaction (day 1) differences between amines were observed. After 25 days, the following decreasing δF value was obtained: i-BA > BA > s-BA > t-BA. Results can be explained on the basis that a higher steric

hindrance has led to a lower ability to produce change in the fluorescence properties of the reaction mixture.

In both the BA and i-BA the -NH_2 group is linked to a primary carbon atom. However, the positive inductive effect of the iso-butyl radical is known to be bigger than the one of the butyl radical (Fieser & Fieser, 1966), so that the corresponding -NH_2 group may react easier (higher nucleophilicity) and produce a greater δF .

Influence of the chain length in primary amines

All the amines tested (MA, EA, PA, BA and OA) showed an increase in the δF value as the reaction time increased (Table 4). A comparison between amines after 25 days of reaction, led to the following decreasing sequence: $\text{OA} > \text{BA}$, $\text{PA} > \text{EA}$, MA. The present results led to the conclusion that the increasing chain length exerts a positive effect on the δF value. Possibly longer chain length provides the amine molecule with a more lipid-type structure, so that interaction with lipid oxidation compounds would be facilitated.

A similar conclusion about the effect of the aldehydes chain length was obtained by Montfoort *et al.* (1987), where a comparative study of fluorescence formation was carried out by exposing phosphatidyl ethanolamine containing liposomes to a variety of aldehydes. The fluorescence formation increased with chain lengths in saturated aldehydes and this effect was attributed to the increasing lipophilic character of the aldehydes.

Influence of the amine content

Model systems containing CLO and amines (BA, DBA and TBA) at different concentrations were tested. The δF was studied and results are given in Table 5.

Although differences were not always statistically significant, δF values for each amine increased with concentration at each reaction time, differences were more marked after reaction for 25 days.

These observations on δF values indicate that greater attention should be given to the measurement of these interaction compounds during processing of food materials with a high content of amine constituents such lysine and other basic amino acids (Navarro, 1991).

CONCLUSIONS

The present investigation has provided a comparison between different kinds of amines in terms of their ability in producing interaction compounds with fluorescence properties similar to those detected during fish processing (sterilisation, cooking and chilling) (Aubourg *et al.*, 1992, 1995 & 1997). Similar bathochromic shifts, accelerated by the temperature and amine reactivity, were observed presumably as a result of a formation of compounds with progressively increasing numbers of double bonds (Pokorný, 1977; Gardner, 1979).

Fluorescent compounds are known to be formed as a result of lipid oxidation in the presence of amine molecules (Montfoort *et al.*, 1987; Lubis & Buckle, 1990; Aubourg, 1993) and a great attention has been given to the oxidation compounds formation (Frankel, 1991). From the present results, it is concluded that amine compounds would also play an important role in the fluorescence formation. The absence of such compounds (CLO controls) has shown to produce very little δF . As a

result, it is concluded that both kinds of compounds (lipid oxidation molecules and amines) are necessary in order to obtain the fluorescent development.

From a qualitative point of view, amines with a lower substitution and steric hindrance, and with a higher chain length have been found to produce a higher fluorescence ratio. From a quantitative point of view, amines had been supposed to play a catalytic effect on the condensation reactions (Suyama *et al.*, 1981; Pokorný *et al.*, 1987). However, the present results indicate that the formation of fluorescent compounds would depend not only on the presence of lipid oxidation products but also on the amine content.

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TABLE 1

Mean fluorescence ratio (δF) values ($\pm$ SD, $n = 3$)* obtained by reaction at 30 °C of cod liver oil (CLO) and 20 mM amines with different substitution degree**

Reacting Mixture	Reacting Time (days)		
	1	7	25
CLO	1.72 ± 0.03 a*	1.72 ± 0.11 a	2.44 ± 0.6 a
CLO + BA	4.93 ± 0.16 c	34.3 ± 1.7 d	144 ± 7.2 d
CLO + DBA	4.76 ± 0.31 c	10.6 ± 0.01 c	32.1 ± 0.9 c
CLO + TBA	2.89 ± 0.15 b	5.45 ± 0.16 b	10.4 ± 0.5 b

* Values in the same column followed by different letters are significantly different ($p < 0.05$). Results correspond to 20 mM amine mixtures. Initial δF value of CLO was 1.75.

** Abbreviations: BA (n-butylamine), DBA (dibutylamine) and TBA (tributylamine).

TABLE 2

Mean fluorescence ratio (δF) values ($\pm$ SD, n = 3)* obtained by reaction at 30 °C of glutaraldehyde (GL) and 40 mM amines with different substitution degree**

Reacting Mixture	Reacting Time (days)		
	1	7	25
GL	3.53 $\pm$ 0.12 a*	3.47 $\pm$ 0.03 a	3.27 $\pm$ 0.02 a
GL + BA	5.49 $\pm$ 0.40 c	9.49 $\pm$ 0.19 d	11.2 $\pm$ 0.26 d
GL + DBA	4.34 $\pm$ 0.02 b	4.88 $\pm$ 0.02 c	6.03 $\pm$ 0.05 c
GL + TBA	3.75 $\pm$ 0.03 a	3.70 $\pm$ 0.01 b	3.78 $\pm$ 0.03 b

* Values in the same column followed by different letters are significantly different ($p < 0.05$). Initial δF value of GL was 3.54.

** Abbreviations as expressed in Table 1.

TABLE 3

Mean fluorescence ratio (δF) values ($\pm$ SD, n = 3)* produced by reaction at 30 °C of cod liver oil (CLO) and 20 mM primary amines with different steric hindrance**

Reacting Mixture	Reacting Time (days)		
	1	7	25
CLO	2.30 $\pm$ 0.07 a*	2.26 $\pm$ 0.55 a	2.37 $\pm$ 0.47 a
CLO + BA	5.85 $\pm$ 0.25 d	17.2 $\pm$ 1.0 d	37.6 $\pm$ 2.7 d
CLO + i-BA	5.80 $\pm$ 0.29 d	17.3 $\pm$ 1.0 d	43.4 $\pm$ 1.7 e
CLO + s-BA	3.35 $\pm$ 0.15 c	6.80 $\pm$ 0.41 c	12.6 $\pm$ 0.5 c
CLO + t-BA	2.80 $\pm$ 0.19 b	4.16 $\pm$ 0.28 b	6.20 $\pm$ 0.45 b

* Values in the same column followed by different letters are significantly different ($p < 0.05$). Initial δF value of CLO was 2.28.

** Abbreviations: i-BA (iso-butylamine), s-BA (sec-butylamine), t-BA (tert-butylamine); BA as indicated in Table 1.

TABLE 4

Mean fluorescence ratio (δF) values ($\pm$ SD, n = 3)* obtained by reaction at 30 °C of cod liver oil (CLO) and 20 mM primary amines with different chain length**

Reacting Mixture	Reacting time (days)		
	1	7	25
CLO	2.30 $\pm$ 0.07 a*	2.26 $\pm$ 0.55 a	2.37 $\pm$ 0.47 a
CLO + MA	6.16 $\pm$ 0.23 c	17.6 $\pm$ 1.5 cd	29.5 $\pm$ 1.7 b
CLO + EA	5.09 $\pm$ 0.04 b	14.5 $\pm$ 0.3 b	31.5 $\pm$ 1.6 b
CLO + PA	5.35 $\pm$ 0.29 bc	16.2 $\pm$ 1.8 bc	35.1 $\pm$ 1.6 c
CLO + BA	5.85 $\pm$ 0.25 c	17.2 $\pm$ 1.0 cd	37.6 $\pm$ 2.7 c
CLO + OA	5.70 $\pm$ 0.26 c	19.7 $\pm$ 1.3 d	44.9 $\pm$ 1.5 d

* Values in the same column followed by different letters are significantly different ($p < 0.05$). Initial δF value of CLO was 2.28.

** Abbreviations: MA (methylamine), EA (ethylamine), PA (propylamine), OA (octylamine); BA as indicated in Table 1.

TABLE 5: Mean fluorescence ratio (δF) values ($\pm$ SD, $n = 3$)* obtained by reaction at 30 °C of cod liver oil (CLO) and different amine concentrations**

Amine	Concentration of amine (mM)	Reacting Time (days)		
		1	7	25
BA	0.2	2.25 $\pm$ 0.24 a*	6.76 $\pm$ 3.6 a	13.8 $\pm$ 4.3 a
"	0.8	2.51 $\pm$ 0.12 a	9.55 $\pm$ 1.0 a	44.4 $\pm$ 3.2 b
"	4	3.31 $\pm$ 0.10 b	16.7 $\pm$ 0.7 b	70.0 $\pm$ 5.1 c
"	20	4.93 $\pm$ 0.45 c	34.3 $\pm$ 1.7 c	144 $\pm$ 7.2 d
DBA	0.2	1.91 $\pm$ 0.08 a	2.02 $\pm$ 0.13 a	3.42 $\pm$ 0.26 a
"	0.8	2.01 $\pm$ 0.09 a	3.13 $\pm$ 0.36 b	6.30 $\pm$ 1.0 b
"	4	2.47 $\pm$ 0.12 b	5.38 $\pm$ 0.43 c	12.8 $\pm$ 0.59 c
"	20	4.76 $\pm$ 0.31 c	10.6 $\pm$ 0.24 d	32.1 $\pm$ 0.24 d
TBA	0.2	1.79 $\pm$ 0.08 a	1.87 $\pm$ 0.09 a	2.24 $\pm$ 0.13 a
"	0.8	1.93 $\pm$ 0.02 ab	2.34 $\pm$ 0.11 b	3.65 $\pm$ 0.6 a
"	4	2.06 $\pm$ 0.09 b	3.02 $\pm$ 0.27 c	6.14 $\pm$ 1.0 b
"	20	2.89 $\pm$ 0.15 c	5.45 $\pm$ 0.16 d	10.4 $\pm$ 0.53 c

* Abbreviations as indicated in Table 1. Four different concentrations (20, 4, 0.8 and 0.2 mM) for each amine were employed. Initial δF value of CLO was 1.75.

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