FOOD CHEMICAL CONTAMINANTS

Relevance of Matrix Effect in Determination of Biogenic Amines in Plaice (*Pleuronectes platessa*) and Whiting (*Merlangus merlangus*)

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Spoilage can be evaluated by separating and determining biogenic amines by various techniques, notably high-performance liquid chromatography. Previous studies have not taken into account how the muscle tissue matrix affects the assay. We demonstrate a matrix effect in plaice and whiting and show that it changes during spoilage. This effect should be taken into account when plotting regression lines relating the quantity of amine to the biogenic amine/internal standard ratio.

Most studies of biogenic amines in fish relate to the toxic effects of histamine, especially in scombroid fish whose muscle tissue is rich in free histidine. The degree of spoilage of fish has been assessed in terms of biogenic amines (cadaverine, histamine, putrescine, tryptamine, tyramine, spermidine, and spermine), which result from decarboxylation of amino acids, essentially by microbial enzymes (1). Gas chromatography (2), micellar electrokinetic chromatography (3), and thin-layer chromatography (4) can be used to separate these amines, and high-performance liquid chromatography (HPLC; 5–7) enables rapid determination of a few parts per million (whence the importance of the method of quantitation). We studied samples that decompose with time, during which period the composition of the matrix also may undergo changes. As the sample matrix may perturb the biological assay (8), its effect on the quantitation should be taken into account.

We sought to optimize the quantitation of biogenic amines in samples containing fish muscle tissue. We used HPLC to separate the amines in 2 fish species, whiting and plaice, at different stages of spoilage.

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METHOD

Reagents

(a) *Standard solutions.*—Putrescine dihydrochloride (put), cadaverine dihydrochloride (cad), histamine dihydrochloride (his), methylamine hydrochloride (met), spermidine trihydrochloride (spd), spermine tetrahydrochloride (spm), tyramine hydrochloride (tyr), and tryptamine hydrochloride (tryp) distilled water were purchased from (Sigma Chemical Co., St. Louis, MO). Use ammonia (NH_4^+) distilled water. Store at 5°C. Prepare 50 ppm (final concentration in fish muscle slurry) stock solution of a mixture of the biogenic amines plus dilutions (25, 12.5, 6.25, 3.12, and 1.56 ppm).

(b) *Dansyl chloride solution.*—Dissolve 750 mg dansyl chloride (DC; Sigma) in 100 mL acetone. Store at –20°C protected from light.

(c) *Internal standard.*—Dissolve 80 mg 1,3-diaminopropane dihydrochloride (diam; Sigma) in 100 mL distilled water and store at 5°C.

(d) *L-Proline solution.*—Dissolve 100 mg L-proline (pro; Sigma) in 1 mL distilled water and store at 5°C.

Apparatus

(a) *Cryostat.*—Heto (Allerod, Denmark).

(b) *Homogenizer.*—Ultraturrax (Labortechnik, Germany).

(c) *Centrifuge.*—Avanti 30, Beckman.

(d) *LC system.*—Kromasil C_{18} 5 μm, 100 Å (25 cm × 4.6 mm) reversed-phase column thermostated at 25°C, fitted with a Brownlee C_{18} 5 μm (3 cm × 4.6 mm) precolumn. Elute with the gradient developed by Malle et al. (1) with 2 pumps (Model LC-6A; Shimadzu, Kyoto, Japan). Class-VP software

was used for programming and integration (Shimadzu). Filter (0.2 μm) and inject with an automatic injector (Model SIL-6B, Shimadzu) cooled with the same cryostat.

Sample Preparation

Two species of whole fish from commercial fishing boats were used: plaice (*Pleuronectes platessa*) and whiting (*Merlangus merlangus*). A homogeneous batch of each species was stored at 0°C in ice for several days. The flesh of a whole fillet was ground in a mixer (Waring) to provide a homogeneous sample for measurement of the different biogenic amines added.

Biogenic Amines Calculation

We used the standard addition method by adding increasing quantities of biogenic amines (1.56, 3.12, 6.25, 12.5, 25, and 50 ppm) to the sample (muscle tissue) and plotting the regression lines used in quantitation, taking into account the matrix. A regression line was used directly to quantitate the different biogenic amines in solution and by the standard addition method. In each case, we used the ratio A_{ba}/A_{is}, where A_{ba} is the integration area corresponding to the amine and A_{is} is that of the internal standard. The correlation between the amount of biogenic amine (Q in ppm) and the A_{ba}/A_{is} ratio was determined from a simple linear regression model without a constant coefficient:

$$Q_k = a(A_{ba}/A_{is})_k + e_k$$

where k is the index of repetition, a is the slope of the line, and e_k is the residual term.

The regression line parameters were determined for amines in solution and in standard solution in distilled water at concentrations of 50, 25, 12.5, 6.25, 3.12, and 1.56 ppm, with 5 measurements per concentration. The regression line parameters for the standard addition method were determined by adding to 5 different fish the following concentrations of each amine: 50, 25, 12.5, 6.25, 3.12, and 1.56 ppm.

Statistical Analysis

SAS, version 6 software was used. The results were calculated by using general linear models, based on the least-squares method. Simple linear regression models without a constant coefficient were used to determine a coefficient of response. Then, models of analysis of covariance were used to study any effects of species and stage of spoilage.

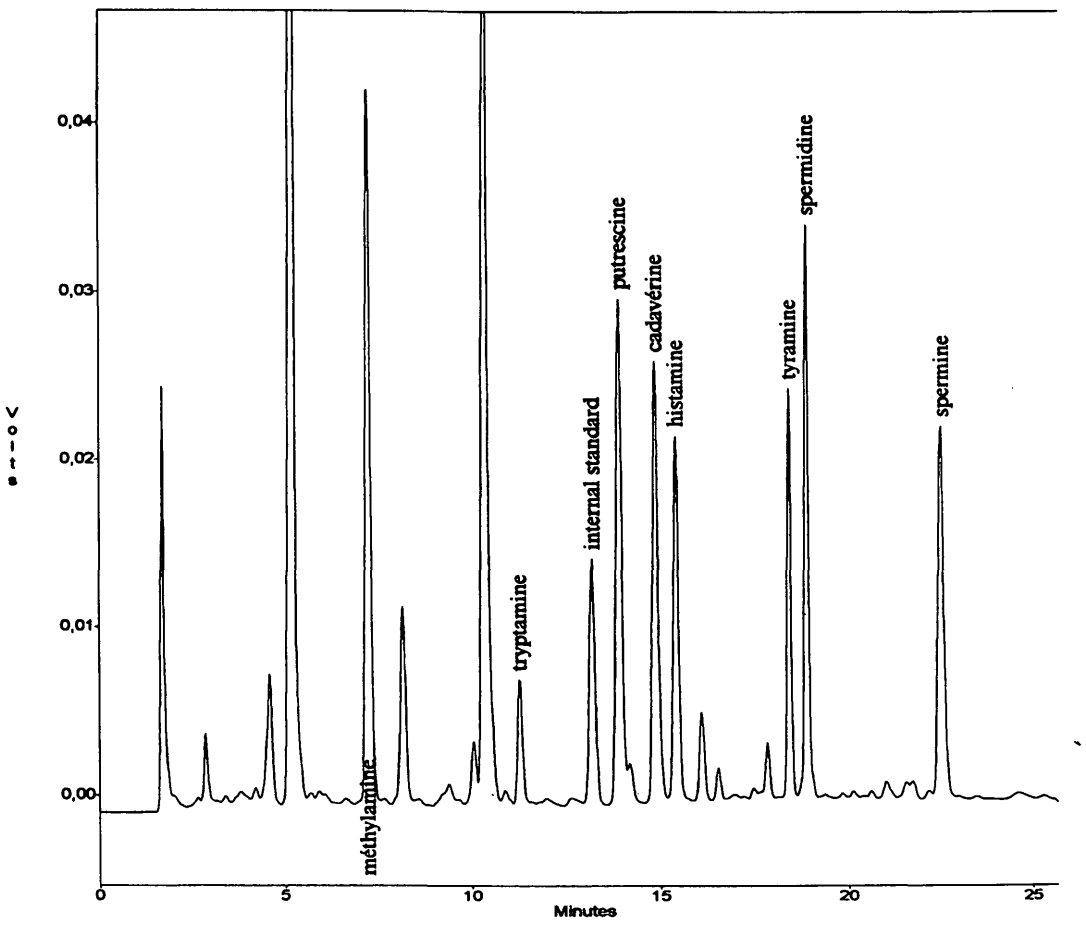

Figure 1. Sample chromatogram of separation of 25 ppm biogenic amines in plaice.

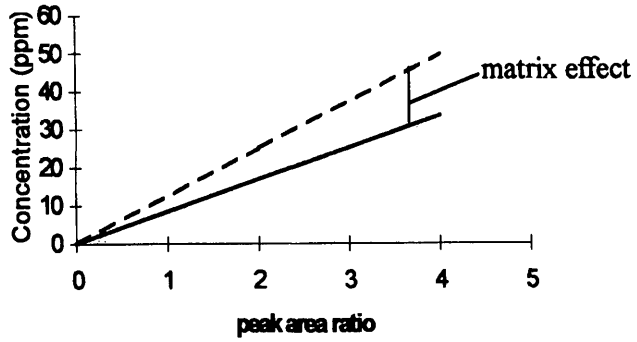

Figure 2. Regression lines for histamine obtained by the method of amines in solution (———) and by the standard addition method (— — —) in whiting.

Protocol

To 5 g fish sample and/or standard solution (50, 25, 12.5, 6.25, 3.12, and 1.56 ppm final concentration), add 10 mL 0.2M perchloric acid and 100 mL internal standard. Cold grind once; centrifuge at 12 500 × *g* for 5 min; transfer to a tube that can be hermetically sealed; add 100 µL supernatant, 200 µL saturated Na_2CO_3, and 400 µL DC solution. Shake and leave in a water bath at 60°C for 5 min. Add 100 µL proline solution, shake, and allow to stand at room temperature for 15 min in the dark. Add 500 µL toluene, shake, recover the organic phase, and evaporate it under a stream of nitrogen. Resuspend the pellet in 200 µL acetonitrile and filter before injection and LC analysis.

Results and Discussion

Calculation of Regression Lines

Diaminopropane can be used in reference to previous studies (9–11) to calculate a response factor for different amines compared with the internal standard (m). The regression parameters of Q for (A_{ba}/A_{is}) are then estimated from the chromatogram, such as that presented in Figure 1. This method of calculation allows losses occurring during the various steps of the protocol to be taken into account directly (losses of amines are taken to be proportionally identical to those of the internal standard).

This approach was applied to determination of the regression lines for putrescine, cadaverine, histamine, spermine, spermidine, tyramine, tryptamine, methylamine, and ammonia in standard solution, and in whiting flesh, by the standard addition method (l).

Figure 2 shows 2 regression lines obtained for the histamine assay. There are significant differences between the slope determined in solution and that obtained by the standard addition method with whiting. The amine solution method gave a value of 25.2 ppm whereas the standard addition method, which took into account the matrix, gave 37.4 ppm (Figure 2) for the same peak area ratio of 3. It is conceivable that biogenic amines are underestimated because they are part of a complex matrix in which interactions occur and may lower the detection sensitivity.

Table 1 shows the coefficients for the regression lines for the biogenic amines in solution and as determined by the standard addition method. The slopes are lower for amines in solution in all cases, but with marked variations for spermine, spermidine, methylamine, and histamine. These variations may distort the quantitation, which is also affected by the matrix. It is interesting to investigate whether changes in the matrix during the course of spoilage, or as a function of the fish species analyzed, result in significant alterations in the quantitation.

Changes in the Regression Lines as a Function of Spoilage and Species

Two species of fish (plaice and whiting) at 3 stages of spoilage (D + 1, D + 4, D + 7; D = day) were studied by the standard addition method. The influence of the 2 qualitative factors—species and spoilage—was tested by using a covariance analysis model, without a constant coefficient, for each amine (12):

$$Q_{ijk} = (a + \alpha_i + \beta_j + \gamma_{ij})\, x_{ijk} + e_{ijk}$$

Table 1. Regression lines for biogenic amines in solution and by the standard addition method in whiting[a]

Biogenic amine	a for standard solution	r^2	a for standard addition method in whiting	r^2
Spermine	8.23	0.9964	16.99	0.9635
Spermidine	9.20	0.9825	13.83	0.9661
Tyramine	13.91	0.9897	15.12	0.9189
Tryptamine	24.2	0.999	29.24	0.9364
Methylamine	2.98	0.9995	5.61	0.9698
Putrescine	6.59	0.9841	8.39	0.9756
Cadaverine	8.69	0.9995	9.95	0.9673
Histamine	8.41	0.9836	12.46	0.9446

[a] a = slope; r^2 = coefficient of determination.

Table 2. Change in slope during spoilage of whiting and plaice[a]

Amine	Day	$Pr(\alpha_1 = \alpha_2 = 0)$	(a + α)		r^2
			Whiting	Plaice	
Cadaverine	1	0.0001[b]	12.46	10.71	0.99
	4	0.4511	10.22	10.41	0.98
	7	0.0004[b]	8.7	9.72	0.97
Histamine	1	0.0001[b]	16.2	12.77	0.99
	4	0.0032	13.28	14.81	0.96
	7	0.021[b]	10.28	11.24	0.96
Methylamine	1	0.0001[b]	7.17	6.19	0.99
	4	0.2449	5.28	5.39	0.99
	7	0.0001[b]	5.04	5.68	0.97
Putrescine	1	0.0001[b]	10.05	9.00	0.99
	4	0.4275[b]	8.47	8.30	0.98
	7	0.0723[b]	7.43	7.75	0.98
Spermidine	1	0.0001[b]	14.95	12.49	0.98
	4	0.0001[b]	16.35	13.47	0.95
	7	0.0069[b]	11.5	10.58	0.97
Spermine	1	0.0001[b]	17.51	13.91	0.97
	4	0.0001[b]	20.82	13.68	0.92
	7	0.0001[b]	14.23	11.52	0.96
Tryptamine	1	0.0001[b]	43.9	33.69	0.98
	4	0.0710	27.72	29.21	0.98
	7	0.0001[b]	24.98	31.47	0.96
Tyramine	1	0.0001[b]	20.9	14.89	0.97
	4	0.3791	17.99	17.38	0.95
	7	0.0071[b]	11.71	13.04	0.96

[a] $Pr(\alpha_1 = \alpha_2 = 0)$ = probability to species effect; r^2 = coefficient of determination; (a + α) = slope of the regression line.
[b] Significant to 1%.

where a is a constant term, α_i is the term corresponding to the species effect i (whiting or plaice), β_j is the term corresponding to the effect of stage (D + 1, D + 4, D + 7), γ_{ij} is the term corresponding to the species-spoilage interaction, k is the repetition index, x_{ijk} is the k^{th} value of the ratio (A_{ba}/A_{is}) for the species i and the stage d, e_{ijk} is the residual term, and $(a + \alpha_i + \beta_j + \gamma_{ij})$ is therefore the slope of the regression line between Q and x. The index showed that the interaction between the species and the degree of spoilage had a significant effect (critical probability associated with $F < 0.001$) for all the amines. The adjustments were satisfactory [all coefficients of determination (r^2) > 0.95].

On the basis of models of analysis of covariance, by amine and by stage of spoilage, where the qualitative factor is the species, $Q_{ik} = (a + \alpha_i)\, x_{ik} + e_{ik}$, $(a + \alpha_i)$ is then the slope of the regression line of Q for x.

It can be shown that (*1*) for the stage of spoilage D + 1, the slopes of the regression lines for all amines are significantly smaller for plaice than for whiting (critical probability associated with $F = 0.001$); (*2*) for stage D + 4, the slopes associated with the species are not significantly different, except for spermidine and spermine where the slope is significantly smaller for plaice than whiting; (*3*) for stage D + 7, the slopes again differ significantly (except for putrescine). The coefficients for spermidine and spermine are lower for plaice than whiting but greater than those for the other amines. Table 2 gives the estimates for the slopes of the regression lines for the 3 stages of spoilage and the 2 species.

Note also that (*1*) except for spermine and spermidine, the slope progressively decreases as the muscle tissue of the whiting decomposes, which is also true for cadaverine, putrescine, and spermine in plaice and (*2*) the slope associated with tryptamine is very high and clearly differs for plaice and whiting. Tryptamine has never been found in whiting or plaice, and just traces have been detected in salmon and sardine (13).

The changes in the regression lines linked to species and to duration of spoilage can be remedied by plotting regression lines for each species studied or plotting regression lines by

Table 3. Overall regression lines obtained by using the standard addition method for whiting and plaice[a]

	Whiting		Plaice	
Biogenic amine	a	r^2	a	r^2
Cadaverine	9.95	0.9673	10.24	0.9935
Histamine	12.46	0.9440	12.693	0.9818
Methylamine	5.61	0.9698	5.71	0.9937
Putrescine	8.39	0.9756	8.29	0.9925
Spermidine	13.83	0.9661	11.89	0.9758
Spermine	16.99	0.9635	12.79	0.9716
Tryptamine	29.24	0.9364	31.26	0.9815
Tyramine	15.12	0.9189	14.71	0.9721

[a] a = slope; r^2 = coefficient of determination.

using several samples at different stages of spoilage to determine a single overall regression line, rather than a sample at a precise moment of spoilage (Table 3). This approach also has the advantage of being applicable to samples of unknown degree of spoilage (which is always the case).

Conclusion

We have demonstrated the usefulness of studying the influence of the matrix during assay of biogenic amines in plaice and whiting. It is essential to use the standard addition method to achieve a quantitation that is closest to reality, rather than the method of direct determination using standard solutions. The species factor generates significant differences in slope determination: amines should therefore be quantitated for each species. In addition, spoilage of muscle tissue causes alterations in the matrix, resulting in changes in the slopes of the regression lines. The slopes should therefore be determined for each amine, over several days of spoilage, to provide an overall estimation.

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