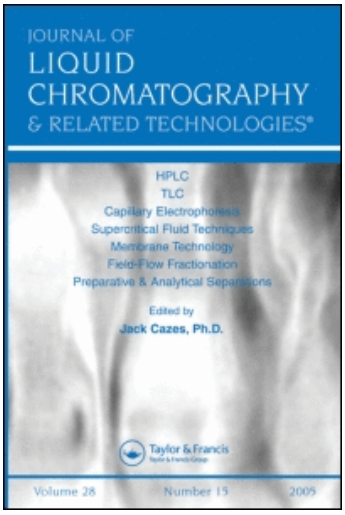

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Thin Layer and High Pressure Liquid Chromatographic Determination of Histamine in Fish Tissues

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THIN LAYER AND HIGH PRESSURE LIQUID CHROMATOGRAPHIC DETERMINATION OF HISTAMINE IN FISH TISSUES

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ABSTRACT

TLC and HPLC techniques have been examined for histamine determination in fish. In case of HPLC determination a comparison of the performance characteristics of UV-Vis and Fluorescence detection is also described. Regression analysis was performed between the two sets of data, in order to examine the relative efficiency of the two techniques for the determination of histamine in fish sample extracts. Either of the two techniques proved sufficient for the determination of histamine in common concentration ranges in fish extracts although fluorescence detection had better sensitivity. The recovery of histamine from fish samples was decreased in comparison to the standard solutions but it was still sufficient for routine analysis of the fish samples.

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INTRODUCTION

Histamine [4-(2-aminoethyl)-imidazole] is produced mainly by kidneys, liver and intestinal mucosa because of histidine decarboxyliosis by the enzyme histidine decarboxylase (1-2). Abnormal secretion and neurotransmitters metabolism (histamine being a by-product) are correlated with several diseases such as pheochromocytoma (3), neuroblastoma (4), schizophrenia (5), malignant hyperthermia (6) and probably hypertension (7). It is generally accepted that histamine acts as endotoxin and plays part in allergic shock (8).

However, besides indigenous production of histamine, it is possible to be noticed elevation of its level in plasma up to 2.6 ppb (9) after consuming foodstuff, especially fish, where histamine is present because of micro-organism action. The excretion of histamine products produced during its metabolism is only 2-3% in the original form while 4-8% is excreted in the form of methylistamine, 9-11% as imidazoloacetic acid, 42-47% as methylimidazoloacetic acid, 16-32% as imidazoloacetic acid ribozide and less than 1% as acetylistamine (10). According to EEC guidelines 91/493, histamine acceptable upper limit in fish is 100 ppm and several investigators have reported that levels vary with the kind of fish and their freshness, for example, in salmon 0-50 ppm, in tuna canned 16-74 ppm, and in anchovy 3.1-13.8 ppm (11-12).

For histamine determination, several techniques have been developed : electrophoretic, radioimmuno-enzymatic, fluorescent and chromatographic (TLC, GC/MS, HPLC) (13-21). In particular, HPLC has been reported as the method of choice for the determination of histamine in case of pig hypothermia and beef stress, while in medicine in case of brain tumour.

In the present study, thin layer and high performance liquid chromatographic techniques have been investigated for histamine determination in fish samples. A comparison of the performance characteristics of UV-Vis and fluorescence detection is, also, described in case of HPLC detection. Also the stability and repeatability of the derivatization procedure for the preparation of the fluorescent derivative of histamine was examined.

MATERIALS AND METHODS

Equipment and Instruments

-Thin layer chromatographic plates 10x10 cm (MERCK No 3156)
-UV chamber 254-366 nm (CAMAG)

-Liquid chromatograph (GILSON, model 802)
-UV-Vis detector (GILSON) at 210 nm
-Fluorescence detector (GILSON model 121)
-Chromatography column (a.*Lichrospher RP-18 5μm and b.Hamilton PRP-X 200*
-Fluorescence filters, excitation 305-395 nm, emission 430-470 nm.

Solvents and Solutions

For TLC:

The developing solvent consisted from ethanol and NH_4OH (80/20 v/v)
The spraying reagents used for colour development were prepared as follows:
1.Iodoplatinate (0.25 g $PtCl_6$ and 5g KI diluted to 100 ml with a solution containing 2 ml c.HCl in deionized water).
2.Ninydrine (0.5 g ninydrine diluted to 100 ml with acetone).
3.Fluorescamine (0.0125 g fluorescamine diluted to 100 ml with acetone).

For HPLC:

The mobile phase used for the absorbance detection was an aqueous solution of 0.4 M KH_2PO_4, pH=4.5.
- Mobile phase for the fluorescence detector 0.05 M NaH_2PO_4 70% and acetonitrile 30%, pH=4.5.
- Derivatization solvents : O-phthalaldehyde (OPA) 0.1% in methanol ,2M NaOH ,1N H_2SO_4.
Standard histamine stock solution of 1000 ppm was prepared in water (169.1 mg histamine hydrochloric/100 ml) and working standards of 0.1, 0.5, 1, 2, 5, 8, 10 ppm in 0.2 N HCl.

Specimen Pretreatment

Histamine isolation was performed according to Mietz and Karnas (11). Five grams of tissue with 10 ml of 5% CCl_3COOH solution were treated and purified with a mixture of n-butanol, n-heptane and chloroform. Histamine is finally isolated with 3 ml 0.2N HCL. From these 3 ml, a) 1.3 and 5μl were injected on TLC plates, b) 20 μl were injected in HPLC with UV detector and c) 100 μl were derivatized for fluorescence detection.

Derivatization Procedure for Fluorescence Detection

A volume of 100 μl from the above hydrochloric solution of histamine was diluted with 900 μl of 0.2 N HCl, treated with 200 μl of 2M NaOH and 100 μl of methanolic solution 0.1% OPA, and were kept in darkness for 4 min. Finally, 200 μl of 1N H_2SO_4 were added and the solution kept for 2 hours. At this time, the specimen was ready for injection. The analysis of the specimens was then executed in the same day.

RESULTS AND DISCUSSION

TLC Identification of Histamine

Histamine was separated from aqueous standard solutions by thin layer chromatography. Three colour developing substances were tested as spraying reagents: iodoplatinate, ninydrine and fluorescamine. The mobile phase employed was a mixture of ethanol/aqueous ammonia (80/20 v/v) and the R_f value was 0.62. The injected volume was 1 μl in all the experiments.

In table 1, the results obtained from the above experiment are presented. All the spraying reagents were proved equally efficient in refer to the detection limit obtained (50 ng absolute), although fluorescamine gave a more clear chromatographic picture. When 0.2 N HCl was used as diluent for the standard solutions the spots were stable for 24 hours only but when HCl was replaced with methanol the stability of the colour was better for longer periods of time. Iodoplatinate reagent coloured the plate with a light brown colour and the spots became dirty. Ninydrine developed a red violet colour and the rate of colour development increased by heating the plates to 60°C.

Performance Characteristics of the Absorption and Fluorescence Determination of Histamine by HPLC

Histamine could be determined after the elution from the chromatographic column either directly by absorbance measurement or by measurement of fluorescence after derivatization. This procedure was described at the experimental section.
In figures 1 and 2, four typical chromatograms obtained from histamine determinations

TABLE 1.
Characteristics of TLC Histamine Determination with Various Spraying Reagents (R_f = 0.62).

Spraying reagents	Developed colour	Remarks
a. Iodoplatinate	Brown	Dirty spots because of light brown colour on the plate surface
b. Ninydrine	Red violet	Heating at 60°C accelerates the colour development
c. Fluorescamine	Yellowish-green	Stable colour when MeOH is used as diluent, well defined spots

are given. In each figure the first chromatogram correspond to standard solutions while the second correspond to fish samples (sardines). In figure 1 the chromatograms were produced by absorption measurements and in figure 2 by fluorescence measurements. It was obvious that the later were almost free from interfering peaks while the picture by absorption detection appeared to be more confused because of the existence of other peaks due to the matrix of the samples.

Regression analysis was performed for both of the determination techniques. The calibration curve by ultraviolet detection was linear up to 10 ppm of histamine. However, the calibration curve by fluorescence detection was extended just to 2 ppm because of its higher sensitivity. The sensitivity expressed as the slope of the calibration curve was five times greater with fluorescence detection than with absorption detection.

In table 2 the two procedures are given in a comparative form as well as the regression data of each working curve.

Critical Comparison of the Absorption and Fluorescence Calibrations in the Determination of Histamine by HPLC

The histamine content of ten different fish samples (anchovy) was determined following the two prescribed techniques and referring to the corresponding calibration

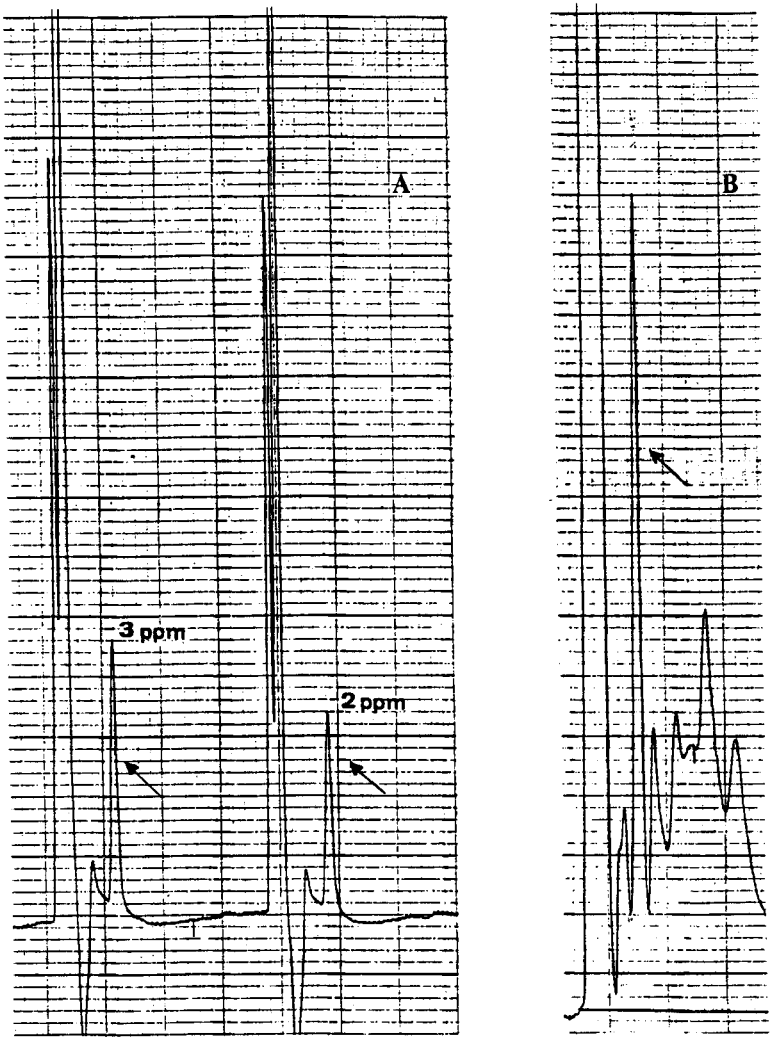

FIGURE 1. Typical chromatograms from HPLC determinations of histamine in standard solutions (A) and in sardine samples (B). The detection was based in absorbance measurements, 0.005 AUFS.

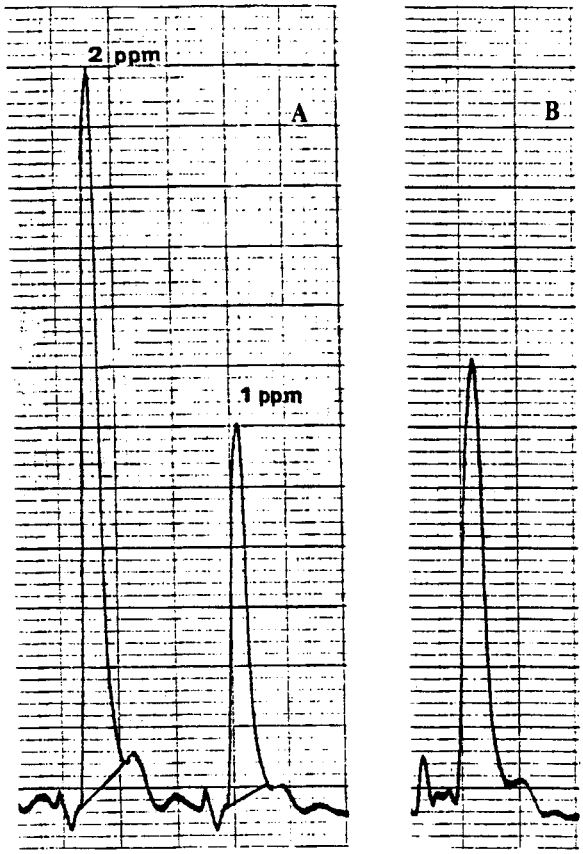

FIGURE 2. Typical chromatograms from HPLC determinations of histamine in standard solutions (A) and in sardine samples (B). The detection was based in fluorescence measurements, 0.02 AUFS. The solutions were diluted 1:10 to 1:15 before their injection, as it is described in the text.

curves (Table 2). Regression analysis was performed between the two sets of data, in order to examine the relative efficiency of the two techniques for the determination of histamine in fish sample extracts. The extracts of fish samples which were measured for fluoresence, needed to be diluted 1:10 to 1:15 with the extraction mixture but for the absorbance measurement they were used directly. The results of these determinations are listed in table 3.

The concentrations calculated by UV-Vis detection were scaled on Y-axis against the concentration calculated by fluoresence detection which were scaled on

TABLE 2.
Comparison of Sensitivity of the two Techniques for the Determination of Histamine in Standard Solutions after High Performance Liquid Chromatographic Elution.

Parameter	Absorbance	Fluorescence
	Chromatographic Data	
Detectors	UV-Vis, 210 nm	Excitation 305-395nm Emission 430-470nm
Chromatographic column	Hamilton, PRP-X200 No 79441 Cationic	Lichrospher RP-18, 5μm
Mobile phase	0.4M KH_2PO_4 100% (pH=4.5)	0.05M NaH_2PO_4 70% Acetonitrile 30% (pH=4.5)
Flow rate	0.5 ml/min	0.5 ml/min
Pressure	0.26 kpsi	1.10 kpsi
Development time	4 min	4 min
	Regression Data	
Concentration Range	0 to 10 ppm	0 to 2 ppm
Intercept value (a)	0.42	-2.31
Confidence Interval of the intercept (95%)	(-5, 6)	(-4.9, 0.25)
Standard deviation of the intercept	2.19	1.26
Slope value (b)	11.756	56.083
Confidence Interval of the slope (95%)	(10.8, 12.7)	(53.5, 58.7)
Standard Deviation of the slope	0.38	1.29
Correlation Coeff.(r)	0.9974	0.9901
Detection limit	10 ng	1 ng

TABLE 3.
Concentration of Histamine in Anchovy Samples Analysed by HPLC and UV-Vis or Fluorescence Detectors.

Sample No.	Concentration(ppm) from absorbance	Concentration (ppm) from fluorescence
1	5.50	5.60
2	7.50	7.35
3	4.50	4.87
4	6.00	5.61
5	3.00	3.36
6	2.25	2.17
7	3.34	3.35
8	3.50	3.55
9	3.75	3.60
10	3.54	3.50

X-axis. In case of equal efficacy between the two techniques (i.e. same sensitivity and precision), the slope of the linear curve should be equal to unit (in this case +1) and the intercept equal to zero. Moreover when the reproducibility of the two techniques is comparable, the correlation coefficient should also be very close to unit (in this case +1). The results of this regression analysis are listed in table 4.

Thus, it was proved that the identical values laid inside the confidence intervals for a 95% significance level. So either of the two techniques could be used for the determination of histamine in such concentration range in fish extracts.

Reproducibility of Derivatization Procedure

The procedure described in the experimental section for the production of a fluorescent product of histamine was repeated six times in the same extract and the variation of the peak heights obtained during the HPLC elution was examined. The mean height of the six determinations was 109.5 ± 16.9 at 99% significant level and the relative standard deviation was 9.38%. Thus, the derivatization procedure was proved sufficiently reproducible.

The next step was to examine the day-to-day stability of the derivative product. Six repetitions of the derivatization procedure were done in a day and the extracts were measured by HPLC. They left stand for 48 hours and then they were analysed

TABLE 4.
Regression Data for the Comparison of the Efficacy of the Two Techniques for the Determination of Histamine in Fish Extracts.

Statistics	Experimental Value	
Overall mean	4.29 ppm	
Experimental Error	0.155	
Relative Experimental Error	3.61 % (<5%)	
Regression Equation	Y = - 0.24 +	0.95X
Standard Deviation	of intercept 0.22	of slope 0.05
Confidence Limits (95%)	-0.27, 0.74	0.85, 1.06
Identical Values	0.0	1.0

again. The two sets of data were statistically tested for their homogeneity. The coefficient of variation was 0.563 and 0.792 for the two days and the pooled coefficient was 0.678. The calculated t-value was 2.46 and the critical values for 95 and 99% significant levels are 2.23 and 3.17 respectively. Thus, for a 99% significance the two sets are homogeneous, although in lower significance they might be different. The conclusion coming from these results is that the fluorescence product must be measured in day in order to avoid probable differentiation in the results.

Recovery Study

The recovery efficiency of the extraction procedure was tested either in standard solutions and in fish samples by absorbance detection. Six different standard solutions and three sardine samples were analysed each time and the results are shown in details in Table 5. The standard solutions were spiked in duplicate with 5, 25 and 50 μg of histamine. One part from each of the three fish samples was spiked with 5 μg of histamine and the other equal part was analysed directly without any addition.

As it can be seen from the above table, although the recovery of histamine from fish samples was decreased in comparison to the standard solutions, it was still sufficient for routine analysis of the fish samples.

TABLE 5.
Recovery of Histamine after Extraction from Standard Solutions and Fish Samples.

Statistics	Standard Solutions	Sardine Samples
Mean Recovery(%)	88.25	75.44
Range of values	80.5-94.5	64.68-82.35
Standard Deviation	5.13	9.44
Standard Error	2.09	5.45
99% Confid. Limits	82.85-93.65	61.38-89.5

CONCLUSIONS

TLC application by three spraying reagents proved sufficient for histamine determination. Fluorescamine as development reagent gave a slightly clearer picture lasting only 24 hours when the standard solution was diluted with 0.2 N HCl but being stable for a longer period when the standard solutions were prepared in methanol. This technique was proved sufficient for the detection of histamine in solutions with concentration of 50 ppm.

Absorbance detection was less sensitive than fluorescence detection. However, for the routine analysis of histamine in fish samples both techniques could be applied with reliable performance characteristics.

Although fluorescence detection was proved very sensitive, it should be kept in mind that it is more time consuming because of an extra procedure needed for the derivatization of histamine.

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