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Short communication

# Histamine and histidine determination in tuna fish samples using high-performance liquid chromatography Derivatization with *o*-phthalaldehyde and fluorescence detection or UV detection of ''free'' species

Valeria Frattini[a], Claudia Lionetti[b,*]

[a]*Presidio Multizonale Igiene e Prevenzione, Azienda U.S.S.L. 7, Oggiono (Lecco), Italy*
[b]*Presidio Multizonale Igiene e Prevenzione, Azienda U.S.S.L. 38, Milan, Italy*

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**Abstract**

A rapid and simple HPLC procedure for the simultaneous determination of histamine and histidine is presented. Sample extraction, extract purification, derivatization and reversed-phase chromatographic determination are described. Also the analysis of ''free'' (i.e., not derivatized) species with UV detection is discussed, and the two procedures are compared. A derivatization step involves more difficulties in terms of linearity and reproducibility because of the great unstability of *o*-phthalaldehyde derivatives but presents many advantages due to the good separation between histamine and histidine as far as the increased sensitivity is concerned. The first results of canned tuna samples analysis are presented and discussed.


*Keywords:* Food analysis; Fish; Histamine; Histidine

## 1. Introduction

Histamine, like some other biogenic amines, is naturally present in many vegetables (tomatoes, bananas, plums) in amounts without toxicological significance. The toxicological aspect becomes relevant with fermented foods (beer, wine, cheese, preserved fish in oil) if the preserving process is performed using hygienically inadequate procedures. In fact, the presence of histamine is due to the bacterial decarboxylation of histidine, the corresponding amino acid, which is present in high amounts especially in fish tissues of the Scomberiscida and Scombridae families, e.g., tuna fish, mackerel, sardine, anchovy etc. [1,2,6,13]. A histamine intake of 70–1000 mg per single meal may cause the so called ''sgombroide poisoning'' that generally reveals itself in a slight form and evolves in a short time but may cause death of some very sensitive subjects [1,2]. Histamine is therefore used as an indicator of the good manufacturing practice and of the preservation state of some food, for instance tuna fish in oil [1].

Many methods for the identification and quantification of histamine have been proposed over the years. They can be divided into two classes:

*Corresponding author.

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(1) Analysis of histamine as such [3–5]: these procedures are generally simple and allow short analysis times, but the chromatographic aspect shows some drawbacks such as the lack of sensitivity and specificity due to the low wavelength of detection.

(2) Analysis of histamine by pre- or post-column derivatization [6–16] with fluorescence detection. The formation of a fluorescent compound enhances the sensitivity and specificity of the analysis. Furthermore, pre-column derivatization also improves the chromatographic behaviour of the analyte.

The aim of the work presented herein was the development of a simple procedure for the identification of histamine in food samples. The procedures proposed have been tested on tuna fish samples. Two different methods were evaluated: (method A) extraction with $HClO_4$ and analysis of histamine as such by high-performance liquid chromatography (HPLC) with UV detection at 210 nm, and (method B) extraction with trichloroacetic acid (TCA) or $HClO_4$ and pre-column derivatization analysis with fluorescence detection.

## 2. Experimental

### 2.1. Chemicals

HPLC-grade solvents were provided by Carlo Erba (Milan, Italy). Histamine and histidine reference standards (98% purity grade) were from Sigma–Aldrich (Milan, Italy). All other chemicals were of analytical grade from Mallinckrodt Baker Italia (Milan, Italy): 10% aqueous TCA, 6 *M* HCl, 0.1 *M* HCl, 0.6 *M* $HClO_4$, 1 *M* NaOH, NaCl, *n*-heptane, butanol, light petroleum (b.p. 46.1–60.6°C), sodium tetraborate heptahydrate ($Na_2B_4O_7 \cdot 7H_2O$), *o*-phthalaldheyde (OPA), β-mercaptoethanol (BME), ethyl acetate, methanol, potassium dihydrogenphosphate ($KH_2PO_4$), 85% $H_3PO_4$, acetonitrile.

Water was obtained from a Milli-Q purification system (Millipore, Milan, Italy).

#### 2.1.1. Extracting solution

Eight ml of 0.1 *M* HCl, 76 ml of 10% aqueous TCA and 16 ml of *n*-heptane.

#### 2.1.2. Derivatizing solution

One g of $Na_2B_4O_7 \cdot 7H_2O$, 50 mg of OPA, 50 μl of BME, 1 ml of methanol were mixed in a 50-ml flask and brought to volume with 1 *M* NaOH (pH 10); this solution was kept in the dark, at 4°C and used within a day.

#### 2.1.3. HPLC eluents

Eluent A was 20 m*M* $KH_2PO_4$ at pH 3, prepared by weighing the salt and obtaining the right pH value by addition of 85% $H_3PO_4$. Eluent B was composed of 700 ml of 20 m*M* $KH_2PO_4$ at pH 4 (for 85% $H_3PO_4$) and 300 ml of acetonitrile.

### 2.2. Equipment

The HPLC system consisted of a LC200 Model binary pump equipped with a 7161 Rheodyne valve (20 μl loop), a LC95 UV–Vis and LS4 fluorescence detectors connected to an LC PLUS integrator/plotter from Perkin-Elmer (Monza, Italy). The analyses were performed by a Spherisorb ODS2 RP-18 25 cm×4.6 mm (5 μm) column from Alltech (Milan, Italy), kept at 34–35°C by a column thermostat from Chrompack (Middelburg, Netherlands).

Two slightly different analytical conditions were used as a function of the method run. Method A, analysis of free species, was performed by eluent A with 0.5 ml/min flow and UV detection at $\lambda$=215 nm. The conditions of method B, analysis of OPA derivatives, were: eluent B, 0.8 ml/min flow and fluorescence detection at $\lambda_{ex}$=315 nm and $\lambda_{em}$=415 nm.

### 2.3. Sample preparation

#### 2.3.1. Extraction

TCA procedure: 5 g of sample are homogenized with 10 ml of TCA extracting solution (Section 2.1.1) and then centrifuged for 15 min at about 13 *g* (*r*=0.3 m). The aqueous layer was recovered and the organic layer was discarded; the procedure was repeated twice on the pelleted sample. The aqueous fractions, collected together, were filtered on paper into a 50-ml flask and then made to volume with the 10% aqueous TCA solution. The extracted solution

is reported to be stable for a week when stored at 4°C [13] but after few days emitted a foul odor, probably due to polyamine formation as already reported [18,19]. In our laboratory this solution was stored at 4°C and used within two days.

$HClO_4$ procedure: in the case of samples with a high fat content or stored in oil it is advisable to remove the fat from the sample by treating 5 g of it with 10-ml of light petroleum, twice or more, (depending on the sample: methods have been performed both on ''natural'' canned tuna and ''in olive oil'' canned tuna). The sample is then centrifuged, the organic layer discarded and the procedure repeated twice or more on the solid. The defatted sample is homogenized with 10 ml of 0.6 *M* $HClO_4$ and filtered. After addition of 10 ml of 0.1 *M* NaOH, the specimen is extracted with 25 ml of butanol (five times). The organic layers joined together are extracted with 20 ml of light petroleum in order to eliminate the residual water and then extracted with 5×10 ml of 0.1 *M* HCl (histamine and histidine as chlorohydrates). In order to optimize the recoveries the light petroleum addition procedure can be repeated twice. The sample is diluted to 50 ml with water and the analysis is performed generally within a day.

### *2.3.2. Derivatization*

Two ml of solutions prepared according to the procedures reported in Section 2.3.1, or standard histamine and histidine solutions, are transferred into a 20-ml test tube and 1 ml of 1 *M* NaOH was added in order to make a strongly basic environment for the derivatization reaction; after about 5 min, 1 ml of derivatizing solution (Section 2.1.2) is added; 5–10 min later, after the solution has reached a yellow (standard) or brown (tuna fish samples) colour, 0.5 g of NaCl are added and the solution is extracted with 2×3 ml of ethyl acetate. The aqueous and organic layers are separated by centrifugation. Histamine-OPA was determined by injecting 20 μl of the organic extract after gentle evaporation under $N_2$ to 100 μl (measured with a Hamilton syringe) and reconstitution to 2 ml with eluent B. The sample should be analyzed in 30–40 min (see Section 3). Histidine-OPA was determined by injecting 20 μl of the aqueous solution.

## 3. Results and discussion

Method A is much simpler and allows shorter analysis times compared to method B, but can be used when the foods involved are poor in substances absorbing in 210–220 nm UV zone. The extraction step must be carried out with $HClO_4$ which is compatible with the UV detection zone. TCA should be avoided because of its high absorbance in the 210–300 nm UV region. Fig. 1 shows the analysis of a tuna fish sample (spiked with histamine standard to a final concentration of 50 ppm) performed according to method A. It can be seen that the method described allows a reasonable separation between the two compounds even in complex matrices like tuna fish in oil over the expiry date. The method sensitivity calculated from the standard is about 0.1 μg (50 ppm in standard solution) while real samples show a higher (about 1 μg) LOD because of the complex clean-up process.

Two parameters are to be taken in account in this method:

(1) The pH should be in the range of 3–4 (3.5 was empirically found to be the best value). A lower

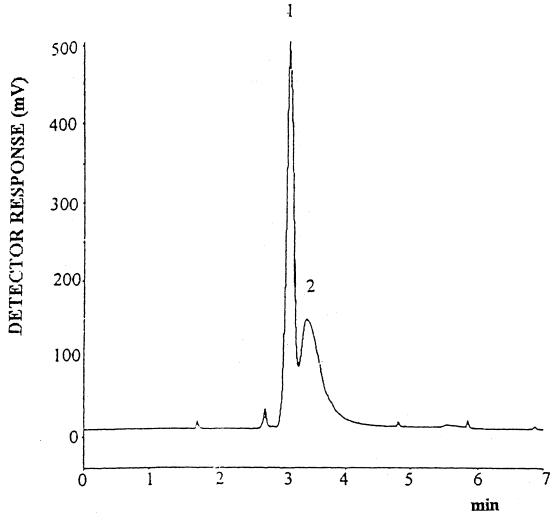


Fig. 1. Reversed-phase HPLC separation of histamine and histidine in tuna fish sample spiked with 50 ppm of histamine. Sample: 20 μl of sample solution (0.1 μg of theoretical histamine); column: Spherisorb ODS2 RP-18 (250×4.6 mm); buffer: 0.020 mol/l $KH_2PO_4$ (pH 3); flow-rate: 0.5 ml/min; detector: Perkin-Elmer LC95 UV–Vis; temperature: 35°C. Peaks: 1=histidine; 2=histamine.

value would not allow the separation between the two molecules while a higher value increases the run time and worsens the peak symmetry dramatically (note that also with the suggested conditions the histamine peak is slightly tailed).

(2) The flow should be around 0.4–0.5 ml/min in order to maintain a reasonable separation between peaks without tailing.

The run time is 6 min, included column regeneration and re-equilibration. Retention times are 3.1 and 3.4 min for histidine and histamine, respectively.

Method B is more complex but shows many advantages because of the increased sensitivity (0.01 μg) due to the fluorescence detection and the real separation between histamine and histidine derivatives. The latter, in high amounts in animal tissues, represents a potential interfering agent. In this case either TCA or $HClO_4$ can be used. This second method consists of: (i) histamine and histidine extraction from samples as chlorohydrates in a high acidic medium ($HClO_4$ or TCA); (ii) extract purification by liquid–liquid extraction in high alkaline medium in the presence of organic solvent and further re-extraction with HCl; (iii) derivatization with OPA, BME in basic medium followed by extraction of OPA derivatives (the $-NH_2$ group of histamine and histidine combines with OPA and BME to form a high fluorescent isoindole derivative); (iv) HPLC analysis with a $C_{18}$ RP column and fluorescence detection.

A standardization of time between derivatization and analysis was necessary in order to evaluate the linearity range (50–100 ppm) and reproducibility, due to the instability of derivatives. The best results were obtained by injecting the derivative 30–40 min after the derivatization.

Method B was evaluated in more detail. The evaluations were carried out on the same samples analyzed with method A. Identical treatment has been applied for standards and blank samples. The histamine-OPA derivative is mainly in the organic phase (ethyl acetate) (Fig. 2a) and presents a rather strong peak even for low concentration (5 ppm in solution). The histidine-OPA derivative does not interfere because it stays in the aqueous phase (Fig. 2b) and furthermore has a different retention time (3.6 compared to 7.5 min).

From the blanks and the fish samples chromatograms comparison, it appears that the presence of fluorescent compounds in the latter may be due to the derivatization reaction with alkylic and biogenic amines that may be present in the sample as a natural content or that can derive from a bad preservation procedure [16,19]. As already mentioned, in order to obtain reproducible quantitative results the HPLC injection must be performed 40 min after the de-

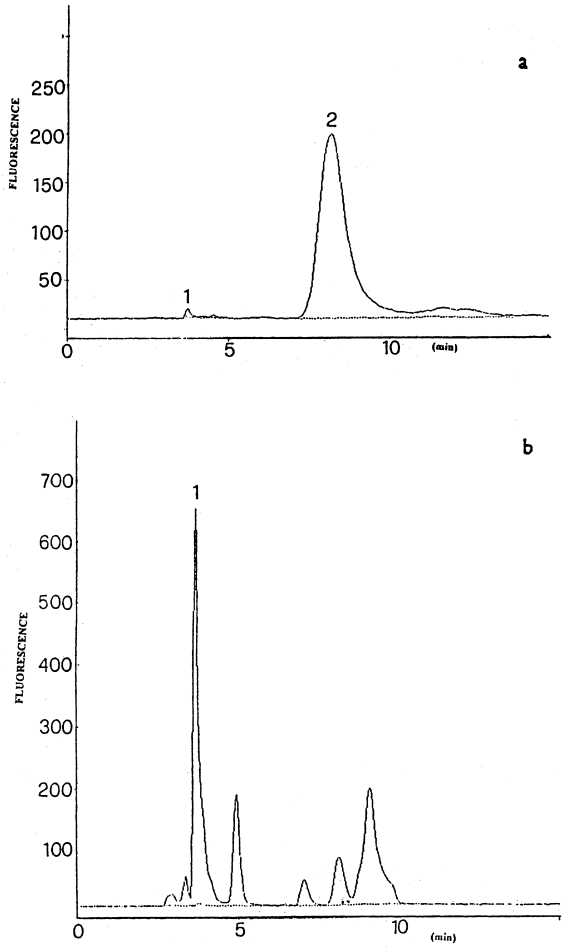


Fig. 2. Reversed-phase HPLC separation of histamine and histidine as OPA derivative in tuna fish sample spiked with 50 ppm of histamine. Sample: 20 μl of sample solution (0.1 μg of theoretical histamine), organic (a) and aqueous (b); column: Spherisorb ODS2 RP-18 (250×4.6 mm); buffer: 0.020 mol/l $KH_2PO_4$ (pH 4) with 300 ml of acetonitrile per l; flow-rate: 0.8 ml/min; detector: Perkin-Elmer LS4, 0.1 μA full-scale, excitation 315 nm, emission 415 nm; temperature: 35°C. Peaks: 1=histidine-OPA derivative; 2=histamine-OPA derivative. The dotted line indicates the analysis of a blank.

Table 1
Comparison of methods A and B

| Procedure | Sensitivity | | Linearity (ppm) | *R* (%) | Actual[a] (ppm) | Experimental[b] (ppm) | R.S.D.[c] (%) |
|---|---|---|---|---|---|---|---|
| | (ppm) | (μg) | | | | | |
| Method A | 50 | 0.1 | 50–500 | 0.9987 | 50 | 48.9 | 1.48 |
| Method B | 5 | 0.01 | 10–100 | 0.9889 | 50 | 48.5 | 1.60 |

Sample of tuna spiked with histamine to a final concentration of 50 ppm has been used for accuracy and precision evaluation of the method.
[a] Theoretical value corresponding to the histamine amount spiked into the sample.
[b] Average value obtained by determination of five separate sample weighings.
[c] Relative standard deviation obtained from 10 replicate measurements of a representative sample solution.

rivatization. In fact, the OPA derivatives are quite instable: a decay of 60% (measured as peak area) was registered after 12 h. The results are comparable with those reported in literature [13]. The robustness of the method has been evaluated too, by precision and accuracy determination. Repeatability has been evaluated by analyzing the same sample twice for 10 consecutive days. Samples were frozen and analyzed as necessary. The accuracy has been determined by spiking histamine-free tuna fish samples with a known amount of histamine and performing five runs. Table 1 reports the sensitivity and linearity parameters as well as the statistical data of method A compared to method B.

Further evaluations on other kinds of food are in progress and the method could be improved to determine polyamines like spermine and putrescine [17].

In the literature [20] the use of determination of biogenic amines and polyamines methods to determine the preservation state of some foods are already reported.

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