Validation of high-performance liquid chromatography (HPLC) method for quantitative analysis of histamine in fish and fishery products

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Abstract: A high-performance liquid chromatography method is described for quantitative determination and validation of histamine in fish and fishery product samples. Histamine is extracted from fish/fishery products by homogenizing with tri-chloro acetic acid, separated with Amberlite CG-50 resin and C18-ODS Hypersil reversed phase column at ambient temperature (25°C). Linear standard curves with high correlation coefficients were obtained. An isocratic elution program was used; the total elution time was 10 min. The method was validated by assessing the following aspects; specificity, repeatability, reproducibility, linearity, recovery, limits of detection, limit of quantification and uncertainty. The validated parameters are in good agreement with method and it is a useful tool for determining histamine in fish and fishery products.

Subjects: Food Analysis; Meat & Poultry; Seafood

Keywords: high performance liquid chromatography (HPLC); histamine; method validation; fish

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1. Introduction

Histamine is a biogenic amine produced by decarboxylation of free histidine. Histamine is generally present at low levels in the human body and can be present in a range of foods such as fish, cheese, meat, wine and fermented foods (Rocco, Lina, Nicola, & Paolo, 2006). Scombroid fish (Teleostea, Scombroidei), such as mackerel and tuna, or clupeid fish (Teleostea, Clupeoidei) such as sardines, anchovies and herrings are often involved in histamine toxicity. Histamine remains one of the problems for exporting tuna and tuna-like species from the tropics and subtropics to international markets. The food and drug administrative (FDA, 1998) set the safety level of histamine 5/100 g to ensure safety of the products. The European Union (EU/EC, 2005) has established that the average content of histamine in fish should not exceed 100 mg/kg and no sample may contain more than 200 mg/kg and fishery products should not exceed 200 mg/kg and no sample may contain more than 400 mg/kg out of nine samples. Mishandling coupled with high temperature abuse are common practices in handling fish in the tropic and subtropics, which significantly enhance histamine formation (Nejib, Moza, Ismail, Ann, & Mohammad, 2005).

Several methods to analyse biogenic amines in food have been described so far, including thin-layer chromatography, the use of the amino acid analyzer, liquid chromatography, gas chromatography and seven several biochemical assays. The method routinely used for histamine involves an extraction with methanol, subsequent ion-exchange chromatography and a chemical reaction with O-Phtaldehyde (OPA) or dansyl chloride under defined conditions to measure the resulting fluorescent reaction products (Jana, Kathleen, & Christine, 2002).

The aim of this study was a modified high-performance liquid chromatography (HPLC) method on the basis of an automated pre-column derivatization described by handouts of 5th regional training course in fish quality assessment methods of seafood safety (2004), South Asian Fisheries Development Center (SEAFDEC), Singapore. In addition, the method was optimized in terms of specificity, repeatability, reproducibility, linearity, recovery, limit of detection (LOD), limit of quantification (LOQ) and uncertainty.

2. Methods

2.1. Apparatus

The HPLC model Shimadzu, SIL 20A (Kyoto, Japan) equipped with LC solution software, quaternary pump and online degasser model LC20AD and injection valve with a loop capacity of 20 μL was used. The detector used was a programmable fluorescence detector model RF10AXL with a 350-nm excitation, 450-nm emission. The histamine compound was determined on reverse-phase ODS Hypersil (150 × 4.6 mm), C18 column.

2.2. Reagents and standards

(a) Histamine standard solution: Weighed 82.9 mg of histamine dihydrochloride (C₅H₉N₃.2HCl; Fluka chemicals, Japan) to the nearest 0.1 mg on an analytical balance and dissolved in HPLC water in a 50-mL one-mark volumetric flask and made up to the mark with HPLC grade water. Then, prepared the 100 and 10 mg/L intermediate standards. Those are prepared fresh weekly. The working standard solution prepared fresh daily on 0.5, 1.0, 1.5, 2.0 and 3.0 mg/L concentration.

(b) Ion-exchange resin (Amberlite CG-50): Amberlite CG-50 (100–200 mesh, H⁺ form, Fluka chemicals, USA) resin is converted to the H-form as follows. Added 100 mL of 1 M HCl per 20 g of resin and stir continuously for 10 min. Let it stand for another 10 min or more. Discarded the upper liquid layer. Repeat this procedure three times. Thoroughly washed the resin with distilled water until free form HCl.
(c) 0.1% O-phthalaldehyde (OPT) in methanol: Dissolved 100.0 mg of O-phthalaldehyde (Fluka chemicals, Austria) in methanol in a 100-mL one-mark volumetric flask and made up to the mark with methanol. Store in an amber bottle in a refrigerator.

2.3. Sample preparation and extraction
Cut whole sample into small pieces and mashed mechanically. Mixed mass well and weighed a 10-g sample into 100-mL beaker and added 20 mL of 10% tri-chloro acetic acid (TCA) and 20 mL of distilled water. Homogenized sample in 2 min using homogenizer (Heidolph-Q1) and transferred the sample into 100-mL volumetric flask, made up using distilled water and stood 10 min. Then, the sample was filtered through Whatman No. 1 filter paper and pipetted out 10 mL of filtrate to 50-mL beaker and adjusted the pH to 4.6 (Hanna, pH 211, USA) and passed through the column filled by Amberlite CG-50 resin. The 8 mL of eluted samples were taken into 25-mL beaker and adjusted the pH to 7 and made up to 10 mL with distilled water.

2.4. Derivatization of sample extracts and standard
Pipetted out 5.0 mL of the column chromatography elute into a 10.0-mL volumetric flask and added 1.00 mL of 1.0 M NaOH and mixed. Then added 0.50 mL of 0.1% OPT and mixed. After that, added 1.50 mL of 1 M H2SO4 just after 4 min and mixed. Made up to the mark with distilled water and mixed thoroughly.

2.5. Chromatographic separation
Set up the HPLC system and follow at least 30 min to stabilize. The HPLC conditions were maintained as follows; column; ODS Hypersil (150 × 4.6 mm), mobile phase; NaCl:Methanol (20:80), adjust to pH 3.1, flow rate; 0.5 mL/min, detection; excitation 350 nm, emission 450 nm.

2.6. Calculation
Histamine content (mg/kg) is calculated using the area under the peaks of standard histamine solution chromatograms.

\[
\text{Histamine content (mg/kg)} = \left( \frac{\text{Measured concentration of histamine in extract (mg/L)}}{\text{Sample weigh (g)}} \right) \times 100
\]

2.7. Method validation
Standard quality control materials (canned fish) T-2742 from FAPAS (Food Analysis Performance Assessment Scheme, the Food and Environment Agency, Sand Hutton, York, UK) were used for quality control in the study. The method validation procedures were followed according to IUPAC technical report, harmonized guidelines for single laboratory validation of methods of analysis (IUPAC, 2002) and EURACHEM/CITAC guide CG 4, quantifying uncertainty in analytical measurement (EURACHEM/CITAC, 2000). In the method validation following parameters were calculated, i.e. specificity, selectivity, precision, accuracy, linearity and range, LOD, LOQ, robustness/ruggedness and uncertainty.

3. Results and discussion
The chromatographic separation of histamine standard, (upper) (1 μg/L) & fish sample (lower) is shown in Figure 1.

The precision of the method was assayed by 6 replicate extraction of pure analytical standard, which contained histamine in low, medium and high concentrations within the shortest possible time period in the same instrument. Calculated amount, standard deviation and relative standard deviation are listed in Table 1.

The accuracy and reproducibility were tested using histamine quality control material (FAPAS T 2742, assigned value is 30.3 mg/kg) at different time periods. The results are given in Table 2.
The LOD was established with six independent sample blanks fortified at a lowest acceptable concentration of histamine (1.00 mg/kg) and measured once each. LOD was calculated using analyte concentration corresponding to a mean blank value +3s. According to that, the LOD was 0.2 mg/kg histamine and LOQ was calculated as LOD × 5 and it was 1 mg/kg of histamine.

The linearity and working range calculated by normal calibration curve was extended to higher and lower ranges (Figure 2).

The dilution factor was 10, hence the working range of this method is calculated as 1.0–250.0 mg/kg, with 0.99 or more correlation coefficient.

Identify variable/interferences which could have a significant effect on method performance. Set up experiments (using reference materials or histamine standards) in order to monitor the effect of
each changed condition on the mean value (percentage of validation). Rank the variables in order of significant effect on method performance. Maintain quality control data in order to control the effect of critical variables. Interfering compounds in the analysis of histamine are cadaverine and putrescine (having a similar chemical structure of histamine) and the effect of those studied. The mixture of histamine, cadaverine and putrescine was analysed by HPLC. Separate three peaks were observed for histamine, cadaverine and putrescine. That means, this method is effective to separate histamine from other similar amine compounds.

The pH of mobile phase and the ambient temperature were identified as critical variables. According to the method, the pH of the mobile phase and ambient temperature are 3.1 and 25°C, respectively. Six histamine standards (100.0 mg/kg) were analysed under normal and change conditions (pH of mobile phase 3.4 and ambient temperature 30°C). The percentage of variation in between replicates was calculated and variation of mobile phase pH and ambient temperature were 2.7 and 4.1%, respectively.

From our work, the efficiency of OPT derivatives extraction calculated from the unfortified and fortified fish samples, yellowfin tuna (fortified 50.0 mg/kg, histamine standard solution) at different time periods were found and the results are given in Table 3. Average recoveries for fortified samples were 86.3% and that is between the AOAC recommended ranges (75–120%).

The main component of uncertainty calculation was associated with the recovery (3.97%) and the uncertainty associated with precision was 0.022%. The expanded uncertainty value of this method was calculated as 11.0% ($k = 2$).

After validation the method, the analytical chemistry laboratory, national aquatic resources research and development agency (NARA), Sri Lanka participated the two proficiency testing scheme (low level and high level histamine in fish matrices) with this method and that results also confirmed that the method is suitable for the determination histamine in fish and fishery products ($z$-score $\sim 1.4$)

| Trial No. | Histamine concentration (mg/kg) |
|----------|--------------------------------|
| 1        | 26.2                           |
| 2        | 24.1                           |
| 3        | 25.5                           |
| 4        | 26.4                           |
| 5        | 28.6                           |
| 6        | 26.8                           |
| Mean     | 26.3                           |

Table 2. Accuracy and reproducibility values in sample

| Trial No. | Histamine concentration (mg/kg) |
|----------|--------------------------------|
| 1        | 26.2                           |
| 2        | 24.1                           |
| 3        | 25.5                           |
| 4        | 26.4                           |
| 5        | 28.6                           |
| 6        | 26.8                           |
| Mean     | 26.3                           |

Standard deviation (s) 1.5
Relative standard deviation (RSD), % 5.7

![Figure 2. The graph of peak area vs. histamine concentration.](image)
was obtained at an assigned value of 212 mg/kg, z-score 0.0 was obtained at an assigned value of 26.8 mg/kg in the Food Analysis Performance Assessment Scheme, Proficiency Testing Report 27137, May–June 2014 and 27126, Oct. 2013, The Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, UK).

4. Conclusion
Considering the results of method validation criteria such as specificity, selectivity, precision, accuracy, linearity and range, LOD, LOQ and uncertainty, this method is suitable for the determination of histamine in fish and fishery product samples. The method was also validated and results comply with ISO 17025 laboratory accreditation criteria.

Table 3. Recovery percentage of histamine fortified samples

| Trial No. | Histamine concentration (mg/kg) | Recovery (%) |
|-----------|---------------------------------|--------------|
| 1         | 43.95                           | 87.9         |
| 2         | 42.76                           | 85.5         |
| 3         | 40.42                           | 80.8         |
| 4         | 53.39                           | 106.8        |
| 5         | 38.99                           | 77.98        |
| 6         | 39.37                           | 78.74        |
| Mean      |                                 | 86.3         |

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