INTRODUCTION

Fish sauce is one of the most popular fermented fish products used as a condiment in South-East Asia. It is known by various names according to the country of origin, for example, nampla in Thailand, patis in the Philippines and noucmam in Vietnam. In 1999, Thailand exported a total of 38,224 metric tons which was valued at around USD 15.94 million, to USA, Japan, Europe and other countries. Fish sauce is traditionally produced by mixing whole fish with salt (3:1–1:1) and fermented for 6–12 months or longer. When most of the fish tissue has solubilized, the liquid fish sauce is drained off and filtered to yield a clear amber solution. Anchovy (Stolephorus spp.), a small red-fleshed pelagic fish which contains high amounts of free histidine, is the most popularly used raw material. Histidine can give rise to a high level of histamine through decarboxylation by microbiological deterioration. Fermented fish products and fish pastes were frequently found to contain high amounts of histamine. For example, histamine levels at 430 mg/L in nampla and even 1,380 mg/L in Korean anchovy sauce were reported. Histamine has been implicated as the causative agent in scombroid poisoning, an allergy-like foodborne illness associated with consumption of poor-quality scombroid (tuna and mackerel) and non-scombroid fish (mahi-mahi, sardines, anchovies etc.). Several scombroid poisoning cases have been reported in USA, Japan, England, and other European countries. The Canadian Fish Inspection Agency set up the maximum limit for histamine in fish sauce at 200 mg/L while the US Food and Drug Administration set it at 500 mg/L.

Many methods for histamine analysis, such as the fluorometric method, gas chromatography and liquid chromatography (LC) were designed for fish flesh. However, there is no official method established for determination of histamine in fish sauce. The purpose of the present study was to develop a method for histamine analysis in fish sauce. This was achieved by modifying the LC method of Walters. As fish sauce contains very high salt levels, additional steps had to be

Original Article

Determination of histamine in fish sauce from Thailand using a solid phase extraction and high-performance liquid chromatography

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ABSTRACT: A clean-up procedure using a solid phase extraction technique and a high-pressure liquid chromatography (HPLC) method for the determination of histamine in fish sauce from Thailand is introduced. The method was tested on fish sauce samples by adding various concentrations of histamine standards: 50–1000 mg/L of fish sauce, five replicates for each concentration. The samples were mixed with methanol and shaken for 1 min, applied to Bond Elut strong cation exchanger, then eluted with a mixed buffer solution of 0.5 M KH2PO4 (pH 6.5):methanol (1:1, v/v). Analysis was carried out by using HPLC with a post-column reaction with o-phthalaldehyde to form a fluorescent derivative. The recovery from fish sauce ranged from 90.5 to 95.5% with the limit of detection at 5 ng of histamine. The analysis of 549 commercial fish sauce samples showed that the samples contained histamine at various levels with the majority in the range of 200–600 mg/L.

KEY WORDS: fish sauce, high-pressure liquid chromatography, histamine, o-phthalaldehyde, solid phase extraction.

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Fish sauce is one of the most popular fermented fish products used as a condiment in South-East Asia. It is known by various names according to the country of origin, for example, nampla in Thailand, patis in the Philippines and noucmam in Vietnam. In 1999, Thailand exported a total of 38,224 metric tons which was valued at around USD 15.94 million, to USA, Japan, Europe and other countries. Fish sauce is traditionally produced by mixing whole fish with salt (3:1–1:1) and fermented for 6–12 months or longer. When most of the fish tissue has solubilized, the liquid fish sauce is drained off and filtered to yield a clear amber solution. Anchovy (Stolephorus spp.), a small red-fleshed pelagic fish which contains high amounts of free histidine, is the most popularly used raw material. Histidine can give rise to a high level of histamine through decarboxylation by microbiological deterioration. Fermented fish products and fish pastes were frequently found to contain high amounts of histamine. For example, histamine levels at 430 mg/L in nampla and even 1,380 mg/L in Korean anchovy sauce were reported. Histamine has been implicated as the causative agent in scombroid poisoning, an allergy-like foodborne illness associated with consumption of poor-quality scombroid (tuna and mackerel) and non-scombroid fish (mahi-mahi, sardines, anchovies etc.). Several scombroid poisoning cases have been reported in USA, Japan, England, and other European countries. The Canadian Fish Inspection Agency set up the maximum limit for histamine in fish sauce at 200 mg/L while the US Food and Drug Administration set it at 500 mg/L.

Many methods for histamine analysis, such as the fluorometric method, gas chromatography and liquid chromatography (LC) were designed for fish flesh. However, there is no official method established for determination of histamine in fish sauce. The purpose of the present study was to develop a method for histamine analysis in fish sauce. This was achieved by modifying the LC method of Walters. As fish sauce contains very high salt levels, additional steps had to be
introduced and some procedures modified in order to avoid damaging the LC apparatus. The modification involves an additional solid phase extraction step in the sample extraction procedure in order to remove salt prior to LC analysis.16

The present paper describes a reliable, rapid, quantitative method for the determination of histamine in fish sauce products. Histamine was extracted from the sample matrix with methanol instead of the solution mixture of methanol and 0.1 N HCl, and the clean up of the extracts was performed using cation exchange Bond Elut (Lida Manufacturing Corp., Kenosha, WI, USA) strong cation exchanger (SCX). The elution solution was optimized so that a wide range of histamine levels could be eluted from the Bond Elut (Lida Manufacturing Corp.) SCX.

**MATERIALS AND METHODS**

**Reagents**

Histamine dihydrochloride used for quantitation and recovery experiments, 2-thiophthalaldehyde (OPA) and mercaptoethanol were obtained from Sigma Chemical Company (St Louis, MO, USA). A stock solution containing 0.1% OPA and 10% mercaptoethanol was prepared in methanol of HPLC grade. Analytical reagent (AR)-grade and HPLC-grade methanol were from J.T.Baker (Phillipsburg, NJ, USA). Potassium dihydrogen phosphate, sodium tetraborate, and other reagents were AR grade from Merck Co. (Whitehouse station, NJ, USA).

**Preparation of standard solutions and reagents**

A standard stock solution of 1000 mg/L of histamine was prepared in methanol and kept stable for 1 month in an amber glass bottle at 4°C. Varying volumes of stock solution were diluted with methanol to give concentrations of 0, 0.5, 1.0, 3.0, and 5.0 mg/L before injecting to HPLC in order to obtain the calibration curve prior to sample extracts analysis. The amount of histamine in the sample was calculated from this standard curve.

2-Thiophthalaldehyde reagent was prepared by mixing 25 mL of 0.1% OPA stock solution with 300 mL of 0.05 M sodium tetraborate solution and 5 mL of 10% mercaptoethanol stock solution and diluted to 500 mL with sodium tetraborate solution (pH 10.0). It was prepared fresh daily and protected from light. The extraction solvent as recommended by Walters11 was a mixed solvent of methanol : 0.1 N HCl (4 : 1, v/v).

**Apparatus and chromatographic conditions**

Analyses were performed with a HPLC system (Waters Associates Inc., Milford, MA, USA), consisting of a Model 510 pump (Waters Associates Inc.), a 717 WISP autosampler (Waters Associates Inc.), thermostated column oven set at 45°C, a stainless steel column (25 cm × 4.6 mm inner diameter (i.d.)), Zorbax SCX 8 μm (Chrompack; Chrompack International B.V., Middelburg, The Netherlands), a fluorescence detector set at 350 nm for excitation wavelength and 444 nm for emission wavelength. The post-column reaction system consisted of a PCRS pump (Waters Associates) that was connected to a mixer (a T-shaped stainless tube) installed between the column outlet and detector. Coils of stainless steel tubing (120 cm long, 0.254 mm i.d.) were used to connect the mixer with the PCRS pump and the column outlet.

For solid phase extraction, Bond Elut SCX 500 mg (3 mL capacity) was purchased from Lida Manufacturing Corp. The mobile phase was 0.1 M KH₂PO₄ (pH 6.0) : methanol (7 : 3, v/v) at 1.2 mL/min. 2-Thiophthalaldehyde reagent was introduced, using a post-column reaction pump at a flow rate of 1.2 mL/min.

**Sample extraction and clean-up procedure**

Due to the naturally high histamine contents in fish sauce, only 1 mL of sample was used. Instead of the blending technique by the Walters method,12 a shaking technique was used for liquid fish sauce. The 1 mL sample was transferred into each of twelve 250 mL centrifuge tubes and shaken for 0, 1, 2, and 3 min with 40 mL of extraction solution of methanol : 0.1 N HCl (4 : 1, v/v) at 1.2 mL/min. 2-Thiophthalaldehyde reagent was introduced, using a post-column reaction pump at a flow rate of 1.2 mL/min.
Histamine in Thai fish sauce

FISHERIES SCIENCE

Histamine in Thai fish sauce

Histamine in commercial fish sauce

Table 1 shows the amount of histamine extracted from fish sauce with methanol : 0.1 N HCl (4 : 1, v/v) after shaking for 0, 1, 2, and 3 min. The histamine contents for each shaking time were 108.5, 117.8, 119.3, and 118.8 mg/L, respectively, and RSD ranged from 0.8 to 2.9%. Histamine contents obtained from the shaking times of 1, 2, and 3 min were significantly higher ($P<0.05$) than without shaking (0 min); however, there was no significant difference ($P>0.05$) in histamine contents obtained from 1, 2, and 3 min extraction. Therefore, shaking time of 1 min was sufficient to extract histamine from fish sauce and was adopted for further experiments.

Table 2 shows the percentage of histamine standard eluted from Bond Elut (Lida Manufacturing Corp., Kenosha, WI, USA) strong cation exchanger (SCX) using 5 mL of the elution buffer of 0.5 M KH$_2$PO$_4$ at various pHs mixed with methanol at a ratio of 1:1 (v/v).

Table 3 shows the percentage recovery of histamine added to fish sauce at the concentration range of 50–1000 mg/L. The recoveries ranged from 0.8 to 2.9%.
90.5 to 95.5% and RSD was below 3.5%. A positive correlation was observed between histamine added and histamine found with a correlation coefficient (r) of 0.9999.

Typical chromatograms of 0.5 mg/L of histamine standard, a blank fish sauce extract and fish sauce extracts added with histamine standard are shown in Fig. 1. For the column used in this method, the retention of histamine was approximately 9.2 min. There were no interfering peaks present in the chromatogram from blank fish sauce at this retention time.

The levels of total nitrogen and histamine in the 549 samples tested by this method are shown in Fig. 2. Most of the samples (85%) contained total nitrogen in the range of 10–25 g/L, and histamine at 100–600 mg/L. There were not many samples containing histamine below 100 mg/L, few above 600 mg/L, and rare above 1000 mg/L. Grade I (31%) and Grade II (33%) fish sauce samples contained a wide range of histamine from 100 mg/L to 1000 mg/L, with the majority at the range of 200–600 mg/L. On the other hand, most of the lower grade (36%) fish sauce samples contained histamine at the range of 100–400 mg/L.

**DISCUSSION**

The shaking technique for extracting histamine from fish sauce was found to be effective, convenient, and inexpensive compared with the use of a homogenizer or blender. In addition, it can be done either using an automatic shaker or simply by hand. The elution buffer with pH between 6.5 and 6.8 gave a higher percentage of recoveries than the elution buffer with lower pH. This was due to the fact that histamine was neutralized and no longer charged at a higher pH and thus eluted faster. However, the elution buffer pH 6.5 seemed to be the most suitable pH although it showed no significant differences (P>0.05) in the recovery with the higher pH. Elution buffers at pH 6.6–6.8 tended to form precipitates when they were mixed with methanol at the ratio of 1:1.

The HPLC method modified for histamine determination in fish sauce was quite simple. It was found to be very sensitive and accurate as confirmed by the results of four rounds of testing under the International Comparison Test organized by the Canadian Food Inspection Agency during 1999–2000, as shown in Table 4.

The wide range of histamine levels in each grade of fish sauce samples indicates that there is no correlation between histamine and total nitrogen contents. Total nitrogen content depends mainly on fish endogenous enzymes and the duration of fermentation, with longer fermentation time producing higher total nitrogen contents whereas histamine production depends on bacterial enzyme reactions from histidine as mentioned earlier. The wide variation of histamine (100–1000 mg/L) in both grades of fish sauce may be due to the differences in fermentation conditions and bacterial strains used.

**Table 3** Recovery of added histamine to fish sauce (n = 5) at a concentration range of 50–1000 mg/L using methanol as extraction solution, and subsequent clean-up procedure

<table>
<thead>
<tr>
<th>Histamine added (mg/L)</th>
<th>Histamine recovereda (mg/L)</th>
<th>Recovery (%)</th>
<th>RSDb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>45.3 ± 1.59</td>
<td>90.5</td>
<td>3.5</td>
</tr>
<tr>
<td>100</td>
<td>93.4 ± 2.80</td>
<td>93.4</td>
<td>3.0</td>
</tr>
<tr>
<td>200</td>
<td>183.2 ± 2.90</td>
<td>91.6</td>
<td>1.6</td>
</tr>
<tr>
<td>500</td>
<td>458.0 ± 1.67</td>
<td>91.6</td>
<td>0.4</td>
</tr>
<tr>
<td>1000</td>
<td>954.8 ± 3.36</td>
<td>95.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

aMean ± SD for five replicates.
bRelative SD.17

**Fig. 1** Representative chromatograms of (a) 0.5 mg/L (25 ng/injection) histamine standard, (b) blank fish sauce extract containing histamine of 35 mg/L (7 ng/injection), and (c) fish sauce extract added with histamine at 250 mg/L (50 ng/injection) : histamine.
to differences in freshness of the raw material and differences in salt-mixing techniques. Polyamine contents in fresh fish were found to be very low, but high levels were found in spoiled fish. Delays in salting and improper salt mixing could allow the growth of spoilage bacteria which may include histamine-forming bacteria. This leads to high levels of histamine remaining in the sauce until the end of fermentation time. The histamine formation in fish products was reported to be faster at low salt contents. For example, histamine contents were higher in semipreserved anchovies than in the preserved products. It is worth noting that none of the lower grade fish sauce contained histamine above 600 mg/L. However, the lower grade fish sauce is usually made from the fish residue after the first grade sauce is pumped out. The fish residue is leached with brine solution several times. Therefore, the lower histamine contents are a result of dilution, not decreased histamine production.

In conclusion, the method described was simple
to perform, and could successfully detect histamine in fish sauce without damaging the LC apparatus. The histamine found in Thai fish sauce varied from 100 to 1000 mg/L, with the majority in the range of 200–600 mg/L.

REFERENCES