Quantification of Biogenic Amines in Fermented Food Products by Reversed-Phase HPLC

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Abstract: A fluorometric derivatization method has been developed for the determination of biogenic amines (BAs) using high performance liquid chromatography (HPLC). The aim of this study is to develop a method for determination of BAs contents in different local fermented food products by fluorescence reversed-phase HPLC. The suitable extraction medium for extraction of BAs from fermented meat and Thai fermented sausage was 3 M perchloric acid and 1.5 M perchloric acid was for fermented rice while 4 N HCl was suitable medium for extraction of BAs from the fermented fish and fermented shell. Optimization of the liquid chromatography conditions led to a simple analytical method for the simultaneous determination of polyamines with high reproducibility and linearity. Application of this method to the determination of polyamines in food samples resulted in overall mean recoveries greater than 75% at fortification values of 20 – 100 μg/g of each BA. The fluorescence reverse-phase HPLC with the BA contents nearly corresponded the HPLC using UV-visible detector. The advantages of the method include derivitization, a simple chromatograph with clean separation of ophthalaldehyde derivatives. A major advantage is that it can be readily implemented in any laboratory with typical reversed-phase high performance liquid chromatography (HPLC) equipment.

Keywords: Biogenic Amine, HPLC, Fermented Food

I. INTRODUCTION

Biogenic amines (BAs) are organic bases characterized by their biological activities present in a number of foods, especially fermented foods. BAs are the basic nitrogenous compounds with aliphatic, aromatic or heterocyclic structure. BAs can also be found in a variety of foods, beverages and fermented foods especially in protein-rich foods that are formed by microbial decarboxylation of the corresponding amino acids. In particular, histamine, tyramine, putrescine and cadaverine produced during the process by degradation reactions.

Consumption of foods containing high amounts of BAs may cause problems such as headaches, nausea, hypotension, hypertension, cardiac palpitation, etc. [1]. BAs are recognized as potentially toxic for humans. The toxicity effects are reported to depend on an individual response and on a simultaneous presence of co-factors, which can act in synergy or in antagonism. For example, the toxicity threshold of histamine is lower in the presence of putrescine and cadaverine [2]. The identification (and quantification) of Bas in various kinds of food has received great attention. A number of chromatographic methods have been proposed for the quantitative determination of amines. Several analytical methods for the determination of BAs in foods such as thin layer chromatography, biosensors, capillary electrophoresis and gas chromatographic
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separation have been reported. Of these HPLC is the most popular and frequently reported for the separation and quantification of BAs. Existing tests for BAs can take and require large amount of sample.

The extraction of amines from a solid matrix in sample can be carried out with water, at room temperature or higher temperatures, so that only free amine are extracted, or in an acid medium, with hydrochloric acid (HCl) [3], perchloric acid (HClO₄; PCA) [4] or with trichloroacetic acid (TCA) [5] so that amines linked to other matrix components can also be extracted. The aim of this study are to develop method for determination of BA contents in different local fermented food products, namely, Nham, Sykrokprure, Khawmak (fermented rice), Plara (fermented fish) and Hoydoung (Fermented shell) with o-phtalaldehyde (OPA) derivatives and ninhydrin derivatives by reversed-phase HPLC.

II. MATERIALS AND METHODS

Samples

Fermented food samples were purchased from retail stored and Nham, Sykrokprure, Khawmak (fermented rice), Plara (fermented fish) and Hoydoung (Fermented shell) representing different commercial brands. All samples were kept at -18ºC prior to analysis. Three samples of each product were analyzed and all determination were replicated three times to estimate mean values and standard deviations.

Extractions

Extraction mediums for BAs from fermented foods were hydrochloric acid (HCl) and perchloric acid (HClO₄; PCA) with varied concentration of 1-4 M with the ratio of sample and acid of 1-3 (w/v) and three extractions were made. Finally, the three extractions were mixed in a Pyrex test tube (Bibbi Sterilin, Staff, UK), and dried at 70 ºC. The residue was dissolved in 0.2 ml of 0.1M HCl aqueous. After cooling the mixture was ready to be derivatized.

Instrumentation

For fluorescence reverse-phase HPLC, chromatographic analysis was carried out using a 1100 Model Liquid Chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with quaternary pump, on-line vacuum degassing system, injector (20µl loop volume), column oven and fluorescence detector. Signals were processed by the Agilent Chemstation for LC, Rev. A 09.03-847. Separation of fatty acids was achieved in a Zorbax XDBC18 C18 main column (15 cm x 4.6 cm i.d., particle size 5µm) thermostated at 30 ºC. A pre-column filled with the same stationary phase was used. The selected excitation and emission wavelengths were 340 and 450 nm, respectively.

For UV-VIS reverse-phase HPLC, Varian liquid chromatograph (Palo Alto, CA, USA) Model 9010 equipped with a Rheodyne Model 7161 manual injector with a 20 µl loop was used. The chromatographic separation was preformed on a Hypersil BDS C18 analytical column (200 mm x 4.6 mm i.d.: 5 µm particle size). Varian UV-VIS spectrophotometer detector Model 9050 UV-VIS was set at 254 nm.

Derivatization procedure

For OPA derivatives of BAs, a volume of 200 µl of 0.1M HCl aqueous solution containing the amino acid was introduced in a Pyrex test tube, and 0.1 ml of borate buffer was added. A volume of 0.4 ml of the OPA/2-ME solution was added. After shaking during 4 min, the mixture is filtered though a 0.45 µm i.d. nylon filter and immediately 20 µl were introduced in the chromatographic system.

For ninhydrin derivatives of BAs, derivatization was performed as follows: 0.5
ml sample extract or mixed standard was mixed with 50 μl of 3 mg/ml internal standard (1,7-diaminohepane). It was made alkaline by adding 100 μl of 2 N NaOH. A 150 μl saturated NaHCO₃ and 1 ml of 10 mg/ml of dansyl chloride in acetone were added to the alkaline extract. The reaction mixture was transferred to a 40 °C incubator for 45 min. Residual dansyl chloride was removed by adding 50 μl of 100% ammonia. After centrifugation, the supernatant was filtered through a 0.45 μm filter.

**Mobile phase**

85% (v/v) methanol and 15% 84 mmol/l triethylamine-acetic acid pH 7.5 was used as mobile phase of OPA-derivatives of BAs.

For ninhydrin derivatives of BAs, eluent A and B were methanol and water, respectively. A solution of 0.1 M sodium was prepared. The gradient program was implemented as follows: time= 0 min, 55% A, 35% B; time = 18 min, 70% A, 30% B; time = 28%, 70% A, 30% B; time = 33 min, 80% A, 20% B; time = 36 min, 10 % A, 0% B; time = 41 min, 100% A, 0% B; and time = 45 min, 55% A, 45% B. The two last steps were to re-equilibrate the column to the initial conditions.

**Recovery of the method**

The recovery of the method was determined by the standard addition technique. Samples were spiked with 100 μg of each amine standard solution in triplicate, then the sample was extracted and derivatized by the method described above. The percentage of recovery was determined by comparing the HPLC peak urea ratio each purified sample extract to the area ratio of the same standard solution, and the percentage of recovery was calculated.

**III. RESULTS AND DISCUSSION**

The concentration of tryptamine, tyramine and β-phenylethylamine were in the range of 0-14, 0-30 and 0-2 μg/ml, respectively (Figure 1).

![Fig 1](image-url)

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>65.2±9.2</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>84.4±6.3</td>
</tr>
<tr>
<td>Histamine</td>
<td>78.2±8.6</td>
</tr>
<tr>
<td>Tyramine</td>
<td>96.2±5.3</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>94.2±6.7</td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>79.3±7.2</td>
</tr>
</tbody>
</table>

Extraction efficiency was examined by determining extraction rates of BAs in the eluate, expressed as a percentage, using a variety of acids (Table 3). The following values were recorded in Nham, Sykrokprue, Khawmak, Prara and Hoydoung, respectively: for putrescine 75.37, 77.52, 72.34, 81.27 and 76.71 %, cadaverine 69.43, 78.24, 59.79, 64.25 and 69.37 % and histamine 58.72, 57.44, 59.42, 67.63 and 57.92 %.

BAs in fermented foods determined by reverse-phase HPLC is shown in Table 4-5.
TABLE 2
RECOVERIES (%) OF BAS ADDED TO VARIOUS FOODS DETERMINED BY OPA DERIVATIVATIZATION.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Put</th>
<th>Cad</th>
<th>Him</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nham</td>
<td>75.3±3.7</td>
<td>69.4±2.4</td>
<td>58.7±4.8</td>
</tr>
<tr>
<td>Sykrokprure</td>
<td>77.5±4.4</td>
<td>78.2±4.5</td>
<td>57.4±2.8</td>
</tr>
<tr>
<td>Khawmak</td>
<td>72.3±1.6</td>
<td>59.7±3.3</td>
<td>59.4±4.3</td>
</tr>
<tr>
<td>Prara</td>
<td>81.2±2.9</td>
<td>64.2±2.4</td>
<td>67.6±1.2</td>
</tr>
<tr>
<td>Hoydoung</td>
<td>76.7±3.4</td>
<td>69.3±1.2</td>
<td>57.9±2.7</td>
</tr>
</tbody>
</table>

TABLE 3
THE ACIDS USED FOR EXTRACTION OF BAS FROM FERMENTED FOOD PRODUCTS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Optimum condition of BAs extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extraction medium</td>
</tr>
<tr>
<td>Nham</td>
<td>3 M PCA</td>
</tr>
<tr>
<td>Sykrokprure</td>
<td>3 M PCA</td>
</tr>
<tr>
<td>Khawmak</td>
<td>1.5 M PCA</td>
</tr>
<tr>
<td>Prara</td>
<td>4 N HCl</td>
</tr>
<tr>
<td>Hoydoung</td>
<td>4 N HCl</td>
</tr>
</tbody>
</table>

BA contents of the sample are shown in Table 2 that were determined from the standard curve of putrescine. As seen from the figure, there are significant differences in the contents of BA in the samples may be because of the influent factors such as materials, pH, salt concentration, storage temperature, production process, acid addition [6] and preservative addition [7].

The suitable medium for extraction of BAs from Nham and Sykrokprure was 3 M PCA and 1.5 M PCA was for Khawmak while 4 N HCl was suitable medium for extraction of BAs from Prara and Hoydoung. BAs content of fermented fish was higher than the other fermented foods in this study. The OPA-derivatized BA could be determined by fluorescence reverse-phase HPLC with the BA contents nearly corresponded the HPLC using UV-visible detector with usually takes several hour for derivatization and cause unstable derivatives resulted to lower recovery and lower sensitivity determination.

Amine contents in fermented foods depend on the kind of material, microorganism associated with conditions of manufacturing production such as pH of ripening, time, temperature and also hygiene processing. Without good manufacturing process and expired products the amines contents seem to be increased. It is noted that cheese contained lowest amine contents compared to the other fermented foods in this study. Silla Santos [8] found cadaverine in reconstituted and sterile non-fat dry milk at 0.0367 mg/kg but did not report the presence of putrescine. However, putrescine, cadaverine, histamine and tyramine were detected in different amount. High amine contents in Plara and Hoykoung might occur from contamination of microorganisms producing amine from decarboxylation activity of amino acids and might be out of shelf life at high temperature storage. Silla Santos [8] reported a toxicity threshold (100 mg/kg) for histamine while, for tyramine, different toxic thresholds (100 and 800 mg/kg) were reported by ten Brink et al. [9]. In general, the amounts of 1,000 mg/kg of total amine is considered toxic for human health [10].

IV. CONCLUSIONS

The present procedure involves liquid-liquid extraction fermented food samples on with acid. The eluate is subjected to derivatization with ninhydrin and OPA, followed by HPLC. This approach has been extensively used in screening applications to
identify BA in fermented foods. The suitable medium for extraction of BAs from Nham and Sykrokprue was 3 M PCA and 1.5 M PCA was for Khawmak while 4 N HCl was suitable medium for extraction of BAs from Plara and Hoykoung. BAs content of Plara was higher than the other fermented foods in this study. The OPA-derivatized BAs could be determined by fluorescence reverse-phase HPLC with the BA contents nearly corresponded the HPLC using UV-visible detector with usually takes several hour for derivatization and causes unstable derivatives resulted to lower recovery and lower sensitivity determination.

**REFERENCES**


